Innovative approaches to understanding and limiting the public health risks of *Cryptosporidium* in animals in Australian drinking water catchments

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Doctor of Philosophy

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UNIVERSITY

Declaration

I declare this thesis is my own account of my research and contains as its main content work which has not previously been submitted for a degree at any tertiary education institution.

Alireza Zahedi

Statement of Contribution

This PhD thesis comprises a number of scientific publications, and each paper has been co-authored by multiple authors. The extent to which the work of others has been used is clearly stated in each chapter and certified by my supervisors.

I declare that I, Alireza Zahedi, have undertaken the majority of the original research presented in these papers and main authored the work.

Alireza Zahedi

Abstract

Cryptosporidium is the most important waterborne pathogen due to its resistance to chlorine in drinking water. The contribution of *Cryptosporidium* to waterborne diseases in Australia is however, unknown. The level of faecal contamination of drinking water catchments with this parasite was assessed by longitudinal analysis of faecal samples collected from marsupials, sheep, cattle and rabbits (n = 5,774) from eleven drinking water catchments across three states; New South Wales (NSW), Queensland (QLD) and Western Australia (WA). Faecal samples were screened by quantitative PCR (qPCR) and typed at two loci using Sanger sequencing. The overall prevalence of *Cryptosporidium* in faecal samples was 18.3% (1,054/5,774; 95% CI, 17.3-19.3). Of these, 873 samples produced clean Sanger sequencing chromatograms, and the remaining 181 samples, which initially produced chromatograms suggesting the presence of multiple different sequences, were re-analysed by Next Generation Sequencing (NGS) to resolve the presence of *Cryptosporidium* and the species composition of mixed infections. The overall prevalence of mixed infection was 1.7% (98/5,774), and in the remaining 83 samples, NGS detected only one species of *Cryptosporidium*. Of the 17 *Cryptosporidium* species and four genotypes detected (Sanger sequencing combined with NGS), 13 are capable of infecting humans; *C. parvum, C. hominis, C. ubiquitum, C. cuniculus, C. meleagridis, C. canis*, *C. felis*, *C. muris*, *C. suis, C. scrofarum, C. bovis, C. erinacei* and *C. fayeri*. Sewage (influent) samples across these states were also collected (n = 730) and screened by qPCR and typed using next generation sequencing (NGS). In sewage samples, the overall *Cryptosporidium* prevalence was 11.4% (83/730); 14.3% (3/21) in NSW, 10.8% (51/470); in QLD and 12.1% (29/239) in WA, and a total of 17 *Cryptosporidium* species and 6 genotypes were detected by NGS, including some of the same zoonotic species detected in animal faecal samples. This study highlights the

public health importance of continued identification of the sources/carriers of human pathogenic strains for accurate risk assessment and optimal catchment management.

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Acknowledgments

Here it is, the finished product of four years of my PhD, completed with sweat, lots of it! But no thesis come together without help and support of others. There have been many people who have walked alongside me over the last couple of years and I would like to convey my heartfelt thanks to each and every of them.

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To my co-supervisor Prof. Ian Robertson who has always been an inspiration and a great support. Thank you for not only stepping in when it was needed, but also for planting the initiative and providing ongoing encouragement for me to undertake this PhD.

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Abbreviations and Symbols

Abbreviations

18S rRNA	18S Ribosomal Ribonucleic acid
ACS	Acyl-Coenzyme A Synthetases
ADWG	The Australian Drinking Water Guidelines
AF	Acid Fast
AGWR	Australian Guidelines for Water Recycling
AIDS	Acquired Immunedeficiency Syndrome
ANOFEL	Association française des enseignants de Parasitologie et Mycologie
approx.	approximately
AUS	Australia
AWQC	Australian Water Quality Centre
BLAST	Basic Local Alignment Search Tool
bp	Base Pair
BSA	Bovine Serum Albumin
BTF	Biotechnology Frontiers
C.	*Cryptosporidium*
CC-QMRA	Climate Change Quantitative Microbial Risk Assessment
CDS	The Coding Sequence
cf	Conferre
Ch	China
CI	Confidence Intervals
Clec	C-type lectin domain
COWP	*Cryptosporidium* oocyst wall protein
CryptoDB	*Cryptosporidium* Data Base
Ct	Cycle Threshold
DAPI	4',6-diamidino-2-phenylindole
DALYs	Disability Adjusted Life Years
dPCR	Digital Polymerase Chain Reaction
ddPCR	Droplet Digital Polymerase Chain Reaction
DNA	Deoxyribonucleic acid

dNTP	Deoxynucleotide triphosphate
DWI	Drinking Water Inspectorate (UK)
et al.	And others
e.g.	*exempli gratia* (for example)
EGK	Eastern Grey Kangaroo
EIA	Enzyme immunoassays
ELISA	Enzyme-Linked Immunosorbent Assay
EPA	Environmental Protection Agency
FAO	Food and Agriculture Organisation
FDA	Food and Drug Administration
Fig	Figure
FRET	Fluorescence Resonance Energy Transfer
g	Gram
G/C	*Giardia*/*Cryptosporidium*
GEMS	The Global Enteric Multicentre Study
GIS	Geographic Information Systems
gp15	*15-kDa glycoprotein*
gp40	*40-kDa glycoprotein*
gp45	*45-kDa glycoprotein*
gp60	*60-kDa glycoprotein*
HBT	Health-based target
HIV	Human Immunodeficiency Virus
HMG-CoA	human 3-hydroxy- 3- methyl- glutaryl- coenzyme A
HSP40	Heat Shock Protein 40
ICAM-1	Inter-Cellular Adhesion Molecule-1
i.e.	id est - in other words
IFA	Immuno-Fluorescent Antibody
IFN-γ	γ-interferon
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IMPDH	Inosine 5′-monophosphate dehydrogenase

IMS	Immunomagnetic Separation
IPCC	Intergovernmental Panel on Climate Change
Kb	Kilo Base
Km	Kilometre
Km^2	Square kilometre
L	Litre
LC-ACS	Long-Chain fatty Acyl-Coenzyme A Synthetases
LP	Linkage Project
LRV	Log reduction value
LT2	Long Term2
LT2 ESWTR	Long-term Stage 2 Enhanced Surface Water Treatment Rule
MAL-ED	Malnutrition and Enteric Diseases
MBL	Mannose-binding lectin
$MgCl_2$	Magnesium chloride
mg	Milligram
min	Minute
miRNA	MicroRNA
ml	Millilitre
MLST	Multi-locus sequence typing
mM	Millimole
MMV	Medicines for Malaria Venture
n	Number
NCBI	National Center for Biotechnology Information
NGS	Next Generation Sequencing
NHMRC	National Health and Medical Research Council
NJ	Neighbor Joining
NK cells	Natural Killer cells
NNDSS	National Notifiable Diseases Surveillance System
NSW	New South Wales
NTZ	Nitazoxanide
ORFs	Open Reading Frames
p.a.	Per Annum

PCM	Phase-Contrast Microscopy
PCR	Polymerase Chain Reaction
QMRA	Quantitative Microbial Risk Assessment
QLD	Queensland
qPCR	Quantitative Polymerase Chain Reaction
RNA	Ribonucleic acid
rRNA	Ribosomal Ribonucleic acid
SABC	State Agricultural Biotechnology Centre
SCID mice	Severe Combined Immune Deficiency Mice
SIV	Simian immunodeficiency virus
SNPs	Single Nucleotide Polymorphisms
sp.	Species
spp.	Several species
SPSS	Statistical Package for Social Studies
STP	Sewage Treatment Plant
SSCP	Single Strand Conformational Polymorphism
Taq	*Thermus aquaticus* deoxyribonucleic acid polymerase
TCA	Thymine, Cytosine, Adenine
TCG	Thymine, Cytosine, Guanine
TCT	Thymine, Cytosine, Thymine
Th1	T-helper 1
TLR4	Toll-Like Receptor 4
U	Unit
UK	United Kingdom
U.S.	United States
USA	United States of America
U.S. EPA	United States Environmental Protection Agency
UTR	Untranslated Region
UV	Ultra violet light
vs	Versus
WA	Western Australia
Water NSW	Water New South Wales

Water RA	Water Research Australia
WSAA	Water Services Association of Australia
WHO	World Health organization
WHO GDWQ	World Health Organization Guidelines for Drinking Water Quality
WSAA	Water Services Association of Australia
WSPs	Water Safety Plans
ZOTUs	Zero-radius operational taxonomic units
°C	Celsius
µL	Microlitre
µm	Micrometre
µM	Micromole
µg	Microgram

Symbols

~	approximately
=	equals
>	greater than
<	less than
-	to
%	percent
×	times
±	plus-minus sign

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List of publications

The following publications are for the basis of my thesis:

Zahedi, A., Gofton, A.W., Greay, T., Oskam, C., Ball, A., Bath, A., Watkinson, A., Robertson, I., Ryan, U., 2018. Profiling the diversity of *Cryptosporidium* species and genotypes in wastewater treatment plants in Australia using Next Generation Sequencing. Sci. Total Environ. 644, 635-648.

Zahedi, A., Monis, P., Gofton, A.W., Oskam, C., Ball, A., Bath, A., Bartkow, M., Robertson, I., Ryan, U., 2018. *Cryptosporidium* species and subtypes in animals inhabiting drinking water catchments in three states across Australia. Water Res. 147, 327-340.

Zahedi, A, Gofton, A.W., Jian, F., Paparini, A., Oskam, C., Ball, A., Robertson, I., Ryan, U., 2017. Next Generation Sequencing uncovers within-host differences in the genetic diversity of *Cryptosporidium gp60* subtypes. Int. J. Parasitol. 10-11, 601-607.

Zahedi, A., Monis, P., Aucote, S., King, B., Paparini, A., Jian, F., Yang, R., Oskam, C., Ball, A., Robertson, I., Ryan, U., 2016. Zoonotic *Cryptosporidium* Species in Animals Inhabiting Sydney Water Catchments. PLoS ONE. 11(12), e0168169.

Ryan, U., **Zahedi, A**., Paparini, A., 2016. *Cryptosporidium* in humans and animals - a one health approach to prophylaxis. Parasite Immunol. 38, 535-547. (Invited review).

Zahedi, A., Paparini, A., Jian, F., Robertson, I., Ryan, U., 2016. Public health significance of zoonotic *Cryptosporidium* species in wildlife: critical insights into better drinking water management. Int. J. Parasitol. Parasites Wildl. 5, 88-109. (Invited review).

The following are a list of publications that arose from additional research I conducted during the course of my PhD:

Greay, T., **Zahedi, A**., Krige, A., Owens, J., Rees, B., Ryan, U., Oskam, C., Irwin, P., 2017. Endemic, exotic and novel apicomplexan parasites discovered during a national study of ticks from companion animals in Australia. Parasit. Vectors. 11, 197.

Zahedi, A., Lee, G.K.C., Greay, T., Walsh, A.L., Blignaut, D.J.C., Ryan, U., 2017. First report of *Cryptosporidium parvum* in a dromedary camel calf from Western Australia, Australia. Acta Parasitol. 63, 422-427.

Hijjawi, N., **Zahedi, A**., Kazaleh, M., Ryan, U., 2017. Prevalence of *Cryptosporidium* species and subtypes in paediatric oncology and non-oncology patients with diarrhoea in Jordan. Infect. Genet. Evol. 55, 127-130.

Zahedi, A., Durmic, Z., Gofton, A.W., Kueh, S., Austen, J., Lawson, M., Callahan, L., Jardine, J., Ryan, U., 2017. *Cryptosporidium homai* n. sp. (Apicomplexa: Cryptosporidiiae) from the guinea pig (*Cavia porcellus*). Vet. Parasitol. 245, 92-101.

Zahedi, A., Field, D., Ryan, U., 2017. Molecular typing of *Giardia duodenalis* in humans in Queensland - first report of Assemblage E. Parasitol. 144, 1154-1161.

Zahedi, A., Phasey, J., Boland, T., Ryan, U., 2016. First report of *Cryptosporidium* species in farmed and wild buffalo from the Northern Territory, Australia. Parasitol. Res. 115, 1349-1353.

List of conference proceedings

Zahedi, A., Gofton, A.W., Jian, F., Paparini, A., Oskam, C., Ball, A., Robertson, I., Ryan, U., 2017. Next Generation Sequencing uncovers within host genetic diversity of *Cryptosporidium gp60* subtypes. Australian Society for Parasitology conference, Blue Mountains, Australia. June 26th-29th, p41.

Zahedi, A., Paparini, A., Watkinson, A., Oskam, C., Robertson, I., Ryan, U., 2016. Molecular characterisation of species and genotypes of *Cryptosporidium* in animals inhabiting 3 main water catchments (Lake Baroon, Logan River, North-Pine River) in South-East Queensland (QLD). International Congress of Tropical Medicine and Malaria, Brisbane, Australia. Australia.18th-22nd September, p178.

Zahedi, A., Phasey J., Boland, T., Ryan, U., 2016. First report of *Cryptosporidium* species in farmed and wild buffalo from the Northern Territory, Australia. International Congress of Tropical Medicine and Malaria, Brisbane, Australia. Australia.18th-22nd September, p74.

Zahedi, A., Paparini, A., Jian, F., Monis, P., King, B., Ball, A., Robertson, I., Ryan, U., 2015. Identification of *Cryptosporidium hominis* in Eastern Grey Kangaroo populations in Sydney catchments. World Association for the Advancement of Veterinary Parasitology (WAAVP). Liverpool UK. August 16th-20th, p69.

Zahedi, A., Paparini, A., Jian, F., Monis, P., King, B., Ball, A., Robertson, I., Ryan, U., 2015. Prevalence and molecular characterisation of *Cryptosporidium* species in animals inhabiting Sydney water catchments. Australian Society for Parasitology conference, Auckland, New Zealand. June 29th-2nd July, p44.

Ethical concerns

ANIMAL ETHICS – Cadaver Notification

As animal faecal samples for this project were collected from the ground and not per rectum, animal ethics approval was not required. Instead, an animal cadaver/tissue notification covering all the samples collected was supplied to the Murdoch University Animal Ethics Committee.

HUMAN ETHICS

This project was conducted under Murdoch University Human Research Ethics Sub-Committee outright approval (2014/159).

General introduction

Aims and scope of the thesis

Diarrhoea is one of the five most common disease-related causes of death worldwide and is responsible for 2.2 million deaths annually, mainly in children younger than five years of age (Kosek et al., 2003; Keusch et al., 2016; WHO, 2017). Yet, due to lack of systematic surveillance in developing countries, the global burden of water associated diarrhoea and gastrointestinal diseases is scant (Ryan et al., 2017). Even in some developed countries such as Australia, where routine disease surveillance systems exist, the majority of water related gastroenteritis cases that occur in the community remains unnotified and the true burden of waterborne diseases is unknown (O'Toole et al., 2015).

With a worldwide distribution, waterborne parasitic protozoan pathogens contribute to almost four billion cases of diarrhoea every year, and therefore are of increasing concern (Wright and Gundry, 2009). Of these, *Cryptosporidium* is considered a major cause of severe waterborne diarrhoea worldwide, which can be life-threatening in immunocompromised individuals, and therefore represents a major public health concern for water utilities even in developed nations including Australia (Ryan et al., 2016). Currently ~ 17 million cases of gastroenteritis are reported in Australia per annum (p.a.), which has been estimated to cost over 1 billion dollars p.a. (Anon, 2006), and as a waterborne pathogen, *Cryptosporidium* accounts for a significant proportion of these cases.

Cryptosporidium is particularly suited to waterborne transmission as the oocyst stage is small in size, highly resistant to chlorine disinfection of drinking water and can survive for months in moist, ambient conditions (King and Monis, 2007; Ryan et al., 2017). It has a very low infectious dose (10-100 oocysts) and is usually shed in very large quantities in faeces. For

example, neonatal calves can excrete up to 30 billion oocysts or more over a 1-2 week period (Kuczynska and Shelton, 1999) and even apparently healthy animals can shed high numbers of oocysts (>5×10^6 oocysts per gram) (Chalmers and Giles, 2010). Water sources, mainly surface water, can become contaminated through direct defaecation of oocyst-contaminated faeces by animals or humans in the water or by surface run off. Therefore, risk assessment and risk management of the drinking water supply in relation to *Cryptosporidium* should be based, firstly, on identifying the sources of *Cryptosporidium*, and whether the species being shed in faeces are human-infectious or not. Despite this, the transmission dynamics of human pathogenic species and genotypes of *Cryptosporidium* from animals to humans through contamination of water sources is not well understood.

Currently, 37 *Cryptosporidium* species are recognized as valid (Jezkova et al., 2016; Zahedi et al., 2017; Čondlová et al., 2018; Kváč et al., 2018). Molecular data indicates that at least 17 of these are infectious to humans, with the zoonotic *C. parvum* and the largely anthroponotic *C. hominis* by far the most common species reported in humans worldwide, responsible for all waterborne outbreaks typed to date, with the exception of a single outbreak in the UK caused by *C. cuniculus* (Xiao, 2010; Puleston et al., 2014). In Australia, cryptosporidiosis is considered as a notifiable infectious disease across all states, with a seasonal pattern of disease notification that has remained consistent (Lal et al., 2015). However, relatively few genotyping studies have been conducted in Australia, but to date, *C. hominis, C. parvum, C. meleagridis* (from birds and humans)*, C. fayeri* (from marsupials), and *C. andersoni* and *C. bovis* (from cattle), have been reported in humans in Australia (cf. Ryan and Power, 2012). However, a review conducted for Water Research Australia (Ryan, 2014), identified that a key knowledge gap in assessing microbial risks to surface waters was the lack of quantitative prevalence and genotyping data on zoonotic *Cryptosporidium* species infecting animal hosts in Australian drinking water catchments over time and space, and their potential

link with water related outbreaks of *Cryptosporidium* in the human population (Ryan, 2014). Therefore, the over-arching aim of this PhD thesis was that sophisticated genetic fingerprinting of faecal samples from animals in drinking water catchments and human sewage can be used to characterise the diversity of *Cryptosporidium* species and genotypes contaminating drinking water catchments and Waste Water Treatment Plants (WWTPs) to better inform risk management. To address this, for the first time in Australia, a comprehensive quantitative survey of *Cryptosporidium* species and genotypes in marsupials, rabbits, cattle and sheep in 11 catchments and in 25 wastewater treatment plants (WWTPs) across three states, over a three-year period to gain a more thorough understanding of the zoonotic risk these parasites pose to humans.

Specifically, this project aimed to:

1. Collect faecal samples from the four most dominant non-rodent animals inhabiting water catchments (marsupials, sheep, rabbits and cattle) as well as from WWTP influent across three states, with very different climatic conditions;
2. Use qPCR to screen the faecal samples and WWTP samples for *Cryptosporidium;*
3. Enumerate *Cryptosporidium* oocysts in faecal samples and WWTP samples by qPCR with droplet digital PCR (ddPCR) calibrated standards;
4. Determine the diversity of *Cryptosporidium* species in animal and WWTP samples using both Sanger and next generation sequencing (NGS) at multiple loci;

The Australian Drinking Water Guidelines 2011 (ADWG) provide a framework for good management of drinking water supplies based on a preventive risk management approach (NHMRC-NRMMC, 2011). Risk management is based on assessing risks and reducing them to acceptable levels to assure safety. The ADWG currently do not have numerical targets for microbial safety of drinking water, but it is anticipated that this discrepancy will soon be

addressed and that a combination of quantitative microbial risk assessment (QMRA) and the metric of Disability Adjusted Life Years (DALYs) will be used to define microbial safety (O'Toole et al., 2015). The economic benefits to the industry, of effective modelling and management of waterborne pathogens, in particular *Cryptosporidium*, is substantial, as currently in Australia, >600 million dollars is expended annually to implement monitoring and management policies, and every water quality incident that is avoided through better management can save up to $100 million (CRC, 2008). Therefore, data generated from this thesis is crucial for more informed and accurate modelling and quantitative microbial risk assessments (QMRA), and more targeted control and risk mitigation strategies.

A note on thesis layout, formatting and style

This multidisciplinary PhD thesis incorporates the fields of molecular biology, parasitology, and public health, and comprises a number of scientific manuscripts, presented as chapters, which have been published in peer reviewed journals. In order to maintain a consistent style and to form a coherent and integrated body of work, each chapter commences with a preface introducing and linking the published work to the overall aims of the thesis, and ends with a summary outlining the main findings of the manuscript as they relate to the thesis. Since the chapters contain fully formed scientific manuscripts, the papers naturally contain their own abstract, introduction, materials and methods, results, and discussion sections.

Chapter one provides a thorough review of *Cryptosporidium* species, particularly zoonotic species detected in wildlife and also focuses on a "One Health" approach to prophylactic prevention of cryptosporidiosis including improved detection, diagnosis and treatment, and the importance of understanding zoonotic transmission.

Chapters two, three and four focus on screening faecal samples for *Cryptosporidium* from animals inhabiting water catchment areas across three states and provide improved molecular diagnostic, quantitation (enumeration) and characterisation tools for *Cryptosporidium* using digital PCR and and next generation sequencing (NGS).

Chapter five focuses on screening, quantification and characterisation of *Cryptosporidium* species in influent from wastewater treatment plants (WWTPs) using NGS, to more accurately determine the prevalence and composition of *Cryptosporidium* species in WWTPs.

Chapter six summarises the general findings of this project, discusses potential gaps in the field, and future directions.

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Chapter One – Literature review: Zoonotic *Cryptosporidium* species from One Health perspective

1.1 Preface

For clarity and consistency, and also to avoid repetition, this chapter consists of an amalgamation of two invited review papers which aim to provide a thorough review of zoonotic *Cryptosporidium* species from a one health perspective.

The two papers are entitled "*Cryptosporidium* in Humans and Animals - a One Health approach to prophylaxis" and "Public health significance of Zoonotic *Cryptosporidium* species in wildlife: Critical insights into better drinking water management".

Some of the tables have been updated to include the most recent literature and a section on the *Cryptosporidium* life cycle has been added. The original papers are presented in appendix 1 and 2.

1.2 Introduction

More than 15% of the world's population has no access to safe drinking water (Cauchie et al., 2014). Waterborne parasitic protozoan diseases with worldwide distribution, result in four billion cases of diarrhoea, with 1.6 million deaths annually (www.who.int) and 62.5 million DALYs worldwide (Wright and Gundry, 2009; WHO, 2009). Yet, despite the latest advances made in water treatment measures, protecting drinking water supplies against waterborne pathogens remains one of the most challenging concerns for the entire drinking water supply chain worldwide (Cotruva et al., 2004; Betancourt and Rose, 2004; Thompson and Smith, 2011; Plutzer, 2013; Burnet et al., 2014). In response to this, in 2009, the World Health Organization (WHO) developed guidelines for water suppliers on how to implement "Water Safety Plans" (WSPs), in the hope of halving the number of people without safe access to drinking water by the end of 2015 (WHO, 2009).

In less developed countries, the lack of basic infrastructure for providing safe drinking water is considered a major cause of poor water quality, which contributes to the spread of endemic/epidemic waterborne diseases. However, even in industrialized nations, highly advanced infrastructures are not yet a protective factor against outbreaks (Cummins et al., 2010; Smith and Nichols, 2010; Castro-Hermida et al., 2011; Burnet et al., 2014; Smolders et al., 2015). This appears to be largely due to a lack of knowledge about the epidemiology and transmission dynamics of waterborne pathogens (e.g. from animals ranging within the catchments), which leads to poor management practices for drinking water catchments (Castro-Hermida et al., 2011; Gormley et al., 2011).

Waterborne parasitic protozoans are responsible for the majority of waterborne outbreaks worldwide, with socio-economic impacts even in developed countries (Cotruva et al., 2004; Pond, 2005; Baldursson and Karanis, 2011; Cauchie et al., 2014). Of these, *Cryptosporidium*

was the aetiological agent in 60.3% (120) of the waterborne protozoan parasitic outbreaks that have been reported worldwide between 2004 and 2010 (Baldursson and Karanis, 2011). For the global water industry, therefore, *Cryptosporidium* represents the major public health concern, as its oocyst (the environmentally stable stage) is able to survive and penetrate routine wastewater treatment and is resistant to inactivation by commonly used drinking water disinfectants (Fayer et al., 2001; Baldursson and Karanis, 2011; Burnet et al., 2014). As a result of these waterborne outbreaks of cryptosporidiosis, *Cryptosporidium* testing in source or finished water is now mandatory in many industrialised nations. For example, the U.S. EPA, working with the U.S. public water supply industry, developed and implemented the Long-term Stage 2 Enhanced Surface Water Treatment Rule (LT2ESWTR), known as LT2, to control *Cryptosporidium* in public water supplies (US EPA, 2006). LT2 requires all public water suppliers using surface water sources serving populations >10,000 to monitor their sources for *Cryptosporidium* by analysing at least 24 consecutive monthly samples. In the UK, the Drinking Water Inspectorate (DWI) requires that water companies carry out risk assessments on all their water supply sites to ascertain the level of risk *Cryptosporidium* poses to the final treated water quality. Those at high risk need additional treatment (in the form of properly controlled coagulation/flocculation filtration systems or membrane or UV treatment systems). The UK regulations also require companies to design and continuously operate adequate treatment and disinfection. A proven failure to comply with this is now an offence (DWI, 2010).

Cryptosporidium species are protozoan parasites that infect a broad range of hosts including humans, and domestic and wild animals worldwide, causing asymptomatic or mild-to-severe gastrointestinal disease in their host species (Monis and Thompson, 2003; Hunter et al., 2007; Xiao, 2010; Ryan and Power, 2012; Kváč et al., 2014a; Ryan et al., 2014; Lukášová et al., 2018).

1.2.1 Clinical symptoms

Human cryptosporidiosis is frequently accompanied by abdominal pain, fever, vomiting, malabsorption and diarrhoea that may sometimes be profuse and prolonged (Chalmers and Davies, 2010; Bouzid et al., 2013). The immune status of the host, both innate and adaptive immunity, has a major impact on the severity of the disease and its prognosis. Immunocompetent individuals typically experience self-limiting diarrhoea and transient gastroenteritis lasting up to two weeks and recover without treatment, suggesting an efficient host antiparasite immune response. Immunocompromised individuals, including HIV/AIDS patients (not treated with antiretroviral therapy), often suffer from intractable diarrhoea, which can be fatal (Current and Garcia, 1991). An effective vaccine for cryptosporidiosis is not yet available.

The Global Enteric Multicenter Study (GEMS) study, which was a three-year matched case-control study of moderate-to-severe diarrhoea in over 22,000 infants and children at seven sites across Africa and Asia aged 0-59 months, found that *Cryptosporidium* was second only to rotavirus as a major cause of severe diarrhoea (Kotloff et al., 2013; Nasrin et al., 2013). More recent matched case–control studies of diarrhoea have confirmed this (Breurec et al., 2016). Similarly, a birth cohort study conducted by a Global Network for the Study of Malnutrition and Enteric Diseases (MAL-ED) has assessed pathogen-specific burdens in diarrhoeal and nondiarrhoeal stool specimens from 2,145 children aged 0-24 months, over five years at eight community sites in Africa, Asia and South America, and identified *Cryptosporidium* spp. as one of the five highest attributable burdens of diarrhoea in the first year of life (Platts-Mills et al., 2015). Globally, cryptosporidiosis is estimated to be responsible for the majority of deaths among children under 5 years of age (Lozano et al., 2012; Striepen, 2013; Shoultz et al., 2016) and *Cryptosporidium* infection in children is also associated with

malnutrition, persistent growth retardation, impaired immune response and cognitive deficits (Mølbak et al., 1997; Guerrant et al., 1999). The mechanism by which *Cryptosporidium* affects child growth seems to be associated with inflammatory damage to the small intestine (Kirkpatrick et al., 2002). Undernutrition (particularly in children) is both a sequela of and a risk factor for cryptosporidiosis, particularly in low-income familes (Macfarlane and Horner-Bryce, 1987; Sallon et al., 1988; Checkley et al., 1997; Bushen et al., 2007; Mondal et al., 2009; Quihui-Cota et al., 2015). FAO's executive summary of the State of Food Insecurity in the World (http://www.fao.org/docrep/018/i3458e/i3458e.pdf) indicates there are 842 million chronically malnourished persons worldwide, which significantly contributes to impaired immunity and thus increased susceptibility to infection with *Cryptosporidium*, perpetuating the cycle of chronic diarrhoea and malnutrition. In developed countries, *Cryptosporidium* is less common and accounts for ~9% of diarrhoeal episodes in children (Fletcher et al., 2012).

1.2.2 Taxonomy and Species in the genus

Until recently, *Cryptosporidium* was classified as a coccidian parasite. However, it has long been speculated that *Cryptosporidium* represents a 'missing link' between the more primitive gregarine parasites and coccidians (Ryan et al., 2016). The similarities between *Cryptosporidium* and gregarines have been supported by extensive microscopic, molecular, genomic and biochemical data (cf. Ryan et al., 2016), which have served as the basis for the formal transfer of *Cryptosporidium* from subclass Coccidia, class Coccidiomorphea to a new subclass, Cryptogregaria, within class Gregarinomorphea (Cavalier-Smith, 2014). The genus *Cryptosporidium* is currently the sole member of Cryptogregaria and is described as comprising epicellular parasites of vertebrates possessing a gregarine-like feeder organelle but lacking an apicoplast (Cavalier-Smith, 2014).

Currently, 37 *Cryptosporidium* species have been recognized as valid (Table 1.1), and more than 17 species have been identified in humans (Table 1.1). Of these, by far the most common species reported in humans worldwide are *C. parvum* and *C. hominis* (Xiao, 2010; Li et al., 2015a; Ryan and Xiao, 2014; Ryan et al., 2015; Holubová et al., 2016; Kváč et al., 2016; Zahedi et al., 2017; Čondlová et al., 2018, Kváč et al., 2018) and have been responsible for the majority of waterborne outbreaks typed to date with the exception of a waterborne outbreak in the UK caused by *C. cuniculus* from rabbits (*Oryctolagus cuniculus*) (Chalmers et al., 2009; Xiao, 2010; Ryan et al., 2014).

Table 1.1. Valid *Cryptosporidium* species confirmed by molecular analysis.

Species name	Author(s)	Type host(s)	Major host(s)	Reports in humans
C. occultus	Kváč et al., 2018	*Rattus norvegicus* (Brown rat)	Rodents (*Apodemus* spp.)	Ong et al., 2002; and unpublished (Acc. No. HQ822146)
C. apodemi	Čondlová et al., 2018	*Apodemus agrarius* (Striped field mouse), *Apodemus flavicollis* (Yellow-necked mouse)	Rodents (*Apodemus* spp.)	None reported
C. ditrichi	Čondlová et al., 2018	*Apodemus flavicollis* (Yellow-necked mouse), *Mus musculus* (Mouse)	Rodents (*Apodemus* spp.)	Unpublished single human infection, reported in Sweden (Acc. No. KU892579)
C. homai	Zahedi et al., 2017	*Cavia porcellus* (Guinea pigs)	Rodents (*Apodemus* spp.)	None reported
C. ducismarci	Traversa et al., 2008; Ježková et al., 2016	*Testudo marginata* (Marginated tortoise), *Python regius* (Ball python), *Chamaeleo calyptratus* (Veiled chameleon), *Malacochersus tornieri* (Pancake tortoise), *Agrionemys* [*Testudo*] *horsfieldii* (Russian tortoise)	Tortoises	None reported
C. testudinis	Ježková et al., 2016	*Agrionemys* [*Testudo*] *horsfieldii* (Russian tortoise), *Chelonoidis chilensis* (Chaco tortoise), *Testudo graeca* Linnaeus (Greek tortoise), *Testudo hermanni* Gmelin (Hermann's tortoise), *Geochelone elegans* (Indian star tortoise), *Stigmochelys pardalis* (Leopard tortoise), *Testudo marginata* (Marginated tortoise), *Astrochelys radiata* (Radiated tortoise), *Psammobates oculifer* (Serrated tortoise), *Python regius* (Ball python)	Tortoises	None reported
C. avium	Holubová et al., 2016	*Cyanoramphus novaezelandiae* (Red-crowned parakeet), *Agapornis roseicollis* (Rosy-faced lovebird), *Gallus gallus* (Chicken), *Amazona*	Birds	None reported

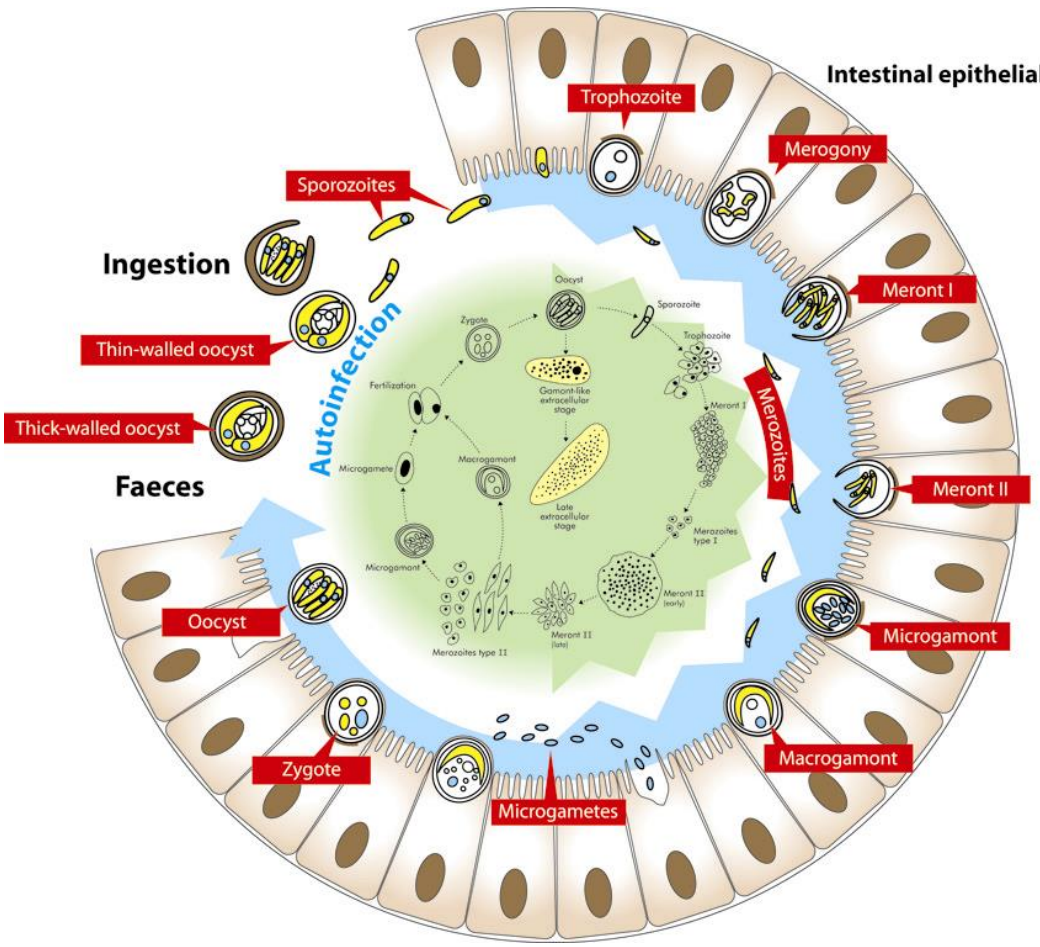
		aestiva (Blue-fronted Amazon parrot), *Lophochroa leadbeateri* (Major Mitchell's cockatoo), *Nymphicus hollandicus* (Cockatiel), *Melopsittacus undulates* (Budgerigar)		
C. proliferans	Kváč et al., 2016	*Equus africanus* (African wild ass), *Equus asinus* (Donkey), *Sciurus carolinensis* (Eastern gray squirrel), *Syncerus caffer* (African buffalo), *Equus caballus* (Horse), *Tachyoryctes splendens* (East African mole rat)	Rodents, Equine	None reported
C. rubeyi	Li et al., 2015a	*Spermophilus beecheyi* (California ground squirrel)	Squirrels	Not reported
C. scophthalmi	Alvarez-Pellitero et al., 2004; Unpublished (Acc. No. KR340588, KR340589)	*Scophthalmus maximus* (Turbot)	Turbot	None reported
C. huwi	Ryan et al., 2015	*Poecilia reticulata* (Guppy), *Paracheirodon innesi* (Neon tetra) and *Puntius tetrazona* (Tiger barb)	Fish	None reported
C. erinacei	Kváč et al., 2014b	*Erinaceus europaeus* (European hedgehog)	Hedgehogs, horses	Kváč et al., 2014a
C. scrofarum	Kváč el al., 2013	*Sus scrofa* (Pig)	Pigs	Kváč et al., 2009a, 2009b
C. viatorum	Elwin et al., 2012a	*Homo sapiens* (Human)	Humans	Elwin et al., 2012a; Insulander et al., 2013
C. tyzzeri	Tyzzer, 1912; Ren et al., 2012	*Mus musculus* (Mouse)	Rodents	Rasková et al., 2013
C. cuniculus	Robinson et al., 2010	*Oryctolagus cuniculus* (European rabbit)	Rabbits	Chalmers et al., 2009; Anonymous, 2010; Molloy et al., 2010; Chalmers et al., 2011a, 2012; Koehler et al., 2014
C. ubiquitum	Fayer et al., 2010	*Bos taurus* (Cattle)	Ruminants, rodents, primates	Commonly reported (cf. Fayer et al., 2010; Elwin et al., 2012b)
C. xiaoi	Fayer et al., 2010	*Ovis aries* (Sheep)	Sheep and goats	Adamu et al., 2014
C. ryanae	Fayer et al., 2008	*Bos taurus* (Cattle)	Cattle	None reported
C. macropodum	Power and Ryan, 2008	*Macropus giganteus* (Eastern Grey kangaroo)	Marsupials	None reported
C. fragile	Jirku et al., 2008	*Duttaphrynus melanostictus* (Toad)	Toads	None reported
C. fayeri	Ryan et al., 2008	*Macropus rufus* (Red kangaroo)	Marsupials	Waldron et al., 2010

C. bovis	Fayer et al., 2005	*Bos taurus* (Cattle)	Cattle	Khan et al., 2010; Ng et al., 2012; Helmy et al., 2013
C. suis	Ryan et al., 2004	*Sus scrofa* (Pig)	Pigs	Xiao et al., 2002a; Leoni et al., 2006; Cama et al., 2007; Wang et al., 2013a
C. galli	Pavalasek, 1999; Ryan et al., 2003	*Spermestidae, Frangillidae, Gallus gallus, Tetrao urogallus, Pinicola enucleator* (Birds)	Birds	None reported
C. hominis	Morgan-Ryan et al., 2002	*Homo sapiens* (Human)	Humans	Most common species in humans
C. molnari	Alvez-Pellitero and Sitja-Bobadilla, 2002	*Sparus aurata* (Gilt-head sea bream) and *Dicentrarchus labrax* (European seabass)	Fish	None reported
C. canis	Fayer et al., 2001	*Canis familiaris* (Dog)	Dogs	Many reports (cf. Lucio-Forster et al., 2010)
C. andersoni	Lindsay et al., 2000	*Bos taurus* (Cattle)	Cattle	Leoni et al., 2006; Morse et al., 2007; Waldron et al., 2011; Agholi et al., 2013; Jiang et al., 2014; Liu et al., 2014a
C. varanii	Pavlasek et al., 1995	*Varanus prasinus* (Emerald Monitor)	Lizards	None reported
C. baileyi	Current et al., 1986	*Gallus gallus* (Chicken)	Birds	None reported
C. parvum	Tyzzer, 1912	*Bos taurus* (Cattle)	Ruminants	Commonly reported in humans
C. meleagridis	Slavin, 1955	*Meleagris gallopavo* (Turkey)	Birds and humans	Commonly reported in humans
C. serpentis	Levin, 1980	*Elaphe guttata, E. subocularis, Sanzinia madagascarensus* (Snakes)	Snakes and lizards	None reported
C. felis	Iseki, 1979	*Felis catis* (Cat)	Cats	Many reports (cf. Lucio-Forster et al., 2010)
C. wrairi	Vetterling et al., 1971	*Cavia porcellus* (Guinea pig)	Guinea pigs	None reported
C. muris	Tyzzer, 1907, 1910	*Mus musculus* (House mouse)	Rodents	Many reports - Guyot et al., 2001; Gatei et al., 2002a; Tiangtip and Jongwutiwes, 2002; Gatei et al., 2003; Palmer et al., 2003; Gatei et al., 2006; Leoni et al., 2006; Muthusamy et al., 2006; Azami et al., 2007; Al-Brikan et al., 2008; Neira et al., 2012; Hasajová et al., 2014; Petrincová et al., 2015; Spanakos et al., 2015

1.2.3 Life cycle

The complex, monoxenous life cycle of *Cryptosporidium* consists of several developmental stages involving both sexual and asexual cycles which are demonstrated in Fig 1.1. The primary site of infection with *C. hominis* and *C. parvum* is the small intestine, with the ileum above the caecal junction being favoured in some animals such mice and calves (Xiao and Fayer, 2008).

Fig 1.1. Diagrammatic representation of the *Cryptosporidium* life cycle (Barta and Thompson, 2006).



Environmentally-resistant oocysts, representing the infective life cycle stage of the parasite, are excreted in the faeces (Xiao and Fayer, 2008). These oocysts are extremely

resilient, due to their thick trilaminar walls, are able to survive many months in a watery environment and are resistant to disinfectants including chlorine in drinking water (Fayer, 2004). Once the oocysts are excreted into the environment, they can be ingested by a host through the faecal-oral route. Excystation occurs in the gastrointestinal tract; this causes the release of four infective sporozoites through a suture opening, which then attaches to the apical membrane of the epithelial host cell and undergo successive rounds of asexual and sexual reproduction (Xiao and Fayer, 2008) (Fig 1.1). Following the adherence of the anterior end of the sporozoite to the luminal surface of an epithelial cell in the microvilli, each sporozoite matures into a trophozoite. Trophozoites undergo asexual proliferation by merogony to form meronts, marking the beginning of the asexual part of the life cycle (Hijjawi et al., 2010). Type I meronts develop six or eight nuclei, each incorporated into a merozoite, which are released from the parasitiphorous vacuole once mature. The mature type I merozoites infect other host cells and either recycle as type I meronts and merozoites, or develop into a type II meront, which produces four merozoites (Hijjawi et al., 2010). Following the release of mature type II merozoites, a new host cell is invaded and the sexual phase in the life cycle (gametogeny) is initiated. Type II merozoites either enlarge and develop into a uni-nucleate macrogamont or undergo cellular fission forming a multi-nucleated microgamont containing 14-16 non-flagellated microgametes.

Microgametes are released from ruptured microgamonts; they penetrate host cells containing macrogamonts and subsequently fertilise the macrogamont forming a zygote (Hijjawi et al., 2010). The zygote undergoes sporogony during which, both thin-walled and thick-walled oocysts are formed, each containing four potentially infective sporozoites. Thin walled oocysts remain within the host leading to autoinfection and persistent infections, thick walled oocysts are shed in the faeces into the environment, for ingestion by a new host.

The presence of gamont-like extracellular stages in the life cycle of *Cryptosporidium* was first observed in a study by Hijjawi et al. (2002) and has since been reported by several investigators (Hijjawi et al., 2004; Rosales et al., 2005; Karanis et al., 2008; Borowski et al., 2010; Koh et al., 2013, 2014; Huang et al., 2014; Aldeyarbi and Karanis, 2016). Where these gamont stages occur in the life cycle and what stages they develop into is not clearly understood (Clode et al., 2015).

1.2.4 Transmission dynamics

Oocysts of *Cryptosporidium* species from humans and animals are ubiquitous in the environment and therefore cryptosporidial infections can be acquired through multiple routes (reviewed by Robertson et al., 2014). Transmission of oocysts is by the faecal-oral route, either directly or indirectly. For humans, direct transmission can be from person to person primarily due to poor hygiene among household members and attendees in day care centres, aged care facilities and other institutions, or from animals to persons such as farmworkers and pet owners. Most indirect transmission is from contaminated drinking or recreational water. Contaminated food can also be a source of transmission, and contamination can occur at every step throughout the food preparation process, from farm to table (Nyachuba, 2010; Budu-Amoako et al., 2011). Findings from animal models, human case reports and a few epidemiological studies suggest that *Cryptosporidium* may also be transmitted via inhalation of aerosolized droplets or by contact with fomites contaminated by coughing (see Sponseller et al., 2014).

Cryptosporidiosis is a highly prevalent and extremely widespread disease (Ryan et al., 2014), and several factors contribute to this. Infected individuals shed large numbers of oocysts, which are environmentally very robust, resistant to inactivation by commonly used drinking water disinfectants including chlorine treatment and are able to survive routine

wastewater treatments (Baldursson and Karanis, 2011; Burnet et al., 2014). *Cryptosporidium* oocysts are highly infectious; in human volunteer studies, as few as 10 or less *Cryptosporidium* oocysts can produce disease in healthy adults (Okhuysen et al., 1999; Chappell et al., 2006). A quantitative risk assessment has estimated that ingestion of a single oocyst of the *C. parvum* IOWA isolate will result in clinical disease in 2.79% of immunologically normal persons (Pouillot et al., 2004). Another contributing factor to the high prevalence and widespread distribution of *Cryptosporidium* is the lack of treatment options. Only one drug, nitazoxanide (NTZ, Alinia; Romark Laboratories, Tampa, FL, United States), has been approved by the US Food and Drug Administration (FDA). This drug, however, exhibits only moderate clinical efficacy in malnourished children and immunocompetent people, and none in immunocompromised individuals like people with HIV (Abubakar et al., 2007; Amadi et al., 2009).

1.2.5 Detection and Diagnosis

The 'One Health' approach to tackle zoonotic diseases, defined as 'One Medicine' by Schwabe (1984), is a worldwide strategy to improve health and well-being through the mitigation and prevention of disease risks that originate at the interface between humans, animals and their various environments. *Cryptosporidium* presents many challenges for detection and diagnosis. The use of different diagnostic methods and the inconsistent application of typing techniques can make direct comparisons difficult or even impossible between clinical, veterinary and environmental testing or between different regions and countries (Chalmers and Katzer, 2013). Detection of *Cryptosporidium* in clinical pathology laboratories is still based mainly on microscopic detection via stains and/or fluorescent antibodies (IFA) and other antigenic detection methods. Although microscopy needs relatively

simple instruments and cheap consumables, it is labour intensive, requires a skilled operator and lacks sensitivity and specificity (Chalmers and Katzer, 2013). Morphological characters for identifying *Cryptosporidium* are few (Fall et al., 2003; Checkley et al., 2015) and differential staining techniques are usually required due to the fact that oocysts are similar in size and shape to yeasts, faecal components and other debris (O'Donoghue, 1995; Fall et al., 2003). Acid fast (AF)-modified Ziehl-Neelsen staining is one of the most common differential staining techniques (O'Donoghue, 1995; Chalmers and Katzer, 2013). However, the detection limits of conventional microscopy for *Cryptosporidium* have been reported to be as low as 10,000 to 50,000 oocysts per gram of human faeces (Weber et al., 1991; 1992), resulting in low levels of infection or sporadic shedding possibly going unnoticed when conventional methods of detection are used. Sporadic shedding is such that studies have shown that three separate faecal samples should be examined for immunocompetent patients and two samples for patients with AIDS for confident diagnosis of cryptosporidial infections using acid-fast staining (Clavel et al., 1995). IFA stains offer superior sensitivity; in some studies, about 97% sensitivity compared with only about 75% sensitivity for acid-fast staining (Chalmers et al., 2011b). However, IFA is more expensive than acid-fast staining and requires a fluorescence microscope and trained staff (Chalmers et al., 2011b). This is particularly problematic in resource-poor areas where cryptosporidiosis is a major health problem. A recent study proposed the use of phase-contrast microscopy (PCM) as a specific and inexpensive method for detection of *Cryptosporidium*; however, this method still lacks sensitivity (Ignatius et al., 2016).

Other antigen detection formats such as enzyme-linked immunosorbent assays (ELISAs), enzyme immunoassays (EIAs) and immunochromatographic (dipstick) assay for *Cryptosporidium* are also commercially available and have the advantage of reducing assay times and being amenable to automation. However, diagnostic sensitivities are variable (70%-

100%) (Garcia and Shimizu, 1997; Johnston et al., 2003; Youn et al., 2009; Chalmers et al., 2011b); some rapid tests have reduced specificity and sensitivity for species other than *C. parvum* or *C. hominis* (Robinson et al., 2010; Agnamey et al., 2011), and confirmation of positive reactions is needed (Youn et al., 2009). Biosensor chips, that detect and quantitate *C. parvum* in real-time via anti-*C. parvum* IgM binding, have also been developed (Kang et al., 2008; Campbell and Mutharasan, 2008); however, detection limits are relatively high (100 or more oocysts) and they have yet to be fully evaluated on water or faecal samples. Another major limitation of both conventional microscopy and antigen detection methods is that they cannot identify to species or subtype level, which is essential for understanding transmission dynamics and outbreaks, in particular for zoonotic species.

Polymerase chain reaction (PCR)-based techniques have permitted specific and sensitive detection and differentiation of *Cryptosporidium* spp. for clinical diagnosis and environmental monitoring (Chalmers et al., 2011b). Real-time or quantitative PCR (qPCR) assays have been developed to quantitate the numbers of *Cryptosporidium* oocysts present in human and animal faecal and water samples (Hadfield et al., 2011; Elwin et al., 2012a; Yang et al., 2013, 2014) with 100% specificity and sensitivities as low as 200 oocysts per gram of faeces, which equates to 2 oocysts per PCR (Hadfield et al., 2011). Multiplex qPCR assays have also been developed for the detection of *Cryptosporidium* and other common causes of diarrhoea such as *Giardia duodenalis* and *Entamoeba histolytica*, which have the advantage of identifying mixed infections (Taniuchi et al., 2013; Van Lint et al., 2013; Nurminen et al., 2015).

The most widely used molecular markers for typing of *Cryptosporidium* isolates are the 18S ribosomal RNA (18S rRNA) gene and the 60-kDa glycoprotein (*gp60*) gene. The latter locus encodes a precursor protein, that is cleaved to produce mature cell surface glycoproteins (*gp45*/*gp40* and *gp15*) implicated in zoite attachment to, and invasion of enterocytes (Strong et al., 2000; Xiao, 2010; Ryan et al., 2014). Most of the genetic heterogeneity in the *gp60* gene

is the variation in the number of a tri-nucleotide repeat (TCA, TCG or TCT) in the 5′ end (*gp40*) of the coding region, although extensive sequence polymorphism is also present in the rest of the gene. The repeats are used to define the subtype families within a species, whereas the remaining polymorphic sites are used to identify subtypes within a subtype family (Strong et al., 2000; Ryan et al., 2014). Miniaturized fluidic devices, which can detect to species level, have also been developed, mainly for the water industry (reviewed by Bridle et al., 2012), but as with antibody-based biosensor chips, have yet to be fully validated and are costly.

1.2.6 Treatment

New drug targets for *Cryptosporidium* are urgently needed, as the only FDA-approved drug, nitazoxanide, does not provide benefit for malnourished children and immunocompromised patients with cryptosporidiosis. However, *Cryptosporidium* has completely lost the plastid-derived apicoplast present in many other apicomplexans, and the remnant mitochondrion lacks the citrate cycle and cytochrome-based respiratory chain (Abrahamsen et al., 2004). Therefore, many classic drug targets are unavailable in *Cryptosporidium*. Progress in developing anticryptosporidial drugs has also been affected by the inability to generate large numbers of *Cryptosporidium* oocysts *in vitro* and an inability to genetically manipulate the organism (Miyamoto and Eckmann, 2015; Ryan and Hijjawi, 2015). The recent development of a hollow-fibre *in vitro* culture system to generate large numbers of oocysts (up to 10^8 oocysts per day) (Morada et al., 2016) and advances in genetically engineering *Cryptosporidium* (Vinayak et al., 2015), will transform the development of novel therapeutics.

To date, the best studied drug target is the bacterial-derived inosine 5′-monophosphate dehydrogenase (IMPDH) gene, as *Cryptosporidium* does not contain guanine salvage enzymes

and is totally dependent on this enzyme to convert adenosine salvaged from the host into guanine nucleotides (Striepen et al., 2004; Kirubakaran et al., 2012; Mandapati et al., 2014). This, coupled with the parasite's high metabolic demand for nucleotides due to the complicated life cycle of this parasite, make IMPDH an important drug target (Umejiego et al., 2008; Maurya et al., 2009; Macpherson et al., 2010; Sharling et al., 2010; Gorla et al., 2012, 2013; Johnson et al., 2013; Jefferies et al., 2015; Kim et al., 2015; Li et al., 2015b).

Other drug targets include long-chain fatty acyl-coenzyme A synthetases (LC-ACS), which are essential in fatty acid metabolism (Abrahamsen et al., 2004), and a recent study reported good efficacy of the ACS inhibitor triacsin C against cryptosporidial infection in mice (Guo et al., 2014). A parasite cysteine protease inhibitor was also effective *in vitro* and in an animal model (Ndao et al., 2013). Other studies have focused on repurposing existing drugs to overcome the prohibitive costs of *de novo* drug development (estimated to be between $500 million and $2 billion per compound successfully brought to market) (Adams and Brantner, 2006). For example, several compounds from the Medicines for Malaria Venture (MMV) Open Access Malaria Box have exhibited activity against *C. parvum* (Bessoff et al., 2014) and drugs such as the human 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase inhibitor, itavastatin and Auranofin (Ridaura®) initially approved for the treatment of rheumatoid arthritis and have been shown to be effective against *Cryptosporidium in vitro* (Bessoff et al., 2013; Debnath et al., 2013), which holds promise for further in vivo testing in animals and humans.

1.2.7 Vaccines

The development of vaccines for cryptosporidiosis, particularly in vulnerable populations such as children and malnourished populations, is urgent, but has been hampered

by an incomplete understanding of the host immune response to *Cryptosporidium* (Mead, 2014; Ludington and Ward, 2015). Therefore, a better understanding of host-parasite interactions is crucial for the development of an effective vaccine (Mead, 2014). Given that adults in highly endemic areas are partly immune to reinfection, and human challenge studies have shown that previous infection or exposure leads to a higher infectious dose [ID_{50}] (Okhuysen et al., 1998; Chappell et al., 1999), development of a successful vaccine should be possible. It is known that both innate and adaptive host response are important in the control of *Cryptosporidium* infection (Takeuchi et al., 2008; Petry et al., 2010; McDonald et al., 2013). Yet the nature of these responses, particularly in humans, is not completely understood (Borad and Ward, 2010; Ludington and Ward, 2015).

Early mediators of innate immune protection include the thick mucus layer of the small intestine, intestinal epithelial cells and chemokines, cytokines and antimicrobial peptides secreted into the intestinal lumen and/or underlying submucosa and bloodstream (Ludington and Ward, 2015). Important cytokines include γ-interferon (IFN-γ), which is secreted early in infection by natural killer (NK) cells, macrophages and dendritic cells, which are thought to play a major role in orchestrating both the innate and adaptive immune responses (McDonald et al., 2013; Ludington and Ward, 2015). T-helper 1 (Th1) inflammatory response and cytokines, such as interleukin 12, 15 and 18, are also important in the resistance and recovery to *Cryptosporidium* infection (Robinson et al., 2001; Dann et al., 2005; McDonald et al., 2006; Ehigiator et al., 2007a; Choudhry et al., 2012; Bedi et al., 2015). Treatment of both immunocompetent and immunodeficient mice with IL-12 before infection prevented or greatly reduced the severity of infection and was attributed to a decrease in IFN-γ reduction (Bedi et al., 2015). Data suggest that IL-15 has an important role in activating an NK cell-mediated pathway that leads to the elimination of *Cryptosporidium* from the intestine (Dann et al., 2005). IL-18 is produced by epithelial cells in the gut and a number of different immune cells and is

upregulated in response to *C. parvum* infection, and it has been proposed that one of the functions of IL-18 is to promote IFN-γ expression by macrophages (McDonald et al., 2006). Toll-like receptors expressed by epithelial cells have been shown to be important in modulation of the host immune response and subsequent parasite clearance (Chen et al., 2005; Barrier et al., 2006; Costa et al., 2011; O'Hara et al., 2011; Lantier et al., 2014; Perez-Cordon et al., 2014; Yang et al., 2015a).

MicroRNA (miRNA) regulation also appears to play an important role in host cell protection against *Cryptosporidium* (Chen et al., 2007; Zhou et al., 2009; Hu et al., 2010; Gong et al., 2011; Zhou et al., 2012; Xie et al., 2014). miRNA are small RNA molecules of 23 nucleotides that result in gene silencing via translational suppression or mRNA degradation and are a mechanism to fine-tune cellular responses to the environment, and may be regulators of host antimicrobial immune responses (Gong et al., 2011). More than 700 miRNAs have been identified in humans and are postulated to control 20%-30% of human genes. miRNA-mediated post-transcriptional gene regulation may regulate expression of genes critical to epithelial antimicrobial defence, and one cellular miRNA (*let-7i*) has been shown to target Toll-like receptor 4 (TLR4) and regulate TLR4-mediated anti-*C. parvum* defence (Chen et al., 2007). Functional manipulation of select miRNA expression levels in epithelial cells has been shown to alter *C. parvum* infection burden *in vitro* (Gong et al., 2011; Zhou et al., 2012). The intercellular adhesion molecule-1 (ICAM-1; CD54) is a 90-kDa member of the Ig superfamily expressed by several cell types including endothelial and epithelial cells and is thought to facilitate adhesion and recognition of lymphocytes at infection sites as ICAM-1 is constitutively present on endothelial and epithelial cells, but its expression is increased by pro-inflammatory cytokines or following microbe infection. Evidence has shown that miR-221-mediated translational suppression controls ICAM-1 expression through targeting the ICAM-1 3′-untranslated region (UTR), in epithelial cells in response to *C. parvum* infection, as

transfection of an miR-221 precursor an invitro model of human biliary cryptosporidiosis abolished *C. parvum*-stimulated ICAM-1 protein expression (Gong et al., 2011).

Mannose-binding lectin (MBL) is an evolutionarily conserved protein, secreted by hepatocytes, that functions in human innate immunity by binding to microbial surfaces and promoting opsonophagocytosis. MBL has been shown to be important in the protection against cryptosporidiosis, as children and HIV-infected adults with mannose-binding lectin deficiency have increased susceptibility to cryptosporidiosis and more severe disease (Kelly et al., 2000; Kirkpatrick et al., 2006; Carmolli et al., 2009). The genetic contribution to deficient or low serum levels of MBL results from polymorphisms in the MBL2 gene (MBL1 is a pseudogene), which create low MBL-producing MBL2 genotypes in ~5% of the world's population (Carmolli et al., 2009). In one study on a cohort of preschool children from Dhaka, Bangladesh, polymorphisms in the MBL2 gene (and corresponding haplotypes) and deficient serum levels of MBL were associated with increased susceptibility to infection with *Cryptosporidium*. MBL deficiency of <500 ng/mL was associated with single and multiple symptomatic episodes of *Cryptosporidium* infection, with an odds ratio (OR) of 7.6 for children with multiple symptomatic infections with *Cryptosporidium* (Carmolli et al., 2009). The mechanism by which MBL controls *Cryptosporidium* infection and protects children from it is still not clearly understood.

Adaptive immunity creates immunological memory after an initial response to *Cryptosporidium* and leads to an enhanced response to subsequent encounters with *Cryptosporidium*. For example, antibodies to the parasite antigen *gp15* were associated with protection against reinfection (Moss et al., 1998). The adaptive immune response to *Cryptosporidium* is characterised as a Th1 response (Ehigiator et al., 2007a) and the importance of the adaptive immune response during *Cryptosporidium* infection is highlighted by the susceptibility of patients with AIDS to cryptosporidiosis, as well as the resolution of infection

observed following CD4+ T lymphocyte cell reconstitution in patients given antiretroviral therapy (Borad and Ward, 2010; O'Connor et al., 2011). Low absolute CD4+ T cell counts in patients with HIV/AIDS were thought to be responsible for persistent and severe cryptosporidiosis; however, research with Simian immunodeficiency virus (SIV)-infected macaques reported that persistent cryptosporidiosis was more dependent on SIV load and profound viral damage to gut lymphoid tissue and rapid depletion of mucosal CD4+ T cells during the acute phase of viral infection, than on declining circulating CD4+ T cell levels during chronic SIV infection (Singh et al., 2011). This suggests that depletion of local CD4+ T cells may be more predictive of disease severity than absolute CD4+ T cell numbers. The importance of other T cells such as CD8+ has not been extensively studied but do appear to play a role in the protection of the host against gastric cryptosporidiosis (Kváč et al., 2011; Ludington and Ward, 2015). The role of humoral immunity in protection from cryptosporidiosis is not well understood, and no clear surrogate marker of protective immunity exists (reviewed in Ludington and Ward, 2015, and Checkley et al., 2015).

The ideal *Cryptosporidium* vaccine should provide rapid lifelong immunity in all vaccinated individuals, be broadly protective against the most common species and subtypes of *Cryptosporidium*, prevent disease transmission, and be readily accessible, stable and cheap (Mead, 2014; Ludington and Ward, 2015). Ensuring cross-reaction against the most common species infecting humans, however, will be difficult, as more than 20 *Cryptosporidium* species and genotypes can infect humans as discussed above. For example, a recent study showed that infection of gnotobiotic pigs with *C. hominis* resulted in complete protection against subsequent infection with *C. hominis*, but incomplete protection against infection with *C. parvum* (Sheoran et al., 2012); therefore, multiple species will need to be targeted to provide sufficient cross-protection. In addition, as children, malnourished, and immunocompromised individuals are the most important vaccine targets, and they may not be able to develop a strong

and sustained immune-mediated protection in response to vaccination. Indeed, malnutrition has been cited as an important factor underlying limited efficacy of vaccines (Savy et al., 2009). It is therefore likely that adjuvants such as TLR ligands (Steinhagen et al., 2011), will be required to enhance the immune response in target populations (Barrier et al., 2006; Lantier et al., 2014).

Several antigens, aimed at raising immunoglobulin G antibodies, are being developed as vaccine candidates (Mead, 2014). Some of the best studied are *gp15* (Preidis et al., 2007; Egorov et al., 2010; Ajjampur et al., 2011; Allison et al., 2011; Sarkar et al., 2012; Lazarus et al., 2015), *cp*15 (Jenkins and Fayer, 1995; Hong-Xuan et al., 2005; Wang et al., 2010a; Liu et al., 2010; Manque et al., 2011; Roche et al., 2013) and *cp*23 (Ehigiator et al., 2007b; Benitez et al., 2009; Liu et al., 2010). The *gp15* antigen is derived from the glycoprotein *gp60*, which is cleaved by a parasite serine proteinase into two surface proteins-*gp15* and *gp40*, both of which play an essential role in parasite motility and attachment to and invasion of host epithelial cells (Boulter-Bitzer et al., 2007), and can stimulate γ-interferon production by peripheral blood mononuclear cells of those previously infected (Preidis et al., 2007). The *gp15* antigen is relatively conserved between *C. parvum* and *C. hominis,* and studies in Bangladesh indicated that there is a significant cross-reactivity between them and that antibodies to *gp15* were associated with shorter duration of illness (Allison et al., 2011). Similarly, in a study in Kenya, AIDS patients without diarrhoea had significantly higher serum IgG levels to *gp15* than those with diarrhoea (Wanyiri et al., 2014).

cp15 is an immunodominant protein present on the oocyst surface and is associated with internal structures and bears no apparent similarity to *gp15* (Boulter-Bitzer et al., 2007). Immunization of pregnant goats with *cp15* vaccines protected offspring (Sagodira et al., 1999). The impact of malnutrition, however, on vaccination was demonstrated in recent research on intranasal vaccination of nourished and malnourished mice, with the *cp15* antigen primed with

a live enteric bacterial vector (Roche et al., 2013). The authors reported that malnutrition blunted antigen-specific cell-mediated responses to *cp15* and that vaccination resulted in only transient reduction in stool shedding of *Cryptosporidium* and was not protective against disease (Roche et al., 2013).

cp23 is an immunodominant protein, geographically conserved among *C. parvum* isolates, is present in both the sporozoite and merozoite stages (Mead, 2014), and antibodies to it are frequently detected following *Cryptosporidium* infection (Priest et al., 2001; Wanyiri et al., 2014). Serum antibodies to both *gp15* and *cp23* are associated with protection from diarrhoea in immunocompetent adult human volunteers infected with *Cryptosporidium* (Moss et al., 1998; Chappell et al., 1999; Riggs, 2002; Frost et al., 2005). Thus, a multivalent vaccine, incorporating multiple antigens or antigenic epitopes, may enhance protection against infection. For example, a divalent *cp23* and *cp15* vaccine prolonged the prepatent period and decreased oocyst shedding in mice vaccinated with the divalent vaccine compared with vaccination with *cp23* alone (Liu et al., 2010). Similarly, a reverse vaccinology approach based on genome mining that included three antigens; the well-characterised *cp15*, a calcium-activated apyrase involved in the invasion process of *Cryptosporidium* and profilin, an agonist of the innate immune system through its recognition by Toll-like receptors, induced specific and potent humoral and cellular immune responses in mice; however, further studies are necessary to verify the protection induced by these antigens (Manque et al., 2011). The development of an effective vaccine against *Cryptosporidium* is still a challenge and a better understanding of which immune responses are necessary for protection is essential to the development of immune-based interventions.

1.3 Sources of human-infectious *Cryptosporidium* species

Due to the morphological similarity of *Cryptosporidium* oocysts from different host species, initial findings of *Cryptosporidium* infections in both domestic and wild animals were assumed to be due to *C. parvum* leading to an overestimation of the potential role of animals as reservoirs of human disease (Appelbee et al., 2005). However, with the assistance of advanced molecular techniques, many of these species in wildlife particularly were identified as host-adapted genotypes (Table 1.2). Of the 37 *Cryptosporidium* species that have been recognized as valid, more than 20 species and genotypes have been identified in humans including *C. hominis*, *C. parvum*, *C. meleagridis*, *C. felis*, *C. canis*, *C. cuniculus*, *C. ubiquitum*, *C. viatorum*, *C. muris*, *C. suis*, *C. fayeri*, *C. andersoni*, *C. bovis*, *C. scrofarum*, *C. tyzzeri*, *C. erinacei* and *Cryptosporidium* horse, mink, skunk and chipmunk I genotypes, with *C. hominis* and *C. parvum* most commonly reported (Xiao, 2010; Ryan et al., 2014). These *Cryptosporidium* spp. infect both immunocompetent and immunocompromised persons (Ryan et al., 2014; Zahedi et al., 2016a). Of these, *C. parvum* and *C. hominis* are by far the most common species reported in humans worldwide (Xiao, 2010; Ryan and Xiao, 2014), and are responsible for most cryptosporidiosis outbreaks, with *C. hominis* responsible for more outbreaks than *C. parvum* in most regions (Xiao, 2010).

1.3.1 *Cryptosporidium hominis*

Although humans are the major host species for *C. hominis*, there have been isolated reports in domestic animals and wildlife hosts including sheep, goats, cattle, a dugong, non-human primates and kangaroos (Morgan et al., 2000; Smith et al., 2005; Giles et al., 2009; Abeywardena et al., 2012; Ye et al., 2012; Connelly et al., 2013; Karim et al., 2014; Koinari et al., 2014; Parsons et al., 2015; Gu et al., 2016; Koehler et al., 2016; Schiller et al., 2016; Zahedi et al., 2016b) and in fish (Koinari et al., 2013) (Table 1.2). *Cryptosporidium hominis*/*C.*

parvum-like sequences were identified in red and black-and-white colobus monkeys in Uganda (Salyer et al., 2012). However, typing was obtained using a short fragment of the *Cryptosporidium* oocyst wall protein (COWP) gene, which is not reliable for differentiating *Cryptosporidium* species. In Australia, a number of recent studies have also identified *C. hominis*/*C. parvum*-like isolates at the 18S locus in marsupials including bandicoots, brushtail possums, eastern grey kangaroos and brush-tailed rock wallabies (Hill et al., 2008; Ng et al., 2011; Dowle et al., 2013; Vermeulen et al., 2015). However, despite efforts, the identification of *C. hominis*/*C. parvum* could not be confirmed at other loci. This might be due to low numbers of oocysts and the multi copy nature of the 18S rRNA gene. Another study reported a *C. hominis*-like sequence at the 18S locus in a wild dingo, but was also unable to confirm this at other loci (Ng et al., 2011).

Subtyping of *C. hominis* at the *gp60* locus has identified nine subtype families (Ia to Ik) (Ryan et al., 2014). To date, few *C. hominis* subtypes have been reported in wild mammals but include subtype IbA9G2 in flying foxes, IbA10G2 in eastern grey kangaroos, IbA12G3 in Rhesus macaques, subtype IbA9G3 and IiA17 in Cynomolgus monkeys and Rhesus monkeys, and subtype IfA12G2 in baboons and Mitumba chimpanzees (Feng et al., 2011a; Karim et al., 2014; Bodager et al., 2015; Parsons et al., 2015; Schiller et al., 2016; Zahedi et al., 2016b).

1.3.2 *Cryptosporidium parvum*

Cryptosporidium parvum was first described in mice (Tyzzer, 1912) and is primarily a parasite of artiodactyls and humans (Xiao, 2010). *Cryptosporidium parvum* has however been frequently reported in wildlife, infecting a broad range of wild species including various rodents, bovids, camelids, equids, canids, non-human primates and marine mammals (Table 1.2) (Morgan et al., 1999a; Matsui et al., 2000; Atwill et al., 2001; Perez and Le Blancq, 2001;

Matsubayashi et al., 2004; Ryan et al., 2004; Appelbee et al., 2005; Feng et al., 2007; Meireles et al., 2007; Paziewska et al., 2007; Starkey et al., 2007; Ziegler et al., 2007; Gómez-Couso et al., 2012; Ye et al., 2012; Abu Samraa et al., 2013; García-Presedo et al., 2013a; Liu et al., 2013; Reboredo-Fernandez et al., 2014; Montecino-Latorre et al., 2015; Wells et al., 2015; Mynářová et al., 2016; Wagnerová et al., 2016).

Few studies have identified *C. parvum* in captive wild mammals but red deer, fallow deer, addaxes, Arabian oryx, gemsboks, orangutans and sable antelopes are among mammals to be infected with *C. parvum* in captivity (Perez and Le Blancq, 2001; Ryan et al., 2003; Hajdusek et al., 2004; Matsubayashi et al., 2004; Abe et al., 2006; Feng et al., 2007; Meireles et al., 2007; Bodager et al., 2015; Wang et al., 2015; Zhao et al., 2015a; Mynářová et al., 2016).

Subtyping of *C. parvum* at the *gp60* locus has identified fourteen subtype families (IIa to IIo (Ryan et al., 2014). Few studies which identified *C. parvum* in wild mammals have conducted typing at the *gp60* locus, but to date, a variety of *C. parvum* subtypes including IIdA15G1, IIdA18G1, IIdA19G1 from golden takins, lemurs, chipmunks and hamsters, IIaA15G2R1, IIaA19G2R1, IIaA19G3R1, IIaA19G4R1, IIaA20G3R1, IIaA20G4R1, IIaA20G3R2 and IIaA21G3R1 from deer and eastern grey kangaroos, IIdA17G1, IIdA18G1I, IdA19G1 and IIdA24G1 from European hedgehogs, IIaA18G3R1 from water buffalo, IIaA16G2R1 and IIaA13G1R1 from Eurasian wild boars, and IIaA13G2R1, IIaA15G2R1, and IIaA17G2R1from mustangs and Chincoteague ponies have been reported (Lv et al., 2009; García-Presedo et al., 2013a; Bodager et al., 2015; Montecino-Latorre et al., 2015; Wagnerová et al., 2015, 2016; Zhao et al., 2015a; Hofmannová et al., 2016; Sangster et al., 2016; Zahedi et al., 2016b, 2016c). The majority of these *C. parvum* subtypes have been reported in humans (Xiao, 2010).

1.3.3 *Cryptosporidium cuniculus*

Cryptosporidium cuniculus (previously known as rabbit genotype) was first reported in rabbits by Inman and Takeuchi (1979), who described the microscopic detection and ultra-structure of endogenous *Cryptosporidium* parasites in the ileum of an asymptomatic female rabbit. Molecular characterisation of *C. cuniculus* was first conducted on rabbit faecal samples from the Czech Republic (Ryan et al., 2003) and *C. cuniculus* was formally re-described as a species in 2010 (Robinson et al., 2010). Since then, it has been described from rabbits across a wide geographic area including Australia, China, the UK, the Czech Republic, Poland, France and Nigeria (Ryan et al., 2003; Nolan et al., 2010; Shi et al., 2010; Chalmers et al., 2011a; Zhang et al., 2012; Nolan et al., 2013; Koehler et al., 2014; Liu et al., 2014b; Puleston et al., 2014; Zahedi et al., 2016b). *Cryptosporidium cuniculus* has a close genetic relationship with *C. hominis* and its zoonotic potential became clear in 2008, when it was responsible for a drinking-water associated outbreak of cryptosporidiosis in the UK (Chalmers et al., 2009; Robinson et al., 2011; Puleston et al., 2014) and has also been identified in many sporadic human cases of cryptosporidiosis (Chalmers et al., 2011c; Robinson et al., 2011; Elwin et al., 2012b; Koehler et al., 2014). It is also the third most commonly identified *Cryptosporidium* species in patients with diarrhoea in the UK (Chalmers et al., 2011a). Subtyping at the *gp60* locus has identified two distinct subtype families, designated Va and Vb (Chalmers et al., 2009). Most cases described in humans relate to clade Va and the first waterborne outbreak was typed as VaA22 (Robinson et al., 2008; Chalmers et al., 2009). *Cryptosporidium cuniculus* has been reported in rabbits and humans (subtypes VaA9-VaA22 and VbA20-VbA37 - see Wang et al., 2012) but has recently been identified in marsupials (subtype VbA26) (and a human - subtype VbA25) in Australia (Nolan et al., 2013; Koehler et al., 2014). The widespread occurrence of *C. cuniculus* genotypes in rabbits and the fact that it has been now been identified

in marsupials in Australia suggests that *C. cuniculus* might be a species more ubiquitous than previously thought, and might be able to spread to other mammals as well as humans. Therefore, there is a need to diligently monitor for *C. cuniculus* in the vicinity of drinking water catchments and in drinking water.

1.3.4 *Cryptosporidium ubiquitum*

Cryptosporidium ubiquitum (previously cervine genotype, cervid, W4 or genotype 3) was first identified by Xiao et al. (2000) in storm water samples in lower New York State (stormwater isolate W4, GenBank accession no. AF262328). Subsequently, Perez and Le Blancq (2001) identified this genotype in white-tailed deer-derived isolates from lower New York State and referred to it as genotype 3. Since then it has been described in a wide variety of hosts worldwide including humans and was formally described as a species in 2010 (Fayer et al., 2010). *Cryptosporidium ubiquitum* is of public health concern because of its wide geographic distribution and broad host range (Li et al., 2014). In addition to domestic animals (in particular sheep) and wildlife, *C. ubiquitum* has been frequently reported from drinking source water, stormwater runoff, stream sediment and wastewater in various geographic locations, suggesting potential contamination of water sources with oocysts of *C. ubiquitum* shed by animals inhabiting water catchments (Nolan et al., 2013; Li et al., 2014). *Cryptosporidium ubiquitum* is considered an emerging zoonotic pathogen (Li et al., 2014), as it has been identified in many human cases of cryptosporidiosis in the United Kingdom, Slovenia, the United States, Canada, Spain, New Zealand, Venezuela and Nigeria (Wong and Ong, 2006; Fayer et al., 2010; Chalmers et al., 2011c; Cieloszyk et al., 2012; Elwin et al., 2012b; Blanco et al., 2015; Qi et al., 2015a).

In wildlife, *C. ubiquitum* has been reported sporadically in rodents, wild ruminants, carnivores, marsupials, hedgehog and primates (Table 1.2) (Perez and Le Blancq, 2001; da Silva et al., 2003; Feng et al., 2007, 2010; Karanis et al., 2007; Ziegler et al., 2007; Cinque et al., 2008; Wang et al., 2008; Fayer et al., 2010; Feng et al., 2011b; Robinson et al., 2011; Abu Samraa et al., 2013; Mi et al., 2013; Murakoshi et al., 2013; Li et al., 2014; Ma et al., 2014; Perec-Matysiak et al., 2015; Qi et al., 2015a, 2015b; Song et al., 2015; Stenger et al., 2015a; Vermeulen et al., 2015; Koehler et al., 2016; Li et al., 2016a).

Cryptosporidium ubiquitum is genetically distant from *C. hominis* and *C. parvum* and until recently, *gp60* homologs had not been sequenced. However, the *gp60* gene of *C. ubiquitum* was identified by whole genome sequencing and six subtype families (XIIa-XIIf) within *C. ubiquitum* have been identified (Li et al., 2014). Application of this new tool to human, animal, and environmental (water) isolates has suggested that sheep and rodents are a key source of *C. ubiquitum* transmission to humans, through either direct human contact with infected animals or by contamination of drinking source water (Li et al., 2014). For example, in the US, all *C. ubiquitum* specimens from humans characterised belonged to the same subtype families found in wild rodents in the US (XIIb, XIIc and XIId) (Li et al., 2014). However, as persons in the United States usually have little direct contact with wild rodents, the authors concluded that transmission of *C. ubiquitum* to humans from rodents was likely to come from drinking untreated water contaminated by wildlife (Li et al., 2014).

1.3.5 *Cryptosporidium muris*

Cryptosporidium muris is a gastric parasite and was first identified in the gastric glands of mice in 1907 by Tyzzer (1907). Since then, molecular tools have shown that it has a wide host range, including various mammals (rodents, canids, felids, suids, giraffida, camelidae,

equids, nonhuman primates and marsupials) and birds (Tables 1.1 and 1.2). *Cryptosporidium muris* is considered a zoonotic species as there have been numerous reports of *C. muris* in humans and one report in human sewage (Guyot et al., 2001; Gatei et al., 2002a; Tiangtip and Jongwutiwes, 2002; Gatei et al., 2003; Hurkova et al., 2003; Palmer et al., 2003; Gatei et al., 2006; Leoni et al., 2006; Muthusamy et al., 2006; Azami et al., 2007; Al-Brikan et al., 2008; Neira et al., 2012; Hasajová et al., 2014; et al., Petrincová et al., 2015; Spanakos et al., 2015).

In a recent human infectivity study, *C. muris* was examined in six healthy adults (Chappell et al., 2015). Volunteers were challenged with 10^5 *C. muris* oocysts and monitored for 6 weeks for infection and/or illness. All six patients became infected with two patients experiencing a self-limited diarrhoeal illness. The number of *C. muris* oocysts shed during the study ranged from 6.7×10^6 to 4.1×10^8, and *C. muris*-infected subjects shed oocysts longer than occurred with other species studied in healthy volunteers. Three volunteers shed oocysts for 7 months (Chappell et al., 2015). The authors concluded that healthy adults are susceptible to *C. muris*, which can cause mild diarrhoea and result in persistent, asymptomatic infection (Chappell et al., 2015), which confirms the zoonotic status of *C. muris* and highlights the public health risks of finding *C. muris* in wildlife in drinking water catchments.

1.3.6 *Cryptosporidium andersoni*

Like *C. muris*, *C. andersoni* is also a gastric parasite and primarily infects the abomasum of cattle and to a lesser extent, sheep and goats (Wang et al., 2012; Ryan et al., 2014). *Cryptosporidium andersoni* produces oocysts that are morphologically similar to, but slightly smaller than those of *C. muris* (7.4-8.8 × 5.8-6.6 µm vs 8.2-9.4 × 6.0-6.8 µm, respectively) and was originally mistakenly identified in cattle as *C. muris* based on its oocyst size. In 2000, it was described as a new species based on the location of endogenous stages in the abomasum,

its host range, and genetic distinctness at multiple loci (Lindsay et al., 2000). It has only occasionally been detected in wild animals (Table 1.2) (Ryan et al., 2004; Lv et al., 2009; Wang et al., 2008; Feng et al., 2010; Wang et al., 2015; Zhao et al., 2015a; Gu et al., 2016; Osman et al., 2017). Several studies have reported that *C. andersoni* is the dominant species in source and tap water (Nichols et al., 2010; Feng et al., 2011b), suggesting that cattle may be the primary source of contamination. Interestingly, in a recent study, it was found at a prevalence of 15.6% (19/122) and 0.5% (1/200) in captive and wild giant pandas, respectively in China (Wang et al., 2015). It is occasionally detected in humans (Leoni et al., 2006; Morse et al., 2007; Waldron et al., 2011; Agholi et al., 2013; Jiang et al., 2014; Liu et al., 2014a). Two studies in China by the same research group have reported that *C. andersoni* was the most prevalent *Cryptosporidium* species detected in humans (Jiang et al., 2014; Liu et al., 2014a). However, further research is required to better understand the zoonotic importance of *C. andersoni*.

1.3.7 *Cryptosporidium canis*

Cryptosporidium canis (previously dog genotype I) was first identified as the dog genotype by Xiao et al. (1999) and described as a species in 2001 (Fayer et al., 2001), on the basis that *C. canis* oocysts were infectious for calves but not mice and were genetically distinct from all other species. *Cryptosporidium canis* and its sub-genotypes (*C. canis* dog genotype, fox genotype and *C. canis* coyote genotype) have been reported in dogs, foxes and coyotes (Table 1.2) (Xiao et al., 2002a; Zhou et al., 2004; Fayer, 2010; Feng, 2010). It has also been reported worldwide in humans (Fayer, 2010; Lucio-Forster et al., 2010; Elwin et al., 2012b; Mahmoudi et al., 2015; Parsons et al., 2015).

1.3.8 *Cryptosporidium erinacei*

Little is known about epidemiology and pathogenicity of zoonotic *C. erinacei* in wildlife. *Cryptosporidium erinacei* (previously known as the hedgehog genotype) was first identified morphologically in a captive four-toed hedgehog (*Ateletrix albiventris*) in 1998 (Graczyk et al., 1998). An isolate from a European hedgehog originating from Denmark was typed in 2002 (Enemark et al., 2002) and shown to be distinct. Subsequent studies have identified *C. erinacei* in hedgehogs, horses and humans (Meredith and Milne, 2009; Dyachenko et al., 2010; Laatamna et al., 2013; Kváč et al., 2014a, 2014b; Hofmannová et al., 2016). At the *gp60* locus, *C. erinacei* isolates are identified as subtype family XIII (Lv et al., 2009; Dyachenko et al., 2010; Laatamna et al., 2013; Kváč et al., 2014b; Hofmannová et al., 2016). Previously reported *C. erinacei* subtypes include XIIIaA19R12 (GQ214081), XIIIaA19R13 (KU679366), XIIIaA20R10 (KF055453), XIIIaA21R10 (GQ214085), XIIIaA22R9 (KC305644), and XIIIaA22R11 (GQ259140) (Kváč et al., 2014b; Hofmannová et al., 2016).

1.3.9 *Cryptosporidium fayeri* and *Cryptosporidium macropodum*

The two main species identified in a wide range of marsupials are *C. fayeri* and *C. macropodum* (previously marsupial genotype I and II) (Table 1.2) (Morgan et al., 1997; Power et al., 2004, 2005; Power and Ryan, 2008; Ryan et al., 2008; Nolan et al., 2010; Power, 2010; Ng et al., 2011; Yang et al., 2011; Ryan and Power, 2012; Nolan et al., 2013; Vermeulen et al., 2015; Zahedi et al., 2016b; Šlapeta et al., 2017). Neither of these species is associated with diarrhoea in their marsupial hosts (Ryan and Power, 2012). *Cryptosporidium macropodum* has not been reported in humans but cryptosporidiosis caused by *C. fayeri* has been reported in a 29-year-old female patient in Australia (Waldron et al., 2010). The woman was

immunocompetent but suffered prolonged gastrointestinal illness. The patient resided in a national forest on the east coast of New South Wales (NSW), Australia, an area where marsupials are abundant. She had frequent contact with partially domesticated marsupials (Waldron et al., 2010). Identification of *C. fayeri* in a human patient is a concern for water catchment authorities in the Sydney region. The main water supply for Sydney, Warragamba Dam, covers 9,050 km^2 and is surrounded by national forest inhabited by diverse and abundant marsupials. At the *gp*60 locus, the subtype family IV has been identified with six subtypes (IVa-IVf) (Power et al., 2009). Subtyping of the human-derived isolate of *C. fayeri* identified IVaA9G4T1R1, which has also been identified in eastern grey kangaroos in Warragamba Dam, suggesting possible zoonotic transmission (Power, 2010; Waldron et al., 2010).

In addition to *C. fayeri* and *C. macropodum*, there have been several other host-adapted genotypes identified in Australian marsupials. Possum genotype I has been described in brushtail possums, a host species found in a range of habitats throughout Australia (Hill et al., 2008) and the novel kangaroo genotype I in western grey kangaroos (Yang et al., 2011). Possum genotype I and kangaroo genotype I have not been reported in humans or other animals and their zoonotic potential is unknown.

1.3.10 *Cryptosporidium meleagridis*

Cryptosporidium meleagridis infects the intestinal (small and large intestine and bursa) epithelial cells of a wide range of birds (Table 1.3) (Ryan and Xiao, 2014). It was first detected in a wild turkey (*Meleagris gallopavo*) by Tyzzer in 1929, but not named as a valid *Cryptosporidium* species until 1955 (Slavin, 1955). *Cryptosporidium meleagridis* oocysts have been experimentally infected into broiler chickens, ducks, turkeys, calves, pigs, rabbits, rats and mice (Darabus and Olariu, 2003; Ryan and Xiao, 2014). It has also been reported as one

of the most commonly detected human-infectious *Cryptosporidium* species in wastewater (Feng et al., 2007, 2011a; Li et al., 2012).

Molecular analysis has revealed that *C. meleagridis* has relatively low host specificity, and many *C. meleagridis* subtypes at other loci have been found in both birds and humans and both anthroponotic and zoonotic transmission routes have been suggested (Cama et al., 2003; Elwin et al., 2012b; Silverlås et al., 2012). *Cryptosporidium meleagridis* has also been identified in deer mice, mountain gorillas, minks and marsupials (Feng et al., 2007; Sak et al., 2014; Vermeulen et al., 2015; Zhang et al., 2016a). It is also the third most prevalent species infecting humans (Morgan et al., 2000; Cama et al., 2003; Gatei et al., 2006; Leoni et al., 2006; Muthusamy et al., 2006; Berrilli et al., 2012; Elwin et al., 2012b; Neira et al., 2012; Silverlås et al., 2012; Kurniawan et al., 2013; Sharma et al., 2013; Adamu et al., 2014; Ghaffari and Kalantari, 2014; Rahmouni et al., 2014; Ryan and Xiao, 2014; Stensvold et al., 2014, 2015; Wang et al., 2014). In some studies, *C. meleagridis* prevalence is similar to that of *C. parvum* (Gatei et al., 2002b; Cama et al., 2007).

Subtyping at the *gp60* locus has identified seven subtype families (IIIa-IIIg) and the likely occurrence of cross-species transmission of *C. meleagridis* between birds and humans (Wang et al., 2014). Human volunteer studies have shown that healthy adults can be infected and become ill after ingesting *C. meleagridis* oocysts (Chappell et al., 2011). In the study by Chappell et al. (2011), five volunteers were challenged with 10^5 *C. meleagridis* oocysts and monitored for six weeks for faecal oocysts and clinical manifestations. Four volunteers had diarrhoea; three had detectable faecal oocysts; and one infected volunteer remained asymptomatic. All infections were self-limiting and oocysts were cleared within 12 days of challenge (Chappell et al., 2011).

The ability of *C. meleagridis* to infect humans and other mammals, and its close relationship to *C. parvum* and *C. hominis* at multiple loci, has led to the suggestion that

mammals actually were the original hosts, and that the species has later adapted to birds (Xiao et al., 2002a). Subtyping at the *gp60* locus has identified seven subtype families (IIIa to IIIg) (Stensvold et al., 2015). Further details on transmission dynamics will be discussed in section 1.3.12.

1.3.11 Other *Cryptosporidium* species and genotypes reported in wild mammals

A number of other *Cryptosporidium* species and genotypes have been identified in wildlife (Table 1.2). Most are host-adapted genotypes that are not of public health significance, however several have been identified in humans (Table 1.2). Of these, *C. viatorum*, the chipmunk genotype I, mink genotype and skunk genotype are considered emerging human pathogens (Xiao et al., 2002b; Jiang et al., 2005; Feltus et al., 2006; Feng et al., 2007; ANOFEL, 2010; Insulander et al., 2013; Lebbad et al., 2013; Guo et al., 2015; Song et al., 2015; Yan et al., 2017). At the *gp60* locus, 15 different subtypes have been identified for *Cryptosporidium* chipmunk genotype I but subtypes differ only in the number of tandem repeats (TCA/TCG/TCT) and comprise a single subtype family (XIVa). Analysis indicates that subtypes from humans and wildlife are genetically similar and zoonotic transmission might play a potential role in human infections (Guo et al., 2015). The skunk and mink genotypes have also been reported in a few human cases of cryptosporidiosis (Robinson et al., 2008; Chalmers et al., 2009; Rengifo-Herrera et al., 2011; Elwin et al., 2012b; Ng-Hublin et al., 2013a; Ebner et al., 2015).

Table 1.2. *Cryptosporidium* species and genotypes identified by molecular tools in wild terrestrial mammals and their zoonotic importance.

***Cryptosporidium* species/genotypes**	**Wildlife hosts**	**Zoonotic importance**	***gp*60 subtypes reported in wildlife**	**References**
C. hominis	Fallow deer (*Dama dama*), Dugong (*Dugong dugon*), Chinchillas (*Chinchilla lanigera*), Baboons (*Pabio anubis*), Chimpanzees (*Pan troglodytes schweinfurthii*), Red colobus (*Procolobus rufomitratus*), Black-and-white colobus (*Colobus guereza*), Rhesus macaque (*Macaca mulatta*), Cynomolgus monkey (*Macaca fascicularis*), Japanese macaque (*Macaca fuscata*), Crab-eating macaque (*Macaca fascicularis),* Francois' leaf monkey (*Trachypithecus francoisi*), Lemurs (*Lemur* spp.), Gibbon (*Hylobatidae* spp.), Bandicoots (*Isoodon obesulus*), Bushtail possums (*Trichosurus vulpecula*), Estern grey kangaroos (*Macropus giganteus*), Brush-tailed rock-wallabies (*Petrogale penicillata*), Wild dingo (*Canis lupus dingo*), Squirrel monkey (*Saimiri sciureus*), European hedgehogs (*Erinaceus europaeus*), Horses (*Equus caballus*), flying fox (*Pteropus poliocephalus*), Eastern grey kangaroos (*Macropus giganteus*); Badger (*Meles meles*), Striped field mouse (*Apodemus agrarius*), Dromedary camel (*Camelus dromedarius*)	Main *Cryptosporidium* species infecting humans	IaA13R7, IaA13R8, IaA14R7, IbA9G2, IbA9G3, IbA10G2, IbA10G2R2, IbA12G3, IdA14, IdA15, IdA15G1, IdA19, IdA20, IeA11G3T3, IfA12G2, IfA16G2, IfA22G2, IiA17, IkA7G4, IkA15G1, IkA16, IkA16G1, IkA20G1	Morgan et al., 2002; Ng et al., 2011; Salyer et al., 2012; Ye et al., 2012; Dowle et al., 2013; Nolan et al., 2013; Karim et al., 2014; Ryan et al., 2014; Ye et al., 2014; Krawczyk et al., 2015; Laatamna et al., 2015; Liu et al., 2015a; Parsons et al., 2015; Gu et al., 2016; Jian et al., 2016; Koehler et al., 2016; Schiller et al., 2016; Zahedi et al., 2016b; Danišová et al., 2017; Deng et al., 2017; Inácio et al., 2017; Mateo et al., 2017; Baroudi et al., 2018
C. parvum	Alpaca (*Vicugna pacos*), Swamp deer (*Cervus duvauceli*), Red deer (*Cervus elaphus*), Roe deer (*Capreolus capreolus*), Fallow deer (*Dama dama*), Addaxes (*Addax nasomaculatus*), Arabian oryx (*Oryx leucoryx*), Gemsboks (*Oryx gazella*), Sable antelopes (*Hippotragus niger*), White-tailed deer (*Odocoileus Virgini*anus), Grey wolf (*Canis lupus*), Racoon dog (*Nyctereutes procyonoides viverrinus*), European rabbit (*Oryctolagus cuniculus*), Nutria (*Myocastor coypus*), Prezewalski's wild horse (*Equus przewalskii*), Eastern grey squirrel (Sciurus carolinensis), Ground Squirrels (*Spermophilus beecheyi*), Siberian chipmunk (*Tamias sibiricus*),	Major	IIaA10G1R1, IIaA13G1R1, IIaA13G2R1, IIaA14G1R1, IIaA14G2R1, IIaA15G2R1, IIaA16G1R1, IIaA16G2R1, IIaA16G3R1, IIaA17G1R1, IIaA17G2R1, IIaA18G3R1, IIaA19G2R1, IIaA19G3R1, IIaA19G4R1, IIaA20G1R1, IIaA20G3R1, IIaA20G3R2, IIaA20G4R1, IIaA21G3R1, IIcA5G3, IIcA5G3a, IIdA15G1, IIdA17G1, IIdA18G1, IIdA19G1,	Morgan et al., 1999a; Matsui et al., 2000; Atwill et al., 2001; Perez and Le Blancq, 2001; Ryan et al., 2003, 2004; Matsubayashi et al., 2004; Ekanayake et al., 2007; Feng et al., 2007; Meireles et al., 2007; Paziewska et al., 2007; Starkey et al., 2007; Ziegler et al., 2007; Cinque et al., 2008; Lv et al., 2009; Feng, 2010; Gómez-Couso et al., 2012; Ravaszova et al., 2012; Ye et al., 2012;

	Hamsters (*Cricetinae*), Wood mice (*Apodemus sylvaticus*), White-footed mouse (*Peromyscus leucopus*), Yellow-bellied marmot (*Marmota flaviventris*), Bamboo rats (*Rhizomys sinensis*), Small brown bat (*Myotis lucifugus*), Campbell hamster (*Phodopus campbelli*), Golden hamster (*Mesocricetus auratus*), Capybara (*Hydrochoerus hydrochaeris*), Racoon dog (*Nictereutes procyonoides viverrinus*), Red fox (*Vulpes vulpes*), Rhesus macaques (*Macaca mulatta*), Toque macaques (*Macaca sinica sinica*), Grey langurs (*Semnopithecus priam thersites*), Purple-faced langurs (*Trachypithecus vetulus philbricki*), Common dolphins (*Delphinus delphis*), Golden takins (*Budorcas taxicolor bedfordi*), Eastern grey kangaroos (*Macropus giganteus*), Asian house rat (R*attus tanezumi*), Brown rat (*Rattus norvegicus*), Bamboo rats (*Rhizomys sinensis*), Water buffalo (*Bubalus bubalis*), Eurasian wild boars (Sus scrofa), European hedgehogs (*Erinaceus europaeus*), Horse (*Equus caballus*), Mustang (*Equus caballus*), Chincoteague ponies (*Equus caballus*), Donkey (*Equus asinus*), Orangutans (*Pongo abelii* and *Pongo pygmaeus*), European hedgehogs (*Erinaceus europaeus*), Striped field mouse (*Apodemus agrarius*), Yellow-necked mouse (*Apodemus flavicollis*), Bank vole (*Myodes glareolus*), Qinghai vole (*Microtus fuscus*), Wild plateau pika (*Ochotona curzoniae*)		IIdA24G1, IIiA10, IIoA13G1, IIpA9 (novel subtype)	Dowle et al., 2013; García-Presedo et al., 2013a; Nolan et al., 2013; Liu et al., 2014; Reboredo-Fernandez et al., 2014; Bodager et al., 2015; Du et al., 2015; Krawczyk et al., 2015; Laatamna et al., 2015; Liu et al., 2015b; Montecino-Latorre et al., 2015; Qi et al., 2015b; Wagnerová et al., 2015; Wang et al., 2015; Wells et al., 2015; Zhao et al., 2015a, 2015b; Hofmannová et al., 2016; Koehler et al., 2016; Mynářová et al., 2016; Sangster et al., 2016; Zahedi et al., 2016b, 2016c; Danišová et al., 2017; Inácio et al., 2017; Mateo et al., 2017; Martins et al., 2018; Zhang et al., 2018
C. cuniculus	European rabbit (*Oryctolagus cuniculus*), Eastern grey kangaroo (*Macropus giganteus*) (single report)	Responsible for several waterborne outbreaks and sporadic cases of cryptosporidiosis in the UK and has been identified in a human in Australia	VaA18, VbA18, VbA19, VbA21, VaA22, VbA24, VbA26, VbA28, VbA29, VbA32, VbA22R4, VbA23R3, VbA24R3, VbA25R4, VbA26R4	Xiao et al., 2002a; Ryan et al., 2003; Nolan et al., 2010; Chalmers, 2012; Robinson et al., 2010; Elwin et al., 2012b; Zhang et al., 2012; Nolan et al., 2013; Kaupke et al., 2014; Koehler et al., 2014; Liu et al., 2014b; Puleston et al., 2014; Koehler et al., 2016; Yang et al., 2016a

C. ubiquitum	Swamp deer (*Cervus duvauceli*), Deer mouse (*Peromyscus*), Eastern grey squirrels (*Sciurus carolinensis*), Red squirrel (*Sciurus vulgaris*), Eastern chipmunk (*Tamias striatus*), Lemur (Lemuroidea), North American beaver (*Castor canadensis*), Woodchuck (*Marmota monax*), Raccoon (*Procyon lotor*), White–tailed deer (*Odocoileus virginianus*), Sika deer (*Cervus Nippon*), Roe deer (*Capreolus capreolus*), Blesbok (*Damaliscus pygargus phillipsi*), Ibex (*Capara sibirica*), Nyala (*Niyala anagasii*), Coquerel's sifaca (*Propithecus coquereli*), Large Japanese field mouse (*Apodemus speciosus*), Striped field mouse (*Apodemus agrarius*), Jeju striped field mouse (*Apodemus chejuensis*), Yak (*Bos grunniens*), Hedgehog (*Erinaceinae* spp.), Wombat (*Vombatidae* spp.), Red fox (*Vulpes vulpes*), Eastern grey squirrel (*Sciurus carolinensis*), Qinghai vole (*Microtus fuscus*)	Emerging human pathogen	XIIa, XIIb, XIIc, XIId, XIIe, XIIf	Perez and Le Blancq, 2001; da Silva et al., 2003; Ryan et al., 2003; Feng et al., 2007; Karanis et al., 2007; Ziegler et al., 2007; Cinque et al., 2008; Wang et al., 2008; Fayer et al., 2010; Feng, 2010, 2012; Robinson et al., 2010, 2011; Abu Samraa et al., 2013; Nolan et al., 2013; Murakoshi et al., 2013; Li et al., 2014; Ma et al., 2014; Perec-Matysiak et al., 2015; Qi et al., 2015a, 2015b; Song et al., 2015; Stenger et al., 2015b; Koehler et al., 2016; Li et al., 2016a, 2016b; Mateo et al., 2017; Prediger et al., 2017; Zhang et al., 2018
C. muris	Wild rats (*Rattus* sp.), Mice (*Mus* sp.), Greater bilblies (*Macroties lagotis*), Girrafes house mice (*Mus musculus*), Eastern grey squirrel (*Sciurus carolinensis*), Golden hamster (*Mesocricetus auratus*), Rock hyrax (*Procavia capensis*), Large footed mouse-eared bat (*Myotis adversus*), Japanese field mouse (*Apodemus argenteus*), Bilbies (*Macrotis lagotis*), Bank voles (*Clethrionomys glareolus*), Campbell hamster (*Phodopus campbelli*), Siberian hamster (*Phodopus sungorus*), Golden hamster (*Mesocricetus auratus*), Mountain goats (*Oreamnos americanus*), Cynomolgus monkeys (*Macaca fascicularis*), East African mole rat (*Tachyoryctes splendens*), Ringed seal (*Pusa hispida*), Donkey (*Giraffa camelopardalis*), Ringed seal (*Phoca hispida*), Large Japanese field mouse (*Apodemus speciosus*), Cynomolgus monkey (*Macaca fascicularis*), Slow loris (*Nycticebus coucang*), Ostriches (*Struthio camelus*), Mountain gorillas (*Gorilla beringei beringei*), Asian house rat	Numerous reports in humans	-	Morgan et al., 1999a; Dubey et al., 2002; Xiao et al., 2002a, 2004a; Warren et al., 2003; Nakai et al., 2004; Hikosaka and Nakai, 2005; Santin et al., 2005; Azami et al., 2007; Al-Brikan et al., 2008; Kváč et al., 2008; Lupo et al., 2008; Lv et al., 2009; Kodádková et al., 2010; Feng, 2010; Yang et al., 2011, 2013; Murakoshi et al., 2013; Ng-Hublin et al., 2013b; Karim et al., 2014, Qi et al., 2014; Sak et al., 2014; Du et al., 2015; Laatamna et al., 2015; Petrincová et al., 2015; Song et al., 2015; Wagnerová et al., 2015; Zhao et al., 2015b; Mynářová et al., 2016; Wait et al., 2017

	(*Rattus tanezumi*), Brown rat (*Rattus norvegicus*), House mouse (*Mus musculus*), horse (*Equus caballus*), Striped field mouse (*Apodemus agrarius*), Jeju striped field mouse (*Apodemus chejuensis*), Orangutans (*Pongo abelii* and *Pongo pygmaeus*), Tasmanian devil (*Sarcophilus harrisii*)			
C. andersoni	Bacterian camel (*Camelus bactrianus*), Dromedary camel (*Camelus dromedarius*), European wisent (*Bison bonasus*), Marmots Campbell hamster (*Phodopus campbelli*), Golden hamster (*Mesocricetus auratus*), Golden takins (*Budorcas taxicolor bedfordi*), Giant panda (*Ailuropoda melanoleuca*), Rhesus macaque (*Macaca mulatta*), American mink (*Mustela vison*), horse (*Equus caballus*), Yak (*Bos grunniens*)	Minor	-	Matsubayashi et al., 2005; Wang et al., 2008; Lv et al., 2009; Stuart et al., 2013; Du et al., 2015; Liu et al., 2015c; Wang et al., 2015; Zhao et al., 2015a; Gu et al., 2016; Li et al., 2016b; Deng et al., 2017
C. felis	Rhesus macaques (*Macaca mulatta*); Pallas's cat (Felis Manul), Red fox (*Vulpes vulpes*)	Numerous reports in humans	-	Lucio-Forster et al., 2010; Ye et al., 2012; Beser et al., 2015; Ebner et al., 2015; Li et al., 2015c; Mateo et al., 2017
C. canis dog genotype	Unidentified fox, Coyote (*Canis latrans*); American minks (*Mustela vison*)	Numerous reports in humans	-	Xiao et al., 2002a; Ryan et al., 2004; Zhou et al., 2004; Trout et al., 2006; Ziegler et al., 2007; Elwin et al., 2012b; Koompapong et al., 2014; Yang et al., 2018
C. canis fox genotype	Fox (*Vulpes vulpes*), Arctic foxes (*Vulpes lagopus*), raccoon dogs (*Nyctereutes procyonoides*), Minks (*Mustela vison*), Red fox (*Vulpes vulpes*), Mongoose (*Herpestes ichneumon*), Qinghai vole (*Microtus fuscus*)	No reports in humans to date	-	Zhou et al., 2004; Swaffer et al., 2014; Zhang et al., 2016a, 2016b; Mateo et al., 2017; Yang et al., 2018; Zhang et al., 2018
C. canis coyote genotype	Coyote (*Canis latrans*)	No reports in humans to date	-	Xiao et al., 2002a; Zhou et al., 2004
C. erinacei	European hedgehog (*Erinaceus europaeus*), Horses	One report in humans	XIIIaA19R12, XIIIaA19R13, XIIIa20R10, XIIIaA21R10, XIIIaA21R11, XIIIaA22R9, XIIIaA22R11	Meredith and Milne, 2009; Dyachenko et al., 2010; Laatamna et al., 2013; Nolan et al., 2013; Kváč et al., 2014a, 2014b; Hofmannová et al., 2016

C. fayeri	Southern brown bandicoot (*Isodon obesulus*), Western-barred bandicoot (*Permeles bougainville*), Koala (*Phascolarctos cincerus*), Red kangaroo (*Macropus rufus*), Eastern grey kangaroo (*Macropus giganteus*), Yellow footed rock wallaby (*Petrogale xanthopus*), Western grey kangaroo (*Macropus fuliginosus*), Tasmanian devil (*Sarcophilus harrisii*), Wombat (*Vombatidae* spp.), Short-beaked echidna (*Tachyglossus aculeatus*)	Minor	IVaA9G4T1R1, IVaA10, IVaA7, IVaA11G3R1, IVbA9G1T1, IVcA8G1T1, IVdA7G1T1, IVfA12G1T1, IVgA10G1T1R1	Power et al., 2005; Ryan et al., 2008; Yang et al., 2008, 2011; Power, 2010; Waldron et al., 2010; Feng et al., 2011b; Nolan et al., 2013; Swaffer et al., 2014;Vermeulen et al., 2015; Koehler et al., 2016; Šlapeta et al., 2017; Wait et al., 2017; Zahedi et al., 2018
Opossum genotype I *(C. fayeri)*	Opossum (*Didelphimorphia*)	No reports in humans to date	XIaA4G1T1	Feng et al., 2011b
Opossum genotype II	Virginia opossum (*Didelphis virginiae*)	No reports in humans to date	-	Xiao et al., 2002b; Oates et al., 2012
C. meleagridis	Mountain gorillas (*Gorilla beringei beringei*), Brush-tailed rock wallabies (*Petrogale penicillata*), Deer mouse (Peromyscus sp.), Minks (*Mustela vison*), Fox (*Vulpes vulpes*)	Major	IIIbA, IIIgA (closest match to IIIeA19G2R1)	Morgan et al., 2000; Cama et al., 2003; Gatei et al., 2006; Leoni et al., 2006; Muthusamy et al., 2006; Feng et al., 2007; Elwin et al., 2012b; Silverlås et al., 2012; Kurniawan et al., 2013; Adamu et al., 2014; Ghaffari and Kalantari, 2014; Rahmouni et al., 2014; Ryan and Xiao, 2014; Sak et al., 2014; Wang et al., 2014; Stensvold et al., 2015; Vermeulen et al., 2015; Zhang et al., 2016a; Yang et al., 2018
C. tyzerri	Mice (*Mus musculus*), Brown rats (*Rattus norvegicus*), Large-footed bat (*Myotus adversus*), Yellow-necked mouse (*Apodemus flavicollis*), Bank vole (*Myodes glareolus*), Common vole (*Microtus arvalis*), Red panda (*Ailurus fulgens*), Leopard (*Panthera pardus*), Takin (*Budorcas taxicolor*), Prairie bison (*Bison bison*), Lesser panda (*Ailurus fulgens*), Black leopards (*Pantera pardus*), Bobcats (*Lynx rufus*), Horse (*Equus caballus*)	Occasionally reported in humans	IXaA5R2, IXaA6R1, IXaA6R2, IXaA6R3, IXbA6, IXbA6R2, IXbA22R9	Morgan et al., 1999a, Xiao et al., 2002a; Bajer et al., 2003; Alves et al., 2005; Foo et al., 2007; Karanis et al., 2007; Ziegler et al., 2007; Lv et al., 2009; Feng et al., 2011b; Carver et al., 2012; Kváč et al., 2012; Ren et al., 2012; Rasková et al., 2013; Silva et al., 2013; Swaffer et al., 2014; Wagnerová et al., 2015

C. macropodum	Red kangaroo (*Macropus rufus*), Eastern grey kangaroo (*Macropus giganteus*), Swamp wallaby (*Wallabia bicolor*), Western grey kangaroos (*Macropus fuliginosus*)	No reports in humans to date	-	Power et al., 2004, 2005; Power and Ryan, 2008; Power, 2010; Yang et al., 2011; Nolan et al., 2013; Koehler et al., 2016; Zahedi et al., 2016b
C. macropodum-like	Wallaby (*Macropus* spp.)	No reports in humans to date	-	Koehler et al., 2016
C. bovis	Yaks, foxes, Gorillas (single report), Roe deer (*Capreolus capreolus*), Yak (*Bos grunniens*)	Occasionally reported in humans	-	Robinson et al., 2011; Helmy et al., 2013; García-Presedo et al., 2013b; Sak et al., 2013; Qin et al., 2014; Qi et al., 2015b; Li et al., 2016b
C. ryanae	Roe deer (*Capreolus capreolus*), Water buffaloes (*Bubalus bubalis*), Yak (*Bos grunniens*)	No reports in humans to date	-	Feng et al., 2012; García-Presedo et al., 2013b; Li et al., 2016b
C. ryanae-like	Deer (*Cervidae* spp.)	No reports in humans to date	-	Koehler et al., 2016
C. wrairi	Guinea pig (*Cavia porcellus*), California ground squirrels (*Spermophilus beecheyi*)	No reports in humans to date	VIIaA13T1, VIIaA17T1, VIIaA16T1	Atwill et al., 2004; Feng et al., 2007, 2011b, Paziewska et al., 2007; Lv et al., 2009
C. scrofarum	Asian house rat (*Rattus tanezumi*), Brown rat (*Rattus norvegicus*), Eurasian wild boars (*Sus scrofa*), Eurasian wild boars (*Sus scrofa*), Striped field mouse (*Apodemus agrarius*), Yellow-necked mouse (*Apodemus flavicollis*), Common shrew (*Sorex araneus*)	Occasionally reported in humans	-	Castro-Hermida et al., 2011; García-Presedo et al 2013a; Němejc et al., 2013; Ng-Hublin et al., 2013b, Bodager et al., 2015; Parsons et al., 2015; Rodriguez-Rivera et al., 2016; Danišová et al., 2017; Li et al., 2017
C. suis	Chimpanzees (*Pan troglodytes schweinfurthii*), Eurasian wild boars (*Sus scrofa*), Rodents, Yellow-necked mouse (*Apodemus flavicollis*)	Occasionally reported in humans	-	Paziewska et al., 2007; Castro-Hermida et al., 2011; Němejc et al., 2012, 2013; García-Presedo et al., 2013a; Bodager et al., 2015; Parsons et al., 2015; Rodriguez-Rivera et al., 2016; Danišová et al., 2017
C. occultus	Asian house rat (*Rattus tanezumi*), Yak (*Bos grunniens*), Brown rat (*Rattus norvegicus*)	No reports in humans to date	-	Ong et al., 2002; Ng-Hublin et al., 2013b; Li et al., 2016b; Kváč et al., 2018

C. rubeyi	California ground squirrel (*Otospermophilus. beecheyi*), Belding's ground squirrel (*Urocitellus. beldingi*), Golden Mantled ground squirrel (*Callospermophilus. lateralis*)	No reports in humans to date	-	Pereira et al., 2010; Li et al., 2015a
C. viatorum	Australian swamp rat (*Rattus lutreolus*)	reported in humans	XVbA2G1	Koehler et al., 2018
C. occultus	Brown rat (*Rattus norvegicus*)	No reports in humans to date	-	Kváč et al., 2018
Bear genotype	Black bear (*Ursus americanus*)	No reports in humans to date	-	Xiao et al., 2000
Bat genotype I	Chinese rufous horseshoe bat (*Rhinolophus sinicus*), Stoliczka's trident bat (*Aselliscus stoliczkanus*)	No reports in humans to date	-	Wang et al., 2013b
Bat genotype II	Chinese rufous horseshoe bat (*Rhinolophus sinicus*), Fulvus roundleaf bat (*Hipposideros fulvus*), Leschenault's rousette (*Rousettus leschenaultii*)	No reports in humans to date	-	Wang et al., 2013b
Bat genotype III	Big brow bat (*Eptesicus fuscus*)	No reports in humans to date	-	Kváč et al., 2015
Bat genotype IV	Western barbastelle (*Barbastella barbastellus*)	No reports in humans to date	-	Kváč et al., 2015
Bat genotype V	Lesser short-nosed fruit bat (*Cynopterus brachyotis*)	No reports in humans to date	-	Murakoshi et al., 2016
Bat genotype VI	Cave nectar bat (*Eonycteris spelaean*)	No reports in humans to date	-	Murakoshi et al., 2016
Bat genotype VII	Philippine forest horseshoe bat (*Rhinolophus inops*)	No reports in humans to date	-	Murakoshi et al., 2016
Bat genotype VIII	flying fox (*Pteropus poliocephalus*)	No reports in humans to date	-	Schiller et al., 2016
Bat genotypeIX	flying fox (*Pteropus poliocephalus*)	No reports in humans to date	-	Schiller et al., 2016
Bat genotype X	flying fox (*Pteropus poliocephalus*)	No reports in humans to date	-	Schiller et al., 2016
Bat genotype XI	flying fox (*Pteropus poliocephalus*)	No reports in humans to date	-	Schiller et al., 2016
Beaver genotype	North American beaver (*Castor canadensis*)	No reports in humans to date	-	Feng et al., 2007
Brushtail possum I	Brushtail possum (*Trichasuris vulpecula*)	No reports in humans to date	-	Hill et al., 2008; Barbosa et al., 2017

Chipmunk genotype I	Chipmunk sp. (*Tamias* sp.), Eastern grey squirrel (*Sciurus carolinensis*), Deer mice (*Peromyscus maniculatus*), Striped field mouse (*Apodemus agrarius*), Pallas's squirrel (*Callosciurus erythraeus*)	Emerging human pathogen	XIVa16G2T2, XIVaA18G2T1, XIVaA18G2T2	Jiang et al., 2005; Feltus et al., 2006; Feng et al., 2007; ANOFEL, 2010; Insulander et al., 2013; Lebbad et al., 2013; Guo et al., 2015; Song et al., 2015; Prediger et al., 2017
Chipmunk genotype II	Eastern chipmunk (*Ramias striatus*)	No reports in humans to date	-	Feng et al., 2007; Stenger et al., 2015b
Chipmunk genotype III	Siberian chipmunk (*Tamias sibiricus*)	No reports in humans to date	-	Lv et al., 2009
Deer genotype	White–tailed deer (*Odocoileus virginianus*), Sika deer (*Cervus nippon*), Roe deer (*Capreolus capreolus*), Red deer (*Cervus elaphus*), Hokkaido sika deer (*Cervus nippon yesoensis*)	No reports in humans to date	-	Xiao et al. 2002b; Feng et al. 2007; Jellison et al. 2009; Robinson et al., 2011; Santin and Fayer 2015; Wells et al. 2015; Kato et al., 2016; Koehler et al., 2016
Deer mouse genotype I	Deer mouse (*Peromyscus*)	No reports in humans to date	-	Xiao et al., 2002b; Feng et al., 2007, 2011b
Deer mouse genotype II	Deer mouse (*Peromyscus*)	No reports in humans to date	-	Xiao et al., 2002b; Feng et al., 2007
Deer mouse genotype III	Deer mouse (*Peromyscus*)	No reports in humans to date	-	Feng et al., 2007; Stenger et al., 2015b
Deer mouse genotype IV	Deer mouse (*Peromyscus*)	No reports in humans to date	-	Feng et al., 2007
Ferret genotype	Ferret (*Mustelidae*), Siberian chipmunk (*Tamias sibiricus*), River otters (*Lontra canadensis*), Black-footed ferret (*Mustela nigripes*), Red squirrel (*Sciurus vulgaris*), Chipmunk sp (*Tamias* sp.), Eurasian red squirrel (*Sciurus vulgaris*)	No reports in humans to date	VIIIaA5G2, VIIIbA9G1R1, VIIIbA11G1R1, VIIIcA12G2R1, VIIIdA16G1R1	Xiao et al., 2002a; Abe and Iseki, 2003; Gaydos et al., 2007; Kváč et al., 2008; Lv et al., 2009; Feng et al., 2011b; Li et al., 2016a; Prediger et al., 2017
Giant panda genotype	Giant panda (*Ailuropoda melanoleuca*)	No reports in humans to date	-	Liu et al., 2013
Squirrel genotypes I-III	Golden-mantled ground squirrels (*Callospermophilus lateralis*), Belding's ground squirrels (*Urocitellus beldingi*), California ground squirrels (*Otospermophilus beecheyi*), Blacktailed prairie dog (*Cynomys ludovicianus*)	No reports in humans to date	-	Atwill et al., 2004; Pereira et al., 2010; Stenger et al., 2015b
Hamster genotype	Siberian hamster (*Phodopus sungorus*)	No reports in humans to date	-	Lv et al., 2009

Horse genotype	Przewalski's wild horse (*Equus przewalski*), Four-toed hedgehog (*Atelerix albiventris*)	Identified in humans in the UK and New Mexico	VIaA11G3, VIaA15G4, VIbA13	Ryan et al., 2003; Robinson et al., 2008; Xiao et al., 2009; Abe and Matsubara, 2015; Wagnerová etal., 2015
Kangaroo genotype I	Eastern grey kangaroo (*Macropus giganteus*)	No reports in humans to date	-	Yang et al., 2011; Koehler et al., 2016
Mink genotype	River otter (*Lontra canadensis*), American minks (*Mustela vison*), Stoat (*Mustela ermine*), Raccoon dog (*Nyctereutes procyonoides*)	Several reports in humans	XaA5G1, XbA5G1R1, XcA5G1R1, XdA4G1	Feng et al., 2007, Wang et al., 2008; Feng et al., 2011b; Ng-Hublin et al., 2013b; Stuart et al., 2013; Ebner et al., 2015; Zhang et al., 2016a; Yang et al., 2018
Monkey genotype	Rhesus macaques (*Macaca mulatta*), Long-tailed macaques (*Macaca fascicularis*)	No reports in humans to date	-	Xiao et al., 1999a; Sricharern et al., 2016
Mouse genotype II	House mouse (*Mus musculus*)	No reports in humans to date	-	Foo et al., 2007; Silva et al., 2013
Mouse genotype III	House mouse (*Mus musculus*)	No reports in humans to date	-	Silva et al., 2013
Muskrat genotype I	Muskrat (*Ondatra zibethicus*), Boreal red-backed vole (*Myodes rutilus*), Bank vole (*Myodes glareolus*)	No reports in humans to date	-	Xiao et al., 2002a; Zhou et al., 2004; Feng et al., 2007; Danišová et al., 2017
Muskrat genotype II	Muskrat (*Ondatra zibethicus*), Red fox (*Vulpus vulpus*), Deer mouse (*Peromyscus maniculatus*), Meadow vole (*Microtus pennsylvanicus*), Striped field mouse (*Apodemus agrarius*)	No reports in humans to date	-	Ziegler et al., 2007; Robinson et al., 2011; Danišová et al., 2017
Naruko genotype	Large Japanese field mouse (*Apodemus speciosus*)	No reports in humans to date	-	Murakoshi et al., 2013
Pika genotype	Wild plateau pika (*Ochotona curzoniae*)	No reports in humans to date	-	Zhang et al., 2018
Qinghai vole genotype	Qinghai vole (*Microtus fuscus*)	No reports in humans to date	-	Zhang et al., 2018
Rat genotype I	Brown rat (*Rattus norvegicus*)	No reports in humans to date	-	Ng-Hublin et al., 2013b
Rat genotype II	Asian house rat (*Rattus tanezum*),Wild black rat (*Rattus rattus*), Brown rat (*Rattus norvegicus*)	No reports in humans to date	-	Lv et al., 2009; Ng-Hublin et al., 2013b; Silva et al., 2013
Rat genotype III	Asian house rat (*Rattus tanezumi*), Wild black rat *(Rattus rattus*)	No reports in humans to date	-	Lv et al., 2009; Paparini et al., 2012; Ng-Hublin et al., 2013b; Silva et al., 2013

Rat genotype IV	Tanezumi rat (*Rattus tanezumi*), Asian house rat (*Rattus tanezumi*), Brown rat (*Rattus norvegicus*)	No reports in humans to date	-	Ng-Hublin et al., 2013b
Seal genotypes I and II	Ringed seals (*Phoca hispida*), Harbor seals (*Phoca vituline*), Hooded seal (*Cystophora cristata*)	No reports in humans to date	-	Santín et al., 2005; Bass et al., 2012
Seal genotype III	Harp seal (*Pagophilus groenlandicus*)	No reports in humans to date	-	Bass et al., 2012
Seal genotype IV (similar to skunk genotype)	Southern elephant seal (*Mirounga leonina*)	No reports in humans to date	-	Rengifo-Herrera et al. 2011, 2013
Seal genotype V (Weddell seal genotype)	Weddel seal (*Leptonychotes weddellii*)	No reports in humans to date	-	Rengifo-Herrera et al., 2013
Skunk/ skunk-like genotype	Striped skunk (*Mephitis mephitis*), Raccoon (*Procyon lotor*), Eastern grey squirrel (*Sciurus carolinensis*), River otter (*Lontra canadensis*), Raccoon (*Procyon lotor*), Southern elephant seal (*Mirounga leonina*), Skunk (*Mephitis mephitis*), American red (*Tamiasciurus hudsonicus*), Fox squirrel (*Sciurus niger*)	Has been reported in humans	XVIaA14R1, XVIbA16G2a, XVIcA10, XVIcA23	Xiao et al., 2002b; Zhou et al., 2004; Feng et al., 2007; Ziegler et al., 2007; Robinson et al., 2008; Chalmers et al., 2009; Feng et al., 2011b; Rengifo-Herrera et al., 2011; Elwin et al., 2012b; Stenger et al., 2015b; Leśniańska et al., 2016; Prediger et al., 2017
Tasmanian devil genotype	Tasmanian devil (*Sarcophilus harrisii*)	No reports in humans to date	-	Wait et al., 2017; Yan et al., 2017
Vole genotype	Meadow vole (*Microtus pennsylvanicus*)	No reports in humans to date	-	Feng et al., 2007
Wildbeast genotype	Black wildbeast (*Connochaetos*)	No reports in humans to date	-	Alves et al., 2005
Novel genotype (closely related to bear genotype)	Striped field mouse (*Apodemus agrarius*), Jeju striped field mouse (*Apodemus chejuensis*)	No reports in humans to date	-	Song et al., 2015

1.3.12 *Cryptosporidium* in birds

The mobility of migratory birds, together with their distribution and ability to form large colonies, makes them potentially suitable to spread pathogens. Due to their easy access to drinking water catchments and other water sources, wild birds are believed to be a potential risk to drinking water safety. The epidemiology of avian cryptosporidiosis, in particular zoonotic *Cryptosporidium* species infecting birds is therefore of public health importance. Currently four avian *Cryptosporidium* spp. are recognised; *C. meleagridis* (discussed in section 1.3.10), *C. baileyi, C. galli* and *C. avium* (Table 1.3) (Ryan and Xiao, 2014; Holubová et al., 2016). Of these four species, only *C. meleagridis* is considered of zoonotic importance. In addition to *C. meleagridis*, other zoonotic species/genotypes of *Cryptosporidium* reported in birds include *C. hominis*, *C. parvum*, *C. muris*, *C. canis*, *C. ubiquitum* and *C. andersoni*, and the *Cryptosporidium* ferret genotype (Zylan et al., 2008; Jellison et al., 2009; Ryan, 2010; Gomes et al., 2012; Reboredo-Fernandez et al., 2015; Koehler et al., 2016; Li et al., 2016a; Helmy et al., 2017; Osman et al., 2017, Azmanis et al., 2018). In addition, eighteen genotypes; avian genotypes I-IV and VI to IX, the black duck genotype, the Eurasian woodcock genotype, finch genotypes I-III and goose genotypes I-V have been reported (Table 1.3). To date, there is no evidence of human cryptosporidiosis caused by these genotypes.

Cryptosporidium baileyi is generally associated with the respiratory form of cryptosporidiosis in birds and has been predominantly reported in broiler chickens. Compared to *C. meleagridis*, *C. baileyi* is capable of infecting a larger spectrum of avian hosts (Table 1.3), targeting various sites of infection mostly associated with the digestive and respiratory tracts (Ryan and Xiao, 2014; Máca and Pavlásek, 2016; Nakagun et al., 2017; Xiang et al., 2017). Experimental cross-transmission of *C. baileyi* to other birds has been successful, however there has been no reports of cross-transmission between birds and other vertebrates

(Lindsay and Blagburn, 1990; Cardozo et al., 2005), except for a single unsubstantiated report of human infection with *C. baileyi*, which did not include any molecular analysis (Ditrich et al., 1991). Therefore, *C. baileyi* is not considered to be of public health significance.

Unlike other avian species, *C. galli* is a gastric species with endogenous developmental stages occurring in the glandular epithelial cells of the proventriculus (Pavlásek, 1999; 2001; Ryan et al., 2003; Ng et al., 2006; Ryan and Xiao, 2014; Máca and Pavlásek, 2016; Osman et al., 2017). It predominantly infects birds of the family Spermestidae, Fringilidiae and domestic chickens (*Gallus gallus*), and seems to be more prevalent among songbirds (Table 1.3). Successful experimental cross-transmission of *C. galli* to other chickens has been reported, however the full extent of its host range is still unknown (Ryan, 2010). It has not been reported in humans.

The most recently described avian species is *C. avium* (formerly avian genotype V) which infects the ileum, caecum, kidney, ureter, and cloaca of avian hosts (Holubová et al., 2016). First reported in cockatiels (*Nymphicus hollandicus*) in Japan (Abe and Makino, 2010) and subsequently in many other bird hosts (Table 1.3), there is no evidence that *C. avium* infects humans. There is a single report of *C. avium* from green iguanas (*Iguana inguana*) (Kik et al., 2011).

Table 1.3. *Cryptosporidium* species and genotypes in avian hosts confirmed by molecular analysis (Modified from Ryan and Xiao, 2014).

Species name	Major host(s)	Site of infection	References
C. avium (Previously avian genotype V)	Red-crowned parakeet (*Cyanoramphus novaezelandiae*), Rosy-faced lovebird (*Agapornis roseicollis*), Chickens (*Gallus gallus*), Blue-fronted Amazon (*Amazona aestiva*), Major Mitchell's cockatoo (*Lophochroa leadbeateri*), Cockatiel (*Nymphicus hollandicus*), Budgerigar (*Melopsittacus undulates*); Fischer's lovebird (*Agapornis fischeri*).	-	Abe and Makino, 2010; Qi et al., 2011; Zhang et al., 2015; Holubová et al., 2016; Li et al., 2016a
C. baileyi	Turkey (Meleagris gallopavo), Chicken (*Gallus gallus*), Brown squail (*Synoicus australis*), Cocktails (*Nymphicus hollandicus*), Whooping crane (*Grus vipio*), Grey-bellied bulbul (*Pycnonotus* spp.), Black vulture (*Coragyps atratus*), Saffron finch (*Sicalis flaveola*), Mixed-bred falcons (*Falco rusticolus Falco cherrug*), Ruddy Shelduck (*Tadorna Paroaria ferruginea*), Red-billed leiothrixes (*Leiothrix lutea*), Pekin ducks (*Anas platyrhynchos*), Buffy-fronted seedeater (*Sporophila frontalis*), Java sparrows (*Padda oryzivora*), Mynas (*Acridotheres tristis*), Zebra finches (*Taeniopygia guttata*), Crested Lark (*Galerida cristana*), Gouldian finch (*Chloebia gouldiae*), Black-billed magpie (*Pica pica*), Ostriches (*Struthio camelus*), Quails (*Coturnixcoturnix japonica*), Red grouse (*Lagopus lagopus scotica*), Red-crowned crane (*Grus japonenis*), Snowy owl (*Bubo scandiacus*), Ring-necked pheasants (*Phasianus colchicus*), scaled quail (*Callipepla squamata*), Atlantic canary (*Serinus canaria*), Budgerigar (*Melopsittacus undulates*), Crested myna (*Acridotheres cristatellus*), Rock dove (*Columba livia*), Swan goose (*Anser cygnoides*)	Cloaca, bursa, trachea	Morgan et al., 2001; Abe and Iseki, 2004; Kimura et al., 2004; Ng et al., 2006; Huber et al., 2007; Nakamura et al., 2009; Abe and Makino, 2010; Wang et al., 2010; Qi et al., 2011; Wang et al., 2012; Baroudi, et al., 2013; Baines et al., 2014; Hamidinejat et al., 2014; Wang et al., 2014; Li et al., 2015c, 2016a; Máca and Pavlásek, 2015, 2016; da Cunha et al., 2017; Helmy et al., 2017; Nakagun et al., 2017; Xiang et al., 2017
C. galli	Chicken (*Gallus gallus*), Finches (*Spermestidae* and *Fringillidae*), Capercaille (*Tetrao urogallu*), Pine grosbeak (*Pinicola enuncleator*), Turqoise parrots (*Neophema pulchella*), Cuban flamingo (*Phoenicopterus ruber ruber*), Rhinoceros hornbill (*Buceros rhinoceros*), Red-cowled cardinal (*Paroaria dominicana*), Zebra finches (*Taeniopygia guttata*), Chocolate parson finches (*Peophila cincta*), Chesnut finches (*Lonchura castaneothorax*), Painted firetail finches (*Ebmlema picta*), Canaries (*Serinus* sp.), Glosters (*Serinus canaria*), Green-winged saltatros (*Saltator similis*), Slate-	Preventriculus	Ryan et al., 2003; Ng et al., 2006; Antunes et al., 2008; Nakamura et al., 2009; da Silva et al., 2010; Qi et al., 2011; Nakamura et al., 2014; Chelladurai et al., 2016; Li et al., 2016a; Máca and Pavlásek, 2016; Osman et al., 2017

	collard seedeater (*Sporophila schistaceca*), Great-billed seed finch (*Oryzoborus maximiliani*), Ultramarine grosbeak (*Cyanocompsa brissonii*), Bohemian waxwing (*Bombycilla garrulous*), Silver-eared Mesia (*Leiothrix argentauris*), Cockatiel (*Nymphicus hollandicus*), Chopi blackbird (*Gnorimopsar chopi*), Green-winged saltator (*Saltator similis*), Rufous-collared sparrow (*Zonotrichia capensis*), Rosella (*Platycercus eximus*), Ring-necked pheasants (*Phasianus colchicus*), American red-winged blackbirds (*Agelaius phoeniceus*), Chinese hwamei (*Garrulax canorus*)		
C. meleagridis	Turkey (*Meleagris gallopavo*), Indian ring-necked parrot (*Psittacula kameri*), Red-legged partridge (*Alectoris rufa*), Cocktails (*Nymphicus hollandicus*), Bohemian waxwing (*Bombycilla garrulous*), Rufous turtle dove (*Streptopelia orientalis*), Fan-tailed pigeon (*Columba livia*), Chicken (*Gallus gallus*), Quails (*Coturnixcoturnix japonica*), Pekin ducks (*Anas platyrhynchos*), Domestic Pigeons (*Columba livia domestica*), European turtle dove (*Streptopelia turtur*), Red-legged partridge (*Alectoris rufa*), Ring-necked pheasants (*Phasianus colchicus*)	Intestine	Morgan et al., 2000; Glaberman et al., 2001; Abe and Iseki, 2004; Abe and Makino 2010; Wang et al., 2010b; Qi et al., 2011; Berrilli et al., 2012; Wang et al., 2012; Baroudi, et al., 2013; Wang et al., 2014; Koompapong et al., 2014; Máca and Pavlásek, 2015; Reboredo-Fernandez et al., 2015; Máca and Pavlásek, 2016; Zahedi et al., 2018
Avian genotype I	Red factor canary (*Serinus canaria*), Canary (*S. canaria*), Indian peafowl (*Pavo cristatus*)	-	Ng et al., 2006; Nakamura et al., 2009
Avian genotype II	Eclectus (*Eclectus roratus*), Galah (*Eolophus roseicapilla*), Cockatiel (*Nymphicus hollandicus*), Major Mitchel Cockatoo (*Cavcatua lead beater*), Ostriches (*Struthio camelus*), White-eyed parakeet (*Aratinga leucophthalma*)	-	Meireles et al., 2006; Ng et al., 2006; Nakamura et al., 2009; Seva-Ada et al., 2011; Nguyen et al., 2013
Avian genotype III	Galah (*Eolophus roseicapilla*), Cockatiel (*Nymphicus hollandicus*), Java sparrow (*Padda oryzivora*), Son conure (*Aratinga solstitialis*), Peach-faced lovebirds (*Agapornis roseicollis*), Seagull (*Larida*e sp), Blue-fronted amazon (*Amazona aestival*), Cockatiel (*Nymphicus hollandicus*), Rufous-collared sparrow (*Zonotrichia capensis*), Lovebird (*Agapornis species*), Toco toucan (*Ramphastus toco*), White-throated toucan (*Ramphastus Tucanus*), Saffron toucanet (*Pteroglossus bailloni*)	-	Ng et al., 2006; Nakamura et al., 2009; Makino et al., 2010; Gomes et al., 2012; Koompapong et al., 2014; Nakamura et al., 2014; Ravich et al., 2014; Li et al., 2015c; Cano et al., 2016; Silva Novaes et al., 2018
Avian genotype IV	Japanese white-eye (*Zosterops japonica)*	-	Abe and Makino, 2010; Qi et al., 2011
Avian genotype VI	American red-winged blackbirds (*Agelaius phoeniceus*)	-	Chelladurai et al., 2016
Avian genotype VII	Turkey (*Meleagris gallopavo*), Chicken (*Gallus gallus*)	-	Helmy et al., 2017

Avian genotype VIII	Turkey (*Meleagris gallopavo*), Chicken (*Gallus gallus*)	-	Helmy et al., 2017
Avian genotype IX	Turkey (*Meleagris gallopavo*), Chicken (*Gallus gallus*)	-	Helmy et al., 2017
Duck genotype	Black duck (*Anus rubripes*), Canada geese (*Branta Canadensis*), Mandarin duck (*Aix galericulata*)	-	Jellsison et al., 2004; Zhou et al., 2004; Cano et al., 2016; da Cunha et al., 2017
Eurasian woodcock genotype	Eurasian woodcock (*Scolopax rusticola*)	-	Ryan et al., 2003; Ng et al., 2006
Finch genotype I	Gouldian finch (*Erythrura gouldiae*)	-	Morgan et al., 2001
Finch genotype II	Red-winged pytilia (*Pytilia phoenicoptera*)	-	Morgan et al., 2001
Finch genotype III	Red-winged pytilia (*Pytilia phoenicoptera*)	-	Morgan et al., 2001
Goose genotype I	Canada geese (*Branta canadensis*)	-	Xiao et al., 2002b; Jellison et al., 2004; Zhou et al., 2004
Goose genotype II	Canada geese (*Branta canadensis*)	-	Jellison et al., 2004; Zhou et al., 2004
Goose genotype III	Canada geese (*Branta canadensis*)	-	Jellison et al., 2004
Goose genotype IV	Canada geese (*Branta canadensis*)	-	Jellison et al., 2004; Cano et al., 2016
Goose genotype V	Canada geese (*Branta canadensis*)	-	Jellison et al., 2004

1.3.13 *Cryptosporidium* in fish

Cryptosporidium has been described in both fresh and marine water piscine species with parasitic stages located either on the stomach or intestinal surface, or deep within the epithelium (Table 1.4). The first account of *Cryptosporidium* in a piscine host was *C. nasorum*, identified in a Naso tang, a tropical fish species (Hoover et al., 1981). However, currently only three species are recognized; *C. molnari*, *C. scophthalmi* and *C. huwi* (previously known as piscine genotype I) (Alvarez-Pellitero and Sitja-Bobadilla, 2002; Alvarez-Pellitero et al., 2004; Palenzuela et al., 2010; Ryan et al., 2015), none of which have been reported in humans. In fish hosts, *Cryptosporidium* fish species and genotypes are typically located either in the stomach or intestine and the parasite can cause clinical manifestations, such as emaciation, decrease in growth rate, anorexia, whitish faeces, abdominal swelling, and ascites (Alvarez-Pellitero et al., 2004; Ryan et al., 2015). Most studies on *Cryptosporidium* in fish have been reported in farmed or aquarium fish (Table 1.4) and little data are currently available regarding the molecular identification of *Cryptosporidium* species and genotypes in wild fish populations and, in particular, in edible fish (Palenzuela et al., 2010; Reid et al., 2010; Barugahare et al., 2011; Gibson-Keuh et al., 2011; Koinari et al., 2013; Certad et al., 2015).

In addition to the three recognised species of *Cryptosporidium* in piscine hosts, numerous *Cryptosporidium* species and genotypes have been reported in fish including; piscine genotypes 2 to 8, unnamed novel genotypes (n = 6), rat genotype III, *C. parvum*, *C. hominis*, *C. xiaoi* and *C. scrofarum* (Table 1.4). Of these, only *C. parvum*, *C. hominis* and *C. scrofarum* are of public health interest. *Cryptosporidium scrofarum* was identified in a whiting (Reid et al., 2010); *C. parvum* was found in School whiting, Nile tilapias, a Silver barb, Arctic char and European whitefish and *C. hominis* was reported in Mackerel scad (Reid et al., 2010; Gibson-Kueh et al., 2011; Koinari et al., 2013; Certad et al., 2015). In one of the most recent studies,

C. parvum was identified in freshwater fish from Lake Geneva (Lac Leman) by both histology and molecular analysis (Certad et al., 2015). In that study, the overall prevalence of *Cryptosporidium* was 36.6% (15/41); the prevalence of *C. parvum* and *C. molnari* was 86.7% (13/15) and 6.7% (1/15), respectively, while 6.7% (1/15) were mixed *C. parvum* and *C. molnari* infections (Certad et al., 2015). Histological analysis identified *C. parvum* developmental stages in the stomach and intestine suggesting that *C. parvum* was infecting the fish, rather than being passively carried, which has important public health implications.

Subtyping of *Cryptosporidium* isolates in fish has identified *C. parvum* subtype IIaA18G3R1 in School whiting from Australia (Reid et al., 2010), three *C. parvum* subtypes (IIaA14G2R1, IIaA15G2R1 and IIaA19G4R1) in Nile tilapia, silver barb and mackerel scad and a *C. hominis* subtype (IdA15G1) in mackerel scad in Papua New Guinea (Koinari et al., 2013), and *C. parvum* subtypes IIaA15G2R1, IIaA16G2R1 and IIaA17G2R1 in Arctic char and European whitefish from France (Certad et al., 2015). All of these *C. parvum* subtypes are zoonotic and are commonly found in cattle and humans (Xiao, 2010). The identification of the *C. hominis* subtype probably reflects human sewage contamination of the water. Clearly further studies in this area are required to better understand the transmission dynamics of *Cryptosporidium* in fish.

Table 1.4. *Cryptosporidium* species reported in fish using molecular tools (Modified from Ryan et al., 2014).

Species	Host	Site of Infection	Reference
C. huwi (previously piscine genotype 1)	Guppy (*Poecilia reticulata*)	Stomach	Ryan et al., 2004; Ryan et al., 2015
C. molnari	Gilthead sea bream (*Sparus aurata*), European sea bass (*Dicentrarchus labrax*), Murray cod (*Maccullochella peelii peelii*)	Stomach (and intestine)	Palenzuela et al., 2010; Barugahare et al., 2011; Certad et al., 2015
C. scophthalmi	Turbot (*Scophthalmus maximus*)	Intestine	Alvarez-Pellitero et al., 2004; Unpublished (Acc. No. KR340588, KR340589)
Piscine genotype 2	Angelfish (*Pterophyllum scalare*)	Stomach	Murphy et al., 2009
Piscine genotype 3	Mullet (*Mugil cephalus*)	Intestine	Reid et al., 2010
Piscine genotype 4	Golden algae eater (*Crossocheilus aymonieri*), Kupang damsel (*Chrysiptera hemicyanes*), Oscar fish (*Astronatus ocellatis*), Neon tetra (*Paracheirodon innesi*)	Intestine	Reid et al., 2010; Morine et al., 2012
Piscine genotype 5	Angelfish (*Pterophyllum scalare*), Butter bream (*Monodactylidae*), Golden algae eater (*Crossocheilus aymonieri*)	-	Zanguee et al., 2010
Piscine genotype 6, piscine genotype 6-like	Guppy (*Poecilia reticulata*), Gourami (*Trichogaster trichopterus*)	-	Zanguee et al., 2010; Morine et al., 2012
Piscine genotype 7	Red eye tetra (*Moenkhausia sanctaefilomenae*)	-	Morine et al., 2012
Piscine genotype 8	Oblong silver biddy (*Gerres oblongus*)	-	Koinari et al., 2013
Rat genotype III, *C. hominis, C. parvum, C. xiaoi* and *C. scrofarum*	Whiting (*Sillago vittata*), Barramundi (*Lates calcarifer*), Arctic char (*Salvelinus alpinus*), Nile tilapias (*Oreochromis niloticus*), Silver barb (*Puntius gonionotus*), Mackerel scad (*Decapterus macarellus*), European whitefish (*Coregonus lavaretus*), School whiting (*Sillago vittata*)	-	Reid et al., 2010; Gibson-Kueh et al., 2011; Koinari et al., 2013; Certad et al., 2015
Novel un-named genotypes (*n*=5)	Orange clownfish (*Amphiprion percula)*, Azure damsel (*Chrysiptera hemicyanea*), Blue tang (*Paracanthurus hepatus*), Platyfish (*Xiphophorus maculatus*), Oscar (*Astronotus ocellatus)*, Goldfish (*Carassius auratus)*	-	Yang et al., 2015b
Novel genotype	Male Koi carp (*Cyprinus carpio*)		Yang et al., 2016b

1.3.14 *Cryptosporidium* in amphibians and reptiles

Little is known about *Cryptosporidium* species infecting amphibians. Of the three orders of amphibians; *Anura*, *Caudata* and *Gymnophonia*, *Cryptosporidium* has been only reported in *Anura* which includes frogs and toads and only one species, *C. fragile* is recognised (Table 1.5) (Jirků et al., 2008). In transmission experiments, *C. fragile* was not infective in one fish species (*Poecilia reticulate*), four amphibian species (*Bufo bufo*, *Rana temporaria*, *Litoria caerulea* and *Xenopus laevis*), one species of reptile (*Pantherophis guttatus*) and SCID mice (Jirků et al., 2008). This species has not been reported in humans.

Cryptosporidium infections are ubiquitous in reptiles and have been reported in more than 57 reptilian species (O'Donoghue, 1995; Ryan and Xiao, 2014). Unlike in other animals in which *Cryptosporidium* infection is usually self-limiting in immunocompetent individuals, cryptosporidiosis in reptiles is frequently chronic and sometimes lethal in some snakes. Both intestinal and gastric cryptosporidiosis have been described in snakes and lizards. To date, four species are recognised; *C. serpentis*, *C. varanii* (previously *C. saurophilum*), *C. testudinis* (previousely tortoise genotype I) and *C. ducismarci* (previously tortoise genotype II) (Levine, 1980; Pavlásek et al., 1995; Koudela and Modry, 1998; Pavlásek and Ryan, 2008; Traversa, 2010; Jezkova et al., 2016); none of which have been reported in humans, but *C. serpentis* has been identified in cattle (Azami et al., 2007; Chen and Qiu, 2012).

Cryptosporidium parvum, *C. muris*, *C. andersoni* and *Cryptosporidium tyzzeri* are also commonly reported in reptiles, particularly snakes but this is thought to be due to mechanical transmission due to predation of infected rodents and is not thought to present a substantial zoonotic risk (Morgan et al., 1999b; Xiao et al., 2004b; Pedraza-Diaz et al., 2009; Díaz et al., 2013; da Silva et al., 2014; Yimming et al., 2016; Osman et al., 2017). In addition, various host-adapted genotypes have been identified including tortoise genotype III and snake

genotypes I and II (cf. Ryan and Xiao, 2014), which have not been reported in humans (Table 1.5) (Xiao et al., 2004b; Pedraza-Diaz et al., 2009; Traversa, 2010; Richter et al., 2011; Seva-Ada et al., 2011; Rinaldi et al., 2012; da Silva et al., 2014; Abe and Matsubara, 2015; Jezkova et al., 2016).

Table 1.5. Amphibian and reptile *Cryptosporidium* species and genotypes and their hosts confirmed by molecular analyses (modified from Ryan et al., 2014).

Species/genotype	Amphibian/Reptile Host species	Site of infection	Reference
C. ducismarci (previously Tortoise genotype II)	Marginated tortoise (*Testudo marginata*), Ball python (*Python regius*), Veiled chameleon (*Chamaeleo calyptratus*), Pancake tortoise (*Malacochersus* tornieri), Russian tortoise (*Agrionemys* [*Testudo*] *horsfieldii*),	Intestine	Alves et al., 2005; Traversa et al., 2008; Pedraza-Díaz et al., 2009; Griffin et al., 2010; Traversa, 2010; Richter et al., 2012; Jezkova et al., 2016
C. fragile	Black-spined toads (*Duttaphrynus melanostictus*)	Stomach	Jirků et al., 2008
C. serpentis	Amazon tree boa (*Corallus hortulanus*), Black rat snake (*Elaphe obsoleta obsolete*), Bornmueller's viper (*Vipera bornmuelleri*), Bull snake (*Pituophis melanoleucus melanoleucus*), California kingsnake *(Lampropeltis getulus californiae),* Cornsnake (*Elaphe guttata guttata*), Common death adder (*Acanthophis antarticus*), Desert monitor (*Varanus griseus*), Eastern / Mainland Tiger snake (*Notechis scutatus*), Frilled lizard (*Chlamydosaurus kingui*), Giant madagascar or Oustalet's chameleon (*Chamaeleo oustaleti*), Leopard gecko (*Eublepharis macularius*), Mexican black kingsnake (*Lampropeltis getulus nigritus*), Milk snake (*Lampropeltis triangulum*), Mountain viper (*Vipera wagneri*), Python (*Python molurus*), Savannah monitor (*Varanus exanthematicus*), Skink (*Mabuya perrotetii*), Taipan (*Oxyuranus scutellatus*), Red-tailed boa (*Boa constrictor constrictor*), Rainbow boa (*Epicrates cenchria cenchria*), Common frog eye gecko (*Teratoscincus scincus*), Eastern kingsnake (*Lampropeltis getula*)	Stomach	Kimbell et al., 1999; Morgan et al., 1999b; Hajdusek et al., 2004; Xiao et al., 2004b; Pedraza-Díaz et al., 2009; Richter et al., 2011; Seva-Ada et al., 2011; Rinaldi et al., 2012; Diaz et al., 2013; da Silva et al., 2014; Abe and Matsubara, 2015; Yimming et al., 2016
C. testudinis (previousely Tortoise genotype I)	Russian tortoise (*Agrionemys* [*Testudo*] *horsfieldii*), chaco tortoise (*Chelonoidis chilensis*), Greek tortoise (*Testudo graeca Linnaeus*), Hermann's tortoise (*Testudo hermanni Gmelin*), Indian star tortoise (*Geochelone elegans*), Leopard tortoise (*Stigmochelys pardalis*), Marginated tortoise (*Testudo marginata*), Radiated tortoise (*Astrochelys radiata*), Serrated tortoise (*Psammobates oculifer*), Ball python (*Python regius*)	Stomach	Xiao et al., 2002b, 2004b, Alves et al., 2005; Pedraza-Díaz et al., 2009; Griffin et al., 2010; Richter et al., 2012; Jezkova et al., 2016
C. varanii	African fat-tailed gecko (*Hemitheconyx caudicinctus*), Leopard gecko (*Eublepharis macularius*), Boa constrictor (*Boa constrictor*), Cornsnake (*Elaphe guttata guttata*),	Intestine and Cloaca	Koudela and Modry, 1998; Morgan et al., 1999b; Hajdusek et al., 2004; Xiao et al., 2004b; Plutzer and Karanis, 2007;

	Desert monitor (*Varanus griseus*), Gecko (*Gekkoninae* sp.), Green iguana (*Iguana iguana*), *Lampropeltis* sp; Louisiana pine snake (*Pituophis ruthveni*), Plated lizard (*Gerrhosaurus* sp.), Schneider's Skink (*Eumeces schneideri*), Taipan (*Oxyuranus scutellatus*), Baron's green racer (*Philodryas baroni*), Yellow anaconda (*Eunectes notaeus*), Cornsnake (*Elaphe guttata guttata*), Mato Grosso lancehead (*Bothrops matogrossensis)*, Veiled chameleon (*Chamaeleo calyptratus*), Chinese wonder gecko (*Teratoscincus roborowskii*)		Pedraza-Díaz et al., 2009; Richter et al., 2011; da Silva et al., 2014; Abe and Matsubara, 2015
Lizard genotype/*C. serpentis*-like	Leopard gecko (*Eublepharis macularius*), Corn snake (*Pantherophis guttatus*), Chinese wonder gecko (*Teratoscincus scincus*)	-	Xiao et al., 2004b; Richter et al., 2011, Abe and Matsubara, 2015
Snake genotype I	New Guinea Viper boa (*Candoia asper*), Japanese grass snakes (*Rhabdophis tigris*)	-	Xiao et al., 2002b; Kuroki et al., 2008
Snake genotype II	Boa constrictor (*Boa constrictor ortoni*)	-	Xiao et al., 2004b
Tortoise genotype III	leopard tortoise (*Stigmochelys pardalis*)	-	Jezkova et al., 2016

1.4 The role of urbanisation in the transmission of zoonotic *Cryptosporidium* species from wildlife

The risk of waterborne outbreaks of cryptosporidiosis depends on a complex interplay of factors, associated with both the environment and the biology and ecology of host and parasite. *Cryptosporidium* detection in an animal faecal sample does not necessarily mean active infection in the host, nor does this guarantee that the parasite prevalence and the host-population dynamics are conducive to an outbreak. For these reasons, the epidemiological potential of detection of *Cryptosporidium* in wildlife cannot be easily and fully extrapolated. An increased epidemiological risk, however, can be identified when there is an overlap between humans and the distribution and dispersal of animal hosts. This is largely due to human encroachment into wildlife populated areas, which, by extension, also includes conversion of natural environments to drinking water catchments. Similarly, urban environments may also represent attractive new habitats for animals harbouring zoonotic *Cryptosporidium* spp. Thus, it is clear that wildlife-associated *Cryptosporidium* is an increasing concern for cryptosporidiosis in humans.

During the last 100 years in many countries of the world, there have been dramatic changes in natural/rural landscapes due to urbanisation (Mackenstedt et al., 2015). Although urbanisation is one of the leading causes of species extinction (McKinney, 2006), for adaptable species, urban and periurban areas can be very attractive due to increased food and water resources (waste food, pet food, garden produce, water tanks etc.) (Mackenstedt et al., 2015). In these environments, wildlife species may reach far higher population densities than in more natural or rural landscapes (Bradley and Altizer, 2007), potentially increasing the faecal-oral transmission of oocysts between wildlife and humans and contamination of drinking water catchments.

Shifting boundaries between wildlife and humans have been responsible for the emergence of species like *C. ubiquitum* and chipmunk genotype I in human populations. For example, squirrels host *C. ubiquitum*, chipmunk genotype I, the skunk genotype and other *Cryptosporidium* genotypes associated with human disease (Feng et al., 2007; Ziegler et al., 2007; Kváč et al., 2008; Stenger et al., 2015b), and because they frequently share habitats with humans they may be a significant reservoir of human infection. Squirrels can reach relatively high densities in suitable habitats, resulting in high rates of environmental loading of *Cryptosporidium* oocysts (Atwill et al., 2001). For example, California ground squirrels can reach densities as high as 92 adults hectare^{-1} (Owings et al., 1977; Boellstorff and Owings, 1995), which when combined with shedding of up to 2 - 10^5 oocysts animal^{-1} day^{-1} results in rates of environmental loading equivalent to 1 - 10^7 oocysts hectare^{-1} day^{-1} (Atwill et al., 2004). Further analysis of squirrel populations however has suggested that most tree squirrels host zoonotic species and genotypes while ground squirrels host species and genotypes that are tribe-specific and unlikely to cause human disease, despite overlapping ranges (Stenger et al., 2015b). This highlights the importance of extensive molecular epidemiological studies of wildlife to better understand the public health risks.

While urban-environment-induced increases in wildlife population densities are conducive to elevated rates of *Cryptosporidium* transmission, the host specificity of some wildlife species and genotypes may limit the potential for spillover of wildlife genotypes to sympatric populations of humans. For example, in Australia, the common brushtail possum is one of the most abundant native marsupials in urban environments, having successfully adapted to utilise anthropogenic resources (Hill et al., 2008). A higher *Cryptosporidium* prevalence in urban compared to woodland possum populations (11.3 versus 5.6%) has been reported, but the majority of possums sampled shed low numbers of host adapted (possum genotype) oocysts (1 to 10^2) (Hill et al., 2008). However, the finding a *C. fayeri* clinical infection in a human,

which had previously been thought to be a host-adapted species (Waldron et al., 2010), highlights our lack of knowledge about the human infectious potential of many species and genotypes of *Cryptosporidium* infecting wildlife.

1.5 *Cryptosporidium* in Australian humans

In Australia, the National Notifiable Diseases Surveillance System (NNDSS) was established in 1990 under the auspices of the Communicable Diseases Network Australia and in 2001, cryptosporidiosis was listed as a national notifiable disease (Blumer et al., 2003). Analysis of national notification rates revealed increased total notification rates in 2002, 2005, 2006, 2009, 2012, 2013, 2015, 2016 and 2017 with the highest number of cases reported in 2016 (5420) (Table 1.6), suggesting that outbreaks may have occurred. The pattern of cryptosporidiosis in Western Australia (WA) is somewhat different with increased notification rates in 2003, 2007, 2011 and 2017 (Table 1.6).

Cryptosporidiosis outbreaks in Australia have predominantly been attributed to contaminated recreational waters (see Ng-Hublin et al., 2018) and genotyping studies have demonstrated that *C. hominis* and *C. parvum* are the dominant species infecting humans but *C. meleagridis*, *C. fayeri*, *C. andersoni*, *C. bovis*, *C. cuniculus*, a novel *Cryptosporidium* species most closely related to *C. wrairi* and the *Cryptosporidium* mink genotype have been reported (Robertson et al., 2002; Chalmers et al., 2005; Jex et al., 2007; Ng et al., 2008; Alagappan et al., 2008; Jex et al., 2008; O'Brien et al., 2008; Waldron et al., 2009a, 2009b; Waldron et al., 2010; Power et al., 2011; Waldron et al., 2011a, 2011b; Ng et al., 2012; Jex et al., 2012; Koehler et al., 2013; Ng-Hublin et al., 2013; Sari et al., 2013 unpublished - KF279538; Yang et al., 2013; Koehler et al., 2014b).

Table 1.6. Number of cryptosporidiosis notifications received from State and Territory health authorities from 2001-2018. ACT = Australian Capital Territory, NSW= New South Wales, NT= Northern Territory, Qld = Queensland, SA = South Australia, Tas = Tasmania, Vic = Victoria and WA = Western Australia.

Year	ACT	NSW	NT	Qld	SA	Tas	Vic	WA	Total
2001	10	195	258	415	65	78	440	164	**1625**
2002	36	306	217	2023	125	47	284	226	**3264**
2003	8	203	96	162	80	26	208	437	**1220**
2004	6	353	111	674	82	18	306	125	**1675**
2005	27	848	83	1366	168	22	513	183	**3210**
2006	79	778	71	699	202	28	1097	252	**3206**
2007	9	545	111	431	449	37	622	608	**2812**
2008	11	485	102	695	60	36	451	163	**2003**
2009	106	1463	151	1458	106	67	1038	235	**4624**
2010	12	349	97	301	48	100	434	141	**1482**
2011	13	360	94	465	128	42	260	449	**1811**
2012	19	684	235	1371	162	42	461	168	**3142**
2013	39	1109	89	769	135	74	1265	372	**3852**
2014	30	420	87	668	224	33	636	310	**2408**
2015	26	1052	123	1314	419	19	855	255	**4063**
2016	48	1203	280	2368	433	32	811	245	**5420**
2017	83	1252	90	1287	351	44	1183	400	**4690**
2018	19	441	75	771	137	14	497	69	**2023**

1.6 Risk management

A key part of a One Health approach to *Cryptosporidium* prophylaxis is a better understanding of environmental, epidemiological and aetiological factors associated with cryptosporidial infections to enable more targeted risk management. The far-reaching One Health strategy aims at integrating multidisciplinary knowledge and evidence, and at coordinating the interventions, to create a global synergism catering for all aspects of health care for humans, animals and the environment (the One Health Triad).

It has been shown that an important host risk factor includes HIV status. *Cryptosporidium* is an important pathogen regardless of HIV prevalence (Kotloff et al., 2013); however, HIV-positive children are between 3 and 18 times more likely to have

Cryptosporidium than those who were HIV negative (Tumwine et al., 2005; Mbae et al., 2013; Tellevik et al., 2015). With the widespread availability of antiretroviral therapy, particularly in industrialized countries, the incidence of cryptosporidiosis has decreased among people living with AIDS (Kaplan et al., 2000). However, the increasing number of transplant recipients and those receiving immunosuppressive drugs may contribute significantly to the burden in the future (Bonatti et al., 2012; Desoubeaux et al., 2012). Malnutrition is also a risk factor for both diarrhoea and prolonged diarrhoea caused by *Cryptosporidium*, with significantly higher rates of infection in malnourished children after controlling for HIV status (Amadi et al., 2001; Haque et al., 2009; Moore et al., 2010; Mondal et al., 2012). An unknown number of individuals experience asymptomatic *Cryptosporidium* infection (Houpt et al., 2005). This clinically silent infection may remain undetected and untreated, and therefore may contribute not only to parasite transmission but also to malnutrition and the associated clinical sequelae. Breastfeeding may provide some protection, as a recent study of Bangladeshi infants reported that protection from *Cryptosporidium* infection was associated with high anti-*Cryptosporidium* IgA in breastmilk (Korpe et al., 2013).

Under an environmental perspective, the prophylaxis of waterborne cryptosporidiosis must consider optimal management (or design) of source, recycled and recreational waters. Protection of source water and swimming pools is a key element of *Cryptosporidium* prevention as contamination of drinking water and swimming pools is a major mode of transmission (Baldursson and Karanis, 2011; Widerström et al., 2014; Jacob et al., 2015; Ryan et al., 2017) and is often achieved by restricting the access to catchments and water bodies, while swimming pools are designed and monitored according to construction standards and guidelines. Infection prevention and management, however, can only be achieved through a deep understanding of the routes of transmission, sources of contamination (human and animal), disease prevalence in the population and the risk factors in the final host.

The link between *Cryptosporidium* in drinking water and sporadic infections is well documented (Baldursson et al., 2011; Widerström et al., 2014; Jacob et al., 2015); however, the association between drinking water contamination and endemic cryptosporidiosis is not well established. For example, some studies report drinking unsafe water as a risk factor for endemic cryptosporidial infection (Leach et al., 2000; Goh et al., 2004) while others report no association (Khalakdina et al., 2003; Roy et al., 2004; Sarkar et al., 2013). Seasonal patterns are also thought to be associated with an increased transmission risk (Checkley et al., 1998; Muchiri et al., 2009), such as when recreational waters are more heavily utilised.

Management of *Cryptosporidium* public health risks specifically for the drinking water industry requires the implementation of a holistic approach including research, monitoring *Cryptosporidium* oocysts in animals and source water, and catchment management (e.g., access protection, vegetation cover, etc.). High-precipitation events favour the transfer and survival of oocysts in surface waters and/or groundwater (Bridgman et al., 1995; Jagai et al., 2009). This may result in contamination of source water and increased risk of cryptosporidiosis depending on the source of contamination (Jagai et al., 2009). Indeed, the average odds of identifying *Cryptosporidium* oocysts in fresh surface waters is 2.61 (95% CI, 1.63-4.21; I^2=16%) times higher during and after extreme weather events (Young et al., 2015). Shifts in precipitation patterns (intensity and location) is one of the climate change predictions for the future (Pachauri, 2008), and this will clearly impact both waterborne and foodborne transmissions of *Cryptosporidium,* and therefore, future human exposures may differ significantly from current patterns as the climate changes (Schijven et al., 2013). Hydrodynamic modelling has been shown to represent a valid and cost-effective support, for decision-making and understanding of events (Hoyer et al., 2015). Quantitative microbial risk assessment (QMRA) is another widely used tool to estimate health impacts from exposure to *Cryptosporidium* and other pathogens (Young et al., 2015) and has been applied to climate

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1.5 *Cryptosporidium* in Australian humans

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non-zoonotic species in water samples (Li et al., 2015d). Another study of a drinking water supply in Australia, found no *C. hominis* in any water sample tested, but *Cryptosporidium* genotypes associated with native and non-native wildlife made up 70% of all isolates typed (Swaffer et al., 2014). Similarly, Ruecker et al. (2012) reported that non-zoonotic wildlife species and genotypes of *Cryptosporidium* accounted for 64.3% of *Cryptosporidium* identified in environmental water samples in Canada and that only 7.2% of human infectious species were detected. A low prevalence of *C. hominis* and *C. parvum* was also reported by Nolan et al. (2013) in Melbourne catchments, who detected *C. hominis* and *C. parvum* in only 0.6% of samples, despite screening >2,000 animal faecal samples. However, the human-infectious potential of many wildlife-adapted *Cryptosporidium* is currently unknown and the UK outbreak caused by *C. cuniculus* should act as a caution against assuming these unusual species and genotypes are not significant (Chalmers et al., 2009; Robinson et al., 2011).

Accurate, quantitative identification of *Cryptosporidium* in wildlife excreta is an essential starting point for estimating catchment loads (Davies et al., 2003). Quantitative PCR (qPCR) (real-time PCR) therefore represents an invaluable tool that enables rapid, high-throughput and cost-effective detection and quantitation of *Cryptosporidium* oocysts and is increasingly being used to monitor oocyst shedding by animals in catchments (Yang et al., 2014a). Due to the intrinsic constraints of qPCR, standards of known concentration are required to generate calibration curves used to estimate the concentration of pathogens in a sample (Hindson et al., 2013; Rački et al., 2014). Therefore, the quantification of the target molecules in the unknown sample is only as good as that of the standards used. Droplet digital PCR (ddPCR) (Hindson et al., 2013) is the third-generation implementation of conventional PCR that facilitates the quantitation of nucleic acid targets without the need for calibration curves (Vogelstein and Kinzler, 1999). A recent study compared ddPCR with qPCR for the quantitative detection of *Cryptosporidium* DNA in animal and human faecal samples (Yang et

al., 2014b) and revealed that ddPCR appeared to be less sensitive to inhibitors than qPCR and that inaccurate calibration of qPCR standards resulted in qPCR overestimating the numbers of oocysts present (Yang et al., 2014b). This has important implications for catchment risk management. However, qPCR is cheaper and provides better throughput and therefore using ddPCR to precisely quantify qPCR standards would be one way to combine the advantages of the two technologies and provide more accurate assessment of *Cryptosporidium* catchments loads from wildlife faecal samples.

Besides quantitative considerations, measuring the infectivity is also important for adjusting the risk profile of oocysts from wildlife in source waters (Swaffer et al., 2014). For example, a recent study has shown that the infectivity fraction of oocysts within source water samples in South Australian catchments was low (~3.1%), which provided a much more accurate water quality risk assessment (Swaffer et al., 2014). This low infectivity fraction is consistent with source water infectivity reported by Di Giovanni et al. (1999) of 4.9% and Lalancette et al. (2010) of 0%. The ability to routinely measure oocyst infectivity has been hampered by a number of issues including the distribution and low numbers of oocysts, costs and reproducibility (Di Giovanni and LeChevallier, 2005; Swaffer et al., 2014). However, recent improvements in cell culture immunofluorescence assays have led to the development of a single format assay that provides information on method performance (recovery rate), oocyst number, oocyst infectivity and genotype of infectious oocysts, overcoming many of these obstacles (King et al., 2015). This assay should therefore enable a more comprehensive understanding of *Cryptosporidium* risk for different water sources, assisting in the selection of appropriate risk mitigation measures (King et al., 2015). It is, however important to remember that the detection of non-viable oocysts in the 10-20 L of the water column that is usually sampled, does not mean that other oocysts in the water body are also non-viable.

Factors that affect the viability of *Cryptosporidium* oocyst load in faecal samples from wildlife in the catchment and water (runoffs, water column and sediments), include solar inactivation, desiccation, temperature and residence time in catchments and these dynamics should be factored into risk assessments (Hijen et al., 2006; King and Monis, 2007; Monis et al., 2014). Transport, including hydrodynamically-driven accumulation, settlemement, dispersion, dilution etc. can also affect oocyst concentrations in the water, either positively or negatively. Peak flow periods (when the maximum area of catchment is contributing to stream flow), are a major driver behind the transport of oocysts to surface water. Therefore, monitoring the distribution of *Cryptosporidium* during elevated flow conditions caused by rainfall run-off is important given the demonstrated positive and significant correlation between *Cryptosporidium* concentration with flow and turbidity (Swaffer et al., 2014). Measuring the infectivity of different wildlife derived *Cryptosporidium* species under different climatic conditions is therefore crucial for accurate risk assessment of public health implications, particularly as more extreme precipitation events is predicted globally (IPCC, 2013 - www.ipcc.ch) (Ryan et al., 2014).

There are still many research gaps in our understanding of the public health significance of wildlife in drinking water catchments, and taxonomic and molecular epidemiological studies on *Cryptosporidium* spp. in wildlife, especially those in watersheds are still scarce. Whole genome studies in *Cryptosporidium* species will assist with the development of *gp*60 and other typing tools to better access the zoonotic potential and transmission dynamics of *Cryptosporidium* in wildlife. Morphological and biological data, including pathogenicity and oocyst shedding rates, are not yet available for some common zoonotic *Cryptosporidium* species and genotypes in wildlife. There is also a need to confirm if molecular detection of zoonotic *Cryptosporidium* species in wildlife is commonly associated with actual infections or mechanical transmission (Ryan et al., 2014). *Cryptosporidium cuniculus* is the only species

besides *C. hominis* and *C. parvum*, known to be associated with a waterborne outbreak of human cryptosporidiosis, yet little is known about the prevalence and oocyst shedding rates of *C. cuniculus* in rabbits.

The evolution of methods to enumerate and genotype oocysts, and determine oocyst infectivity provides much-needed tools to refine the human health risk from wildlife in catchments and future studies will provide water quality managers with much more accurate and informed data for modelling and quantitative microbial risk assessments (QMRA) of wildlife in various catchments.

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Chapter Two - Zoonotic *Cryptosporidium* species in animals inhabiting Sydney water catchments

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2.1 Preface

This chapter consists of a published paper in PLos One, entitled "Zoonotic *Cryptosporidium* species in animals inhabiting Sydney water catchments". The text of this manuscript is the same as the published version of the paper found in the Appendices (*see* Appendix 3) except for modifications, such as in-thesis referencing. This chapter develops, optimises and validates a pilot methodology to conduct a comprehensive quantitative survey of species and genotypes of *Cryptosporidium* in animal faecal samples. More specifically it aims to: (1) use qPCR to screen a subset of faecal samples (n = 952) collected from animals inhabiting within the WaterNSW area of operation for the presence of *Cryptosporidium* spp.; (2) enumerate *Cryptosporidium* oocysts in faecal samples by qPCR with droplet digital PCR (ddPCR) calibrated standards; (3) identify species and subtypes of *Cryptosporidium* at multiple loci.

2.2 Statement of contribution

As lead author on this manuscript, **AZ** conducted the majority of the laboratory work (with the exception of the independent confirmation by the Australian Water Quality Centre (AWQC) staff members; **PM**, **SA** and **BK**, and enumeration of *Cryptosporidium* oocysts by microscopy by Australian Laboratory Services (Scoresby, Vic), validated and analysed the data, and wrote the first and final drafts of this manuscript. **FJ** provided assistance in PCR set ups and **AP** helped with data analysis. **YR** and **CO** were involved in data validation and manuscript draft preparation. **IR** assisted with statistical analysis. **AB** organised sample collection and helped with funding acquisition and methodology. **UR** supervised the project and was directly involved in project administration, conceptualisation, funding acquisition, methodology, data

validation, review and editing the manuscript. All authors critically reviewed and approved the final version of the manuscript.

AZ: 75%

2.3 Abstract

Cryptosporidium is one of the most common zoonotic waterborne parasitic diseases worldwide and represents a major public health concern of water utilities in developed nations. As animals in catchments can shed human-infectious *Cryptosporidium* oocysts, determining the potential role of animals in dissemination of zoonotic *Cryptosporidium* to drinking water sources is crucial. In the present study, a total of 952 animal faecal samples from four dominant species (kangaroos, rabbits, cattle and sheep) inhabiting Sydney's drinking water catchments were screened for the presence of *Cryptosporidium* using a quantitative PCR (qPCR) and positives sequenced at multiple loci. *Cryptosporidium* species were detected in 3.6% (21/576) of kangaroos, 7.0% (10/142) of cattle, 2.3% (3/128) of sheep and 13.2% (14/106) of rabbit samples screened. Sequence analysis of a region of the 18S rRNA locus identified *C. macropodum* and *C. hominis* in 4 and 17 isolates from kangaroos respectively, *C. hominis* and *C. parvum* in 6 and 4 isolates respectively each from cattle, *C. ubiquitum* in 3 isolates from sheep and *C. cuniculus* in 14 isolates from rabbits. All the *Cryptosporidium* species identified were zoonotic species with the exception of *C. macropodum*. Subtyping using the 5′ half of *gp60* identified *C. hominis* IbA10G2 ($n = 12$) and IdA15G1 ($n = 2$) in kangaroo faecal samples; *C. hominis* IbA10G2 ($n = 4$) and *C. parvum* IIaA18G3R1 ($n = 4$) in cattle faecal samples, *C. ubiquitum* subtype XIIa ($n = 1$) in sheep and *C. cuniculus* VbA23 ($n = 9$) in rabbits. Additional analysis of a subset of samples using primers targeting conserved regions of the MIC1 gene and the 3′ end of *gp60* suggests that the *C. hominis* detected in these animals represent substantial variants that failed to amplify as expected. The significance of this finding requires further investigation but might be reflective of the ability of this *C. hominis* variant to infect animals. The finding of zoonotic *Cryptosporidium* species in these animals may have important

implications for the management of drinking water catchments to minimize risk to public health.

2.4 Introduction

Cryptosporidium is one of the most prevalent waterborne parasitic infections (Baldursson and Karanis, 2011) and represents a public health concern of water utilities in developed countries, including Australia. Currently, 37 *Cryptosporidium* species have been recognised based on biological and molecular characteristics including two recently described species; *C. proliferans* and *C. avium* (Ryan and Hijjawi, 2015; Li et al., 2015; Kváč et al., 2016; Holubová et al., 2016; Zahedi et al., 2017a; Čondlová et al., 2018; Kváč et al., 2018.). Of these, *C. parvum* and *C. hominis* have been responsible for all waterborne outbreaks typed to date, with the exception of a single outbreak in the UK caused by *C. cuniculus* (Xiao, 2010; Chalmers et al., 2009; Puleston et al., 2014).

In Australia, marsupials, rabbits, sheep and cattle are the dominant animals inhabiting drinking water catchments and can contribute large volumes of manure to water sources (Ryan and Power, 2012). Therefore, it is important to understand the potential contribution from these animals in terms of *Cryptosporidium* oocyst loads into surface water. A number of genotyping studies have been conducted on animals in Australian water catchments to date and have reported a range of species including *C. parvum*, *C. hominis*, *C. cuniculus*, *C. ubiquitum*, *C. bovis*, *C. ryanae*, *C. canis*, *C. macropodum*, *C. fayeri*, *C. xiaoi*, *C. scrofarum*, and *C. andersoni* (Power et al., 2004; Cinque et al., 2008; McCarthy et al., 2008; Nolan et al., 2010, 2013; Ng et al., 2011a, 2011b; Yang et al., 2011; Abeywardena et al., 2013a, 2013b; Koehler et al., 2014a; Yang et al., 2014a; Abeywardena et al., 2015). To date, in humans in Australia, *C. hominis*, *C. parvum*, *C. meleagridis*, *C. fayeri*, *C. andersoni*, *C. bovis*, *C. cuniculus*, a novel

Cryptosporidium species most closely related to *C. wrairi* and the *Cryptosporidium* mink genotype have been reported (Robertson et al., 2002; Chalmers et al., 2005; Jex et al., 2007; Ng et al., 2008; Alagappan et al., 2008; Jex et al., 2008; O'Brien et al., 2008; Waldron et al., 2009a, 2009b; Waldron et al., 2010; Power et al., 2011; Waldron et al., 2011a, 2011b; Ng et al., 2012; Jex et al., 2012; Koehler et al., 2013; Ng-Hublin et al., 2013; Sari et al., 2013 unpublished - KF279538; Yang et al., 2013; Koehler et al., 2014b). The aim of the present study was to use molecular tools to identify the *Cryptosporidium* sp. infecting the kangaroos, rabbits, cattle and sheep population inhabiting Sydney's drinking water catchments and so better understand the potential health risks they pose.

2.5 Materials and Methods

2.5.1 Sample collection and processing

Animal faecal samples were collected by WaterNSW staff from watersheds within the WaterNSW area of operations. Sampling was carried out either on land owned by WaterNSW or on private land owned by farmers who gave permission to WaterNSW staff to conduct this study on their property. To minimize cross-contamination and avoid resampling the same animals, animals were observed defaecating and then samples were collected randomly from freshly deposited faeces from the ground, using a scrapper to expose and scoop from the center of the scat pile. Samples were collected on a monthly interval over an 18 months period (July, 2013 to February, 2015) into individual 75 ml faecal collection pots, and stored at 4°C until required (no animal was sacrificed). As faecal samples were collected from the ground and not per rectum, animal ethics approval was not required. Instead, an animal cadaver/tissue notification covering all the samples collected was supplied to the Murdoch University Animal

Ethics Committee. The animal sources of the faecal samples were confirmed by watching the host defaecate prior to collection and also with the aid of a scat and tracking manual published for Australian animals (Triggs, 2004). Faecal samples were collected from two previously identified hotspot zones from eastern grey kangaroos (*Macropus giganteus*) (*n* = 576), cattle (*n* = 142), sheep (*n* = 128) and rabbits (*n* = 106). This study did not involve collecting samples from endangered or protected animal species. Samples were shipped to Murdoch University and stored at 4°C until required.

2.5.2 Enumeration of *Cryptosporidium* oocysts in faecal samples

Enumeration of *Cryptosporidium* oocysts by microscopy was conducted in duplicate for a subset of samples (*n* = 8) by Australian Laboratory Services (Scoresby, Victoria). To quantify recovery efficiency, each individual faecal composite or homogenate was seeded with ColorSeed (Biotechnology Frontiers Ltd. [BTF], Sydney, Australia). *Cryptosporidium* oocysts were purified from faecal samples using immunomagnetic separation (IMS) employing the Dynal GC Combo kit (Dynal, Oslo, Norway) as described by Cox et al. (2005). Oocysts were stained with Easystain and 4',6'-diamidino-2-phenylindole (DAPI; 0.8 $\mu g.ml^{-1}$) (Biotechnology Frontiers Ltd. [BTF], Sydney, Australia) and examined with an Axioskop epifluorescence microscope (Zeiss, Germany) using filter set 09 (blue light excitation) for Easystain (BTF), filter set 02 (UV light excitation) for DAPI staining, and filter set 15 (green light excitation) for ColorSeed (BTF). The identification criteria described in U.S. EPA method 1623 (US EPA, 2012) were used for Easystain-labeled and DAPI-stained objects.

2.5.3 DNA isolation

Genomic DNA was extracted from 250mg of each faecal sample using a Power Soil DNA Kit (MO BIO, Carlsbad, California). A negative control (no faecal sample) was used in each extraction group.

2.5.4 PCR amplification of the 18S rRNA gene

All samples were screened for the presence of *Cryptosporidium* at the 18S rRNA locus using a quantitative PCR (qPCR) previously described (King et al., 2005; Yang et al., 2014b). qPCR standards were *Cryptosporidium* oocysts (purified and haemocytometer counted), diluted to a concentration of 10,000 oocysts/µl. DNA was extracted from this stock using a Powersoil DNA extraction kit (MO BIO, Carlsbad, California, USA). The 10,000 oocyst/µl DNA stock was then serially diluted to create oocyst DNA concentrations equivalent to 1,000, 100, 10, 1 oocysts/µl DNA respectively to be used for standard curve generation using Rotor-Gene 6.0.14 software. Absolute numbers of *Cryptosporidium* oocysts in these standards were determined using droplet digital PCR (ddPCR) at the 18S locus using the same primer set and these ddPCR calibrated standards were used for qPCR as previously described (Yang et al., 2014b). Each 10 µl PCR mixture contained 1x KAPA Taq buffer (KAPA Biosystems), 3.75 mM $MgCl_2$, 400 µM of each dNTP, 0.5 µM 18SiF primer, 0.5 µM 18SiR primer, 0.2 µM probe and 1U/reaction Kapa DNA polymerase (KAPA Biosystems). The PCR cycling conditions consisted of one pre-melt cycle at 95°C for 6 min and then 50 cycles of 94°C for 20 sec and 60°C for 90 sec.

Samples that were positive by qPCR were amplified at the 18S locus using primers which produced a 611 bp product (Table 2.1) as previously described (Silva et al., 2013) with minor modifications; the annealing temperature used in the present study was 57°C for 30 sec and the number of cycles was increased from 39 to 47 cycles for both primary and secondary reactions.

PCR contamination controls were used including negative controls and separation of preparation and amplification areas. A spike analysis (addition of 0.5 µl of positive control DNA into each sample) at the 18S locus by qPCR, was conducted on randomly selected negative samples from each group of DNA extractions to determine if negative results were due to PCR inhibition, by comparing the *Ct* of the spike and the positive control (both with same amount of DNA).

Table 2.1. List of primers used in this study to amplify 18S, lectin (*Clec*), *gp60*, lib13, MIC1 gene loci.

Gene	Forward Primer	Reverse Primer	Reference
18S	5′ ACCTATCAGCTTTAGACG GTAGGGTAT 3′	5′ TTCTCATAAGGTGCT GAAGGAGTAAGG 3′	Silva et al., 2013
	5′ ACAGGGAGGTAGTGA CAAGAAATAACA 3′	5′ AAGGAGTAAGGA ACAACCTCCA 3′	
lectin (*Clec*)	5′ TCAACTAACGAAGGAG GGGA 3’	5′ GTGGTGTAGAAT CGTGGCCT 3′	Present study
	5′ CCAACATACCATCCTTTG G 3′	5′ GTGGTGTAGAATCGT GGCCT 3′	
gp60	5′ ATAGTCTCGCTGTATTC 3′	5′ GCAGAGGAACCA GCATC 3′	Peng et al., 2003; Zhou et al., 2003
	5′ TCCGCTGTATTCTCAGCC 3′	5′ GAGATATATCTTGGT GCG 3′	
18S	5′ TTCTAGAGCTAATACATG CG 3′	5′ CCCATTTCCTTCGA AACAGGA 3′	Xiao et al., 2000; Webber et al., 2014
	5′ CCCATTTCCTTCGA AACAGGA 3′	5′ CTCATAAGGTGCTG AAGGAGTA 3′	
gp60	5′ ATAGTCTCCGCTGTATTC 3′	5′ GGA AGG AAC GAT GTA TCT 3′	Alves et al., 2003; Webber et al., 2014
	5′ GGAAGGGTTGTATTTATT AGATAAAG 3′	5′ GCA GAG GAA CCA GCATC 3′	
lib 13	5′ TCCTTGAAATGAATATTT GTGACTCG 3′	5′ AAATGTGGTAGTT GCGGTTGAAA 3′	Hadfield et al., 2011
	Probe: VIC-CTTACTTCGTGGCGGCGT MGB-NFQ		
MIC1	5′ TGCAGCACAAACAGTAG ATGTG 3′	5′ ATAAGGATCTGCC AAAGGAACA 3′	Webber et al., 2014
	5′ ACCGGAATTGATGAGAA ATCTG 3′	5′ CATTGAAAGGTTGA CCTGGAT 3′	

2.5.5 PCR amplification of the lectin (*Clec*) gene

Samples that were typed as *C. parvum*, *C. hominis* and *C. cuniculus* at the 18S locus were also typed using sequence analysis at a unique *Cryptosporidium* specific gene (*Clec*) that codes for a novel mucin-like glycoprotein that contains a C-type lectin domain (Morgan et al., 1997; Bhalchandra et al., 2013). Hemi-nested primers were designed for this study using MacVector 12.6 (http://www.macvector.com). The external primers Lectin F1 5′ TCAACTAACGAAGGAGGGGA 3′ and Lectin R1 5′ GTGGTGTAGAATCGTGGCCT 3′ produced a fragment size of 668 bp for *C. hominis* and 656 bp for *C. parvum*. The secondary reaction consisted of primers, Lectin F2 5′ CCAACATACCATCCTTTGG 3′ and Lectin R1 5′ GTGGTGTAGAATCGTGGCCT 3′ (Table 2.1), which produced a fragment of 518 bp for *C. hominis*, 506 bp for *C. parvum* and 498 bp for *C. cuniculus*. The cycling conditions for the primary amplification was 94°C for 3 min, followed by 94°C for 30 sec, 58°C for 30 sec, 72°C for 1 min for 40 cycles, plus 5 min at 72°C for the final extension. The same cycling conditions were used for the secondary PCR, with the exception that the number of cycles was increased to 47 cycles. The 25 µl PCR mixture consisted of 1 µl of DNA, 1x Go Taq PCR buffer (KAPA Biosystems), 200 µM of each dNTP (Promega, Australia), 2mM $MgCl_2$, 0.4 µM of each primer, 0.5 units of Kapa DNA polymerase (KAPA Biosystems). The specificity of this locus for *Cryptosporidium* has been previously confirmed (Yang et al., 2013). Enumeration of *Cryptosporidium* oocysts by qPCR was conducted using a specific *C. hominis* and *C. parvum* assay targeting the *Clec* gene as previously described (Yang et al., 2013).

2.5.6 PCR amplification of the *gp60* gene

Samples that were typed as *C. hominis*, *C. parvum*, *C. cuniculus* and *C. ubiquitum* at the 18S locus were subtyped at the 60 kDa glycoprotein (*gp60*) locus using nested PCR as previously described (Table 2.1) (Strong et al., 2000; Peng et al., 2003; Zhou et al., 2003; Li et al., 2014).

2.5.7 Sequence analysis and phylogenetic analysis

The amplified DNA from secondary PCR products were separated by gel electrophoresis and purified for sequencing using an in-house filter tip method (Yang et al., 2013). Purified PCR products from all three loci, were sequenced independently using an ABI Prism™ Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, California) according to the manufacturer's instructions at 57°C, 58°C and 54°C annealing temperatures for the 18S rRNA, lectin and *gp60* loci, respectively. Sanger sequencing chromatogram files were imported into Geneious Pro 8.1.6 (Kearse et al., 2012), edited, analysed and aligned with reference sequences from GenBank using ClustalW (http://www.clustalw.genome.jp). Distance (Neighbor Joining, NJ), Parsimony and Maximum Likelihood (ML) trees were constructed using MEGA version 7 (Tamura et al., 2011).

2.5.8 Independent confirmation by the Australian Water Quality Centre (AWQC)

A total of eight blinded faecal samples consisting of seven *C. hominis* positives and one *Cryptosporidium* negative were sent to the Australian Water Quality Centre (AWQC) for independent analysis. DNA was extracted using a QIAamp DNA Mini extraction kit (Qiagen,

Australia). Samples were screened using primers targeting the 18S rRNA locus (Xiao et al., 2000 as modified by Webber at al., 2014), the *gp60* locus using producing an approx. 871 bp secondary product (Alves et al., 2003 as modified by Webber at al., 2014) and an approx. 400 bp primary product (Zhou et al., 2003) as well as the *lib13* (Hadfield et al., 2011) and *MIC1* gene loci (Webber at al., 2014) as previously described (Table 2.1). PCRs were conducted on a RotorGene 6000 HRM (Qiagen) or LightCycler 96 (Roche) and amplification of the correct product was determined by DNA melting curve analysis (Webber et al., 2014). Amplicons with atypical DNA melting profiles were further characterised by capillary electrophoresis using a DNA 1000 chip on a Bioanalyzer 2100 (Agilent) as per the manufacturer's instructions. The amplicons from all positive PCRs were purified using a Qiagen PCR purification kit according to the manufacturer's instructions and submitted to the Australian Genome Research Facility for DNA sequencing using BigDye3 chemistry on an Applied Biosystems AB3730xl capillary DNA sequencer. Sequences were analyzed using Geneious Pro 6.1.8 (Biomatters).

2.5.9 PCR amplification of open reading frames flanking *gp60* and *MIC1*

Open reading frames flanking both ends of *gp60* and *MIC1* in the *C. parvum* genome were used in BLAST searches (http://blast.ncbi.nlm.nih.gov/) to obtain homologous *C. hominis* sequences. Alignments of the *C. parvum* and *C. hominis* open reading frame pairs were constructed using Geneious Pro 6.1.8 (Biomatters). Conserved primers were designed for each alignment using the default settings and a target amplicon size of approximately 400 bp. The resulting primers (Table 2.2) were subjected to BLAST searches to verify specificity.

Table 2.2. List of primers designed to amplify regions flanking the 5′ and 3′ ends of MIC1 and *gp60* gene loci.

Gene	Flanking open reading frame	Forward Primer	Reverse Primer	Product size (*C. parvum* and *C. hominis*)
MIC1	cgd6_770 Chro. 60100 (3′ end) hypothetical protein CDS	5′ GGTTGTATGACACC ATCA 3′	5′ TCTCTGGTGTTTGGC CTGAC 3′	511
	cgd6_810 Chro. 60105 (5′ end) BRCT	5′ AGACACCAAGATG GAAAAGGCA 3′	5′ GGGAAGACCTTTTG ATATTGCCC 3′	467
gp60	cgd6_1070 Chro. 60137 (3′ end) conserved hypothetical protein	5′ AGCAAGACCGCAA CTCAAGT 3′	5′ CCCATAGTGCCCAGC TTGAA 3′	430
	cgd6_1090 Chro. 60141 (5′ end) hsp40	5′ TATTTGGAGGTGGG GCCAAG 3′	5′ AAAACGGGTTTAGG GGTGGT 3′	367

Each 25 µl qPCR reaction contained 0.5x GoTaq PCR Buffer (Promega), 1.5 mM $MgCl_2$, 0.2 mM dNTP, 3.3 µM SYTO 9, 100 ng GP32, 0.5 µM forward primer, 0.5 µM reverse primer, 1 unit Promega GoTaq HS, and 2 µl of DNA extract. The qPCR was performed on a Light Cycler96 (Roche), and cycling conditions consisted of one pre-melt cycle at 95°C for 6 min and then 40 cycles of 94°C for 45 sec, 60°C for 45 sec and 72°C for 60 sec. High-resolution DNA melting curve analysis was conducted from 65°C to 97°C using an acquisition rate of 25 reads /°C. *Blastocystis hominis* DNA was used as a negative control and nuclease free water was used as a no template control. Positive controls included *C. parvum* Iowa 2a (BTF, Sydney, Australia) and *C. hominis* IbA10G2 (kindly provided by Ika Sari). Amplicons were sized by capillary electrophoresis using a DNA 1000 chip on a Bioanalyzer 2100 (Agilent) as per the manufacturer's instructions.

2.5.10 Statistical Analysis

The prevalence of *Cryptosporidium* in faecal samples collected from each host species was expressed as the percentage of samples positive by qPCR, with 95% confidence intervals calculated assuming a binomial distribution, using the software Quantitative Parasitology 3.0 (Rózsa et al., 2000). Linear coefficients of determination (R^2) and Spearman's rank correlation coefficient (Spearman's rho) were used for the analysis of agreement (correlation) between oocyst numbers per gram of faeces determined by qPCR calibrated with ddPCR standards and enumeration of *Cryptosporidium* oocysts by microscopy (IMS) using SPSS 21.0 for Windows (SPSS Inc. Chicago, USA).

2.6 Results

2.6.1 Prevalence of *Cryptosporidium* in faecal samples collected from various hosts

The overall PCR prevalence of *Cryptosporidium* species in 952 faecal samples collected from four different host species was 5% (48/952) (Table 2.3). *Cryptosporidium* species were detected in 3.6% (21/576) of the kangaroo faecal samples, 7.0% (10/142) of cattle faeces, 2.3% (3/128) of sheep faeces and 13.2% (14/106) of rabbit faecal samples based on qPCR and sequence analysis of the 18S rRNA locus (Table 2.3).

Table 2.3. Prevalence of *Cryptosporidium* species in faecal samples collected from four different host species in Sydney water catchments*. 95% confidence intervals are given in parenthesis.

Host species	Number of samples	Number positive	Prevalence %	Species and subtype
Eastern grey kangaroo	576	21	3.6 (95% CI, 2.3-5.5)	*C. hominis* (*n* = 17)** IbA10G2 (*n* = 12) IdA15G1 (*n* = 2) *C. macropodum* (*n* =4)
Cattle	142	10	7 (95% CI, 3.4-12.6)	*C. hominis* (*n*= 6)** IbA10G2 (*n* = 4) *C. parvum* (*n* = 4) IIaA18G3R1 (*n* = 4)
Sheep	128	3	2.3 (95% CI, 0.5-6.7)	*C. ubiquitum* (*n* = 3)** XIIa (*n* = 1)
Rabbit	106	14	13.2 (95% CI, 7.4-21.2)	*C. cuniculus* (*n* = 14)** VbA 23 (*n* = 9)
Total	**952**	**48**	**5 (95% CI, 3.7-6.6)**	

* Based on PCR amplification and sequencing at the 18S rRNA gene, with subtyping based on DNA sequence analysis of a 400 bp amplicon from the 5′ end of *gp60.*
** Not all positive samples were successfully typed.

2.6.2 *Cryptosporidium* species detected in various hosts

Sequencing of secondary PCR amplicons at the 18S rRNA locus identified four of the 21 positive isolates from kangaroo faecal samples as *C. macropodum*, while the other 17 isolates were identified as *C. hominis* (100% similarity for 550bp) (Table 2.4). Of the ten positives detected in cattle faecal samples, six were *C. hominis* and four were *C. parvum* (Table 2.4). The three sheep positive samples were identified as *C. ubiquitum* and all fourteen positives detected in rabbit faecal samples were *C. cuniculus* (Table 2.4).

Sequence analysis at the lectin (*Clec*) locus was consistent with 18S gene results. Eleven of 17 *C. hominis* isolates from kangaroos were successfully amplified and confirmed as *C. hominis* sequences. Eight of the 14 positives from rabbits successfully amplified at this locus and were identified as *C. cuniculus*. Four of six *C. hominis* and all four *C. parvum* isolates from cattle were also confirmed at this locus.

Table 2.4. Species and subtypes of *Cryptosporidium* identified at 18S and *gp60* loci in this study.

Host species	**Southing**	**Easting**	**qPCR (18S)**	**18S locus**	***gp*60 locus**
Eastern grey kangaroo 1	-34.18861	150.2918	detected	*C. hominis*	*C. hominis* IbA10G2
Eastern grey kangaroo 2	-34.203794	150.284394	detected	*C. macropodum*	-
Eastern grey kangaroo 3	-34.20207	150.2742	detected	*C. hominis*	*C. hominis* IbA10G2
Eastern grey kangaroo 4	-34.193631	150.273387	detected	*C. macropodum*	-
Eastern grey kangaroo 5	-34.188607	150.291818	detected	*C. macropodum*	-
Eastern grey kangaroo 6	-34.20458	150.2881	detected	*C. hominis*	*C. hominis* IbA10G2
Eastern grey kangaroo 7	-34.61547	150.59756	detected	*C. hominis*	no amplification
Eastern grey kangaroo 8	-34.23796	150.2598	detected	*C. hominis*	*C. hominis* IbA10G2
Eastern grey kangaroo 9	N/A	N/A	detected	*C. hominis*	*C. hominis* IbA10G2
Eastern grey kangaroo 10	N/A	N/A	detected	*C. hominis*	*C. hominis* IbA10G2
Eastern grey kangaroo 11	N/A	N/A	detected	*C. hominis*	*C. hominis* IbA10G2
Eastern grey kangaroo 12	N/A	N/A	detected	*C. hominis*	*C. hominis* IbA10G2
Eastern grey kangaroo 13	-34.61686	150.68794	detected	*C. hominis*	*C. hominis* IbA10G2
Eastern grey kangaroo 14	-34.63269	150.619	detected	*C. hominis*	*C. hominis* IbA10G2
Eastern grey kangaroo 15	-34.63269	150.61897	detected	*C. hominis*	no amplification
Eastern grey kangaroo 16	-34.61422	150.59331	detected	*C. hominis*	*C. hominis* IbA15G1
Eastern grey kangaroo 17	-34.61415	150.59376	detected	*C. hominis*	*C. hominis* IbA10G2
Eastern grey kangaroo 18	-34.61686	150.68794	detected	*C. hominis*	no amplification
Eastern grey kangaroo 19	-31.60846	150.60819	detected	*C. macropodum*	-
Eastern grey kangaroo 20	-34.61472	150.68475	detected	*C. hominis*	*C. hominis* IbA10G2
Eastern grey kangaroo 21	-34.61472	150.68475	detected	*C. hominis*	*C. hominis* IbA15G1
Cattle 1	-34.61278	150.585	detected	*C. hominis*	no amplification
Cattle 2	-34.60429	150.60170	detected	*C. hominis*	*C. hominis* IbA10G2
Cattle 3	-34.61283	150.58514	detected	*C. hominis*	no amplification
Cattle 4	-34.60429	150.60170	detected	*C. parvum*	*C. parvum* IIaA18G3R1
Cattle 5	-34.60642	150.60126	detected	*C. parvum*	*C. parvum* IIaA18G3R1
Cattle 6	-34.61373	150.5876	detected	*C. parvum*	*C. parvum* IIaA18G3R1
Cattle 7	-34.61373	150.5876	detected	*C. hominis*	*C. hominis* IbA10G2
Cattle 8	-34.6195	150.5242	detected	*C. hominis*	*C. hominis* IbA10G2
Cattle 9	-34.60429	150.60170	detected	*C. hominis*	*C. hominis* IbA10G2
Cattle 10	-34.63269	150.619	detected	*C. parvum*	*C. parvum* IIaA18G3R1
Sheep 1	-34.61556	150.68353	detected	*C. ubiquitum*	no amplification
Sheep 2	-34.61556	150.68353	detected	*C. ubiquitum*	no amplification
Sheep 3	-34.61743	150.68674	detected	*C. ubiquitum*	*C. ubiquitum* XIIa
Rabbit 1	-34.61954	150.62169	detected	*C. cuniculus*	no amplification
Rabbit 2	-34.61959	150.62172	detected	*C. cuniculus*	*C. cuniculus* VbA23
Rabbit 3	-34.61937	150.62178	detected	*C. cuniculus*	*C. cuniculus* VbA23
Rabbit 4	-34.61479	150.68492	detected	*C. cuniculus*	*C. cuniculus* VbA23
Rabbit 5	-34.61954	150.62169	detected	*C. cuniculus*	no amplification
Rabbit 6	-34.6195	150.52415	detected	*C. cuniculus*	no amplification
Rabbit 7	-34.61937	150.62178	detected	*C. cuniculus*	*C. cuniculus* VbA23
Rabbit 8	-34.61283	150.58514	detected	*C. cuniculus*	*C. cuniculus* VbA23
Rabbit 9	-34.61556	150.68353	detected	*C. cuniculus*	*C. cuniculus* VbA23
Rabbit 10	-34.61278	150.585	detected	*C. cuniculus*	no amplification
Rabbit 11	-34.61479	150.68492	detected	*C. cuniculus*	*C. cuniculus* VbA23
Rabbit 12	-34.60429	150.60170	detected	*C. cuniculus*	*C. cuniculus* VbA23
Rabbit 13	-34.18951	150.2885	detected	*C. cuniculus*	no amplification
Rabbit 14	-34.6327	150.619	detected	*C. cuniculus*	*C. cuniculus* VbA23

Sequences at the *gp60* locus were obtained for 14 kangaroo and four cattle isolates that were typed as *C. hominis* at the 18S rRNA locus. These samples failed to amplify at the *gp60*

locus using the primers of Strong et al. (2000) or Alves et al. (2003), which amplify an approx. 832 bp fragment, but were successfully amplified using the nested primers by Zhou et al. (2003), which amplify a 400 bp product. In approx. 50% of samples, the primary reaction did not produce a visible band by gel electrophoresis but a band of the correct size was visible for the secondary PCR, which was then confirmed by sequencing.

The *C. hominis* subtypes IbA10G2 and IdA15G1 were identified in 12 and 2 kangaroo samples respectively and the IbA10G2 subtype was also identified in four cattle samples (Table 2.4 and Fig 2.1A). The four *C. parvum* isolates from cattle were identified as subtype IIaA18G3R1 and the *C. cuniculus* isolates were subtyped as VbA23 ($n = 9$) (Table 2.4 and Fig 2.1B and 2.1D). Of the three *C. ubiquitum* positive isolates at 18S locus, only one isolate was successfully subtyped and identified as *C. ubiquitum* subtype XIIa (Table 2.4 and Fig 2.1C). Nucleotide sequences reported in this paper are available in the GenBank database under accession numbers; KX375346, KX375347, KX375348, KX375349, KX375350, KX375351, KX375352, KX375353, KX375354 and KX375355.

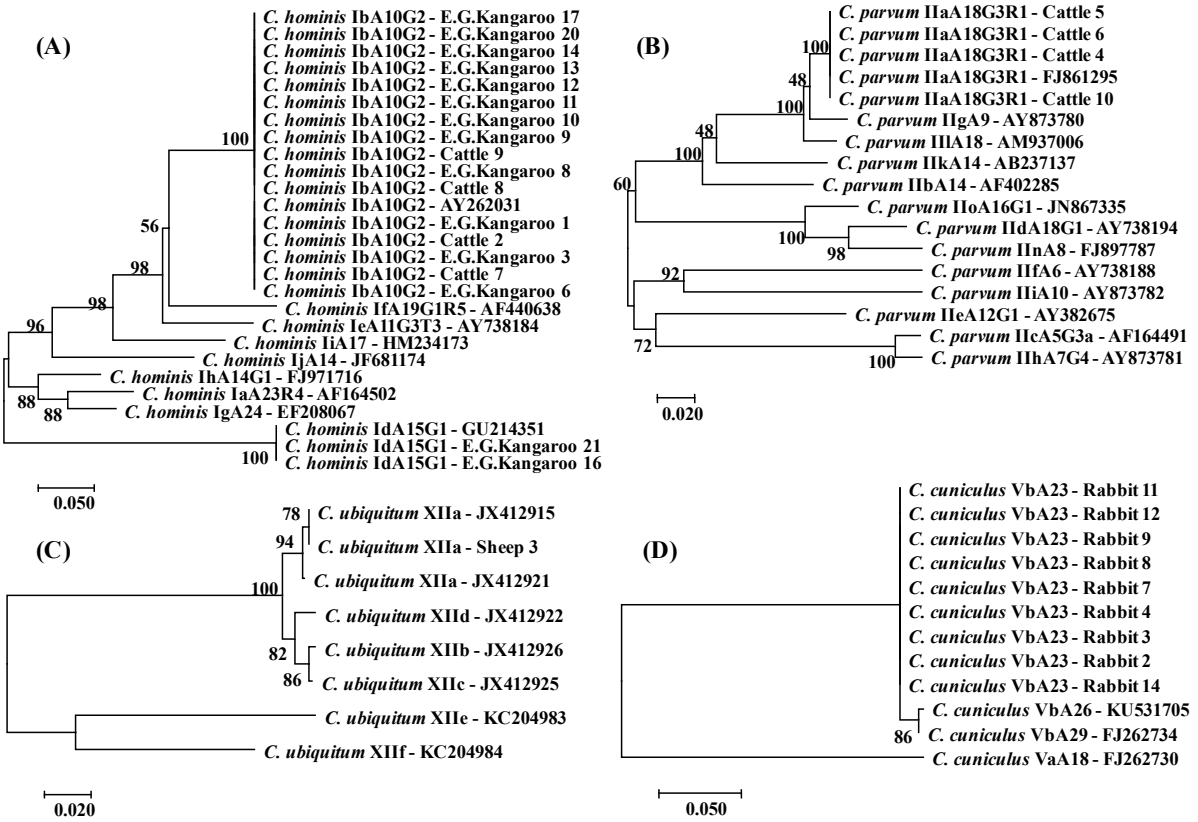

Fig 2.1. Phylogenetic relationships of *Cryptosporidium* subtypes inferred from Neighbor Joining (NJ) analysis of Kimura's distances calculated from pair-wise comparisons of *gp60* sequences. (A) Relationships among *C. hominis* subtypes. (B) Relationships among *C. parvum* subtypes. (C) Relationships between *C. ubiquitum* subtypes. (D) Relationships between *C. cuniculus* subtypes. Percentage support (>50%) from 1000 pseudoreplicates from NJ analyses is indicated at the left of the supported node.

2.6.3 Independent confirmation by the Australian Water Quality Centre (AWQC)

Blind independent analysis conducted by AWQC using the 18S rRNA nested PCR of Xiao et al. (2000) identified *C. hominis* in six samples, corresponding with the six positive samples from kangaroos, and failed to detect *Cryptosporidium* in the other two samples, one of which corresponded with the negative sample. Amplification of a region of *gp60* using the protocol described by Alves et al. (2003) failed to produce an amplicon for either the primary or secondary reactions. Amplification of *gp60* using the protocol described by Zhou et al. (2003), failed to amplify the correct-sized product for the primary PCR but produced amplicons

of the correct size for the secondary PCR for the six positive samples, which when sequenced were confirmed as *C. hominis* subtype IbA10G2. Amplification at the *lib13* locus was also successful for the six positive samples, which were confirmed as *C. hominis*. Amplification at the *MIC1* locus failed to produce any amplicons. The *gp60* and *MIC1* amplification failures were further investigated using PCR assays designed to target open reading frames (ORFs) flanking these two loci. All four primer sets produced strong amplification of the correctly sized fragments for the *C. parvum* and *C. hominis* control DNA. The cgd6-1070 ORF (located downstream of *gp60* in *C. parvum*), and cgd6-810 ORF (upstream of *MIC1*), both amplified from four of the six samples previously identified as *C. hominis*. In the case of the other 2 ORFs, weak amplification was observed for one sample for cgd6-1090 (upstream of *gp60*) and for two samples for cgd6-770 (downstream of *MIC1*). While only single bands were observed for the *C. parvum* and *C. hominis* controls, most of the faecal sample extracts produced multiple bands.

2.6.4 Enumeration of *Cryptosporidium* oocysts in faecal samples

Oocyst numbers per gram of faeces for all PCR positive samples were determined using qPCR at the *Clec* locus for 18 *C. hominis* and 4 *C. parvum* positives and for a subset of samples ($n = 8$) using microscopy (Table 2.5). For the 8 samples for which both microscopy and qPCR data were available, there was poor correlation between the two methods ($R^2 \approx 0.0095$ and ρ (rho) = 0.2026) (Table 2.5). Based on qPCR, the highest numbers of oocysts was detected in eastern grey kangaroo isolate 12 (16,890 oocysts/g^{-1}), which was identified as *C. hominis* subtype IbA10G2. No oocysts (<2g^{-1}) were detected by microscopy in this sample.

Table 2.5. *Cryptosporidium* oocyst numbers in positive samples per gram of faeces (g^{-1}) determined using microscopy and qPCR. Note: microscopy data was only available for 12 samples.

Host species	*Cryptosporidium* species (18S)	Oocyst numbers/g^{-1} microscopy	% Oocyst recovery	Oocyst numbers/g^{-1} qPCR
Eastern grey kangaroo 1	*C. hominis*	210	54	11,337
Eastern grey kangaroo 3	*C. hominis*	11,076	78	5,458
Eastern grey kangaroo 6	*C. hominis*	<2	61	9,528
Eastern grey kangaroo 8	*C. hominis*	<2	45	262
Eastern grey kangaroo 9	*C. hominis*	<2	74	648
Eastern grey kangaroo 10	*C. hominis*	<2	51	8,735
Eastern grey kangaroo 11	*C. hominis*	<2	67	131
Eastern grey kangaroo 12	*C. hominis*	<2	60	16,890
Eastern grey kangaroo 13	*C. hominis*	-	-	26
Eastern grey kangaroo 14	*C. hominis*	-	-	5,458
Eastern grey kangaroo 16	*C. hominis*	-	-	7,570
Eastern grey kangaroo 17	*C. hominis*	-	-	9,626
Eastern grey kangaroo 20	*C. hominis*	-	-	8,735
Eastern grey kangaroo 21	*C. hominis*	-	-	173
Cattle 2	*C. hominis*	-	-	144
Cattle 4	*C. parvum*	-	-	936
Cattle 5	*C. parvum*	-	-	1,819
Cattle 6	*C. parvum*	-	-	2,197
Cattle 7	*C. hominis*	-	-	4,205
Cattle 8	*C. hominis*	-	-	10,827
Cattle 9	*C. hominis*	-	-	15,804
Cattle 10	*C. parvum*	-	-	1,190

2.7 Discussion

The present study described the prevalence and molecular characterisation of *Cryptosporidium* species in faecal samples collected from kangaroo, cattle, sheep and rabbit faecal samples from Sydney's drinking water catchments. The overall prevalence of *Cryptosporidium* species in the faecal samples collected from four animal hosts was 5% and was 3.6% in kangaroos, 7% in cattle, 2.3% in sheep and 13.2% in rabbits. Overall, the prevalence of infection with *Cryptosporidium* was generally lower than that reported previously in Sydney catchments; 25.8% (Cox et al., 2005), 6.7% (Power et al., 2005) and 8.5% (Ng et al., 2011b) and Western Australian catchments; 6.7% (McCarthy et al., 2008). In the study by Ng et al. (2011b), the prevalence in eastern grey kangaroos was much higher (16.9%-27/160) than the 3.6% prevalence in kangaroo faecal samples in the present study. The overall prevalence of *Cryptosporidium* species in faecal samples collected from different species in

the present study was similar to the 2.8% (56/2,009) prevalence identified in faecal samples from animals in Melbourne water catchments (Nolan et al., 2013). The lower prevalence in the present study and the Melbourne study may be a consequence of testing a greater numbers of samples, seasonal and/or yearly variation in prevalence and/or proximity to agricultural land.

Based on sequence analysis using the 18S rRNA locus, a total of five *Cryptosporidium* species were identified; *C. macropodum* (*n* = 4), *C. hominis* (*n* = 23), *C. parvum* (*n* = 4), *C. ubiquitum* (*n* = 3) and *C. cuniculus* (*n* = 14). The prospect of livestock and wildlife being reservoirs for *C. hominis* has human-health implications, so to verify this finding, a subset of faecal samples was subjected to blinded independent analysis. This additional testing initially identified *C. hominis* following sequence analysis of a large fragment of the 18S rRNA gene amplified using the Xiao et al. (2000) nested PCR. It is noteworthy that the Xiao outer 18S PCR produced a clear amplification signal (threshold cycles between 24 and 29 for positive samples), suggesting the presence of reasonable numbers of oocysts with no evidence of PCR inhibition for this relatively large amplicon (approx. 1.2 kilobases). The *lib13* Taqman assay also identified *C. hominis* in these same samples. However, amplification of *gp60* using the Alves et al. (2003) nested PCR failed to amplify any *Cryptosporidium*, either as a nested PCR or by direct amplification using the inner primer set. Application of the Zhou et al. (2003) outer *gp60* primers (which are equivalent to the pairing of the Alves outer forward and inner reverse primers) also appeared to be unsuccessful (only four samples produced a band close to the expected size), but the Zhou *gp60* inner PCR amplified the correctly sized amplicon, which was confirmed to be *C. hominis* IbA10G2.

The failure to amplify *gp60* using the Alves et al. (2003) and Strong et al. (2000) assays was unexpected, especially considering the high degree of conservation for the primer binding sites across the *C. parvum* and *C. hominis gp60* subtypes and the successful amplification of the large 18S rRNA gene fragment, which demonstrates that the DNA quantity and quality was

sufficient for amplification within the first round of PCR. The lack of amplification at other loci is unlikely to be due to PCR inhibition, as spike analysis indicated no inhibition. To investigate this further, a published PCR assay targeting the *MIC1* locus from both *C. parvum* and *C. hominis* (Webber et al., 2014) was also tested and failed to amplify the expected fragment from these samples. The *MIC1* gene encodes a thrombospondin-like domain-containing protein, which is secreted in sporozoites prior to host cell attachment and localized to the apical complex after microneme discharge (Putignani et al., 2008). As secreted proteins often play a critical role in determining virulence and host specificity in host-pathogen relationships, it has been hypothesised that *MIC1* may play a role in the differences in host range observed between *C. parvum* and *C. hominis* (Webber et al., 2014). Previous analysis of the CryptoDB has identified that both the *gp60* and *MIC1* loci are on chromosome 6 and in close proximity (≈60 kb) (Webber et al., 2014), and it has previously been reported that these two genes are genetically linked (Cacciò et al., 2001). Given that three different *gp60* reverse primers appear to have failed, as well as failure of at least one of the *MIC1* primers, it would require the occurrence of multiple individual single nucleotide polymorphisms for the results to be accounted for by point mutations. Alternatively, a truncation or rearrangement on chromosome 6 affecting the 3' end of *gp60* and *MIC1* could affect these PCR assays. To test for any deletions affecting these loci, PCR assays were developed targeting flanking ORFs. The PCR assays targeting two ORFs in the region between *MIC1* and *gp60* (based on the *C. parvum* chromosome 6 map) were positive for some of the samples tested, suggesting that a wholesale deletion is not the cause for the failure to amplify *MIC1* or the entire *gp60*. The other two PCR assays produced equivocal results in the samples, although they yielded strong amplification in the positive controls. The variable sample results may have been due to a combination of the low amount of *Cryptosporidium* DNA present and non-specific amplification from other DNA in the sample extracts. The latter is likely, considering that the

positive controls produced a single amplicon, whereas most of the sample extracts yielded multiple fragments of different sizes.

Sequencing of chromosome six or the entire genome of this variant *C. hominis* is required to determine the underlying cause for the failure to amplify *MIC1* or the larger *gp60* region. Considering the role of *gp60* in host cell adhesion and the hypothesised role of *MIC1* in infection, it is possible that changes or loss of key genes involved in host specificity could explain the success of this particular variant of *C. hominis* in infecting hosts other than humans. If the function of these genes has been altered to better support infection in non-human hosts, then the infectivity of this variant in humans needs to be re-evaluated.

Of the detected species, all but *C. macropodum* have been reported to cause infection in humans at varying frequencies (Xiao, 2010; Ryan and Power, 2012). *Cryptosporidium hominis* and *C. parvum* are responsible for the majority of human infections worldwide (Xiao, 2010; Zahedi et al., 2016). In the present study, the prevalence of the variant *C. hominis* in kangaroo and cattle faecal samples was 2.9% (95% CI, 1.7%-4.7%) and 4.2% (95% CI, 1.6%-9%) respectively, and the prevalence of *C. parvum* in cattle faecal samples was 2.8% (95% CI, 0.8%-7.1%). Both of these parasites have been linked to numerous waterborne outbreaks around the world (Xiao, 2010; Baldursson and Karanis, 2011) and although this prevalence is relatively low, both these host species represent a risk of waterborne transmission to humans. A number of previous studies have identified *C. hominis/C. parvum*-like isolates at the 18S rRNA locus in marsupials including bandicoots (*Isoodon obesulus*), brushtail possums (*Trichosurus vulpecula*), eastern grey kangaroos (*Macropus giganteus*) and brush-tailed rock-wallabies (*Petrogale penicillata*) (Hill et al., 2008; Dowle et al., 2013; Vermeulen et al., 2015). However, in those studies, despite efforts, the identification of *C. hominis/C. parvum* could not be confirmed at other loci. This may be due to low numbers of oocysts and the multi-copy nature of the 18S rRNA gene, which provides better sensitivity at this locus. Alternatively,

failure to confirm identity in these other studies could be due the presence of variants with substantial differences in the diagnostic loci used, causing those PCR assays to fail. Such is the case in the present study, which for the first time has identified a novel *C. hominis* in kangaroo faecal samples based on analysis of multiple loci (18S rRNA, *Clec*, *MIC1*, *lib13* and *gp60*).

Cryptosporidium cuniculus, the most prevalent species detected here (13.2%), has been previously identified in rabbits, humans and a kangaroo in Australia (Nolan et al., 2010, 2013; Sari et al., 2013 unpublished - KF279538; Koehler et al., 2014a). It was implicated in a waterborne outbreak of cryptosporidiosis in humans in England in 2008 (Chalmers et al., 2009; Puleston et al., 2014) and has been linked to a number of sporadic human cases across the UK (Chalmers et al., 2011; Elwin et al., 2012), Nigeria (Molloy et al., 2010) and France (ANOFEL, 2010). *Cryptosporidium ubiquitum* was detected in three sheep samples and is a common human pathogen (Xiao, 2010), but has not been identified in Australia in the limited typing of Australian human *Cryptosporidium* isolates that has been conducted to date (Ryan and Power, 2012), however it has been identified in surface waters in Australia (Monis et al., unpublished).

Subtyping at the *gp60* locus identified the *C. hominis* subtype IbA10G2 in twelve kangaroo and four cattle faecal samples. This is a dominant subtype responsible for *C. hominis* associated outbreaks of cryptosporidiosis in the United States, Europe and Australia (Xiao, 2010; Ng et al., 2010, 2015; Segura et al., 2015). *Cryptosporidium hominis* has previously been reported in cattle in New Zealand (Abeywardena et al., 2012), Scotland (Smith et al., 2005), India (Feng et al., 2007) and Korea (Park et al., 2006). Subtyping at the *gp60* locus identified IbA10G2 (Smith et al., 2005; Abeywardena et al., 2012), and IdA15G1 (Feng et al., 2007). It has been suggested that the IbA10G2 infects cattle naturally in particular circumstances and thus could act as a zoonotic infection source in some instances (Smith et al., 2005). Interestingly, the studies that detected IbA10G2 in cattle, used PCR-based assays that only sequenced the 5' end of *gp60*, similar to the assay used in this study, so it is possible that these

reports also represent detection of a variant *C. hominis gp60*. This is the first report of the same subtype of *C. hominis* in kangaroos and cattle in the same catchment. In two kangaroo samples, the *C. hominis* IdA15G1 subtype was identified. This is also a common *C. hominis* subtype identified in humans worldwide (O'Brien et al., 2008; Sharma et al., 2013; Feng et al., 2014; Guo et al., 2015; Segura et al., 2015). The source and human health significance of the novel *C. hominis* detected in kangaroo and cattle samples in the present study is currently unknown. Environmental pollution from human and domestic animal faeces such as contamination of watersheds due to anthropogenic and agricultural activities conducted in the catchment area, in particular livestock farming, could be a potential source for wildlife infections with *C. hominis*. However, further studies are required to better understand the involvement of humans and livestock in the epidemiology of zoonotic *Cryptosporidium* species in wildlife.

The *C. parvum* subtype IIaA18G3R1 was identified in four cattle samples. IIaA18G3R1 is also a common subtype in both humans and cattle worldwide and has been reported widely in both calves and humans in Australia (Ryan and Power, 2012). Subtyping of the single *C. ubiquitum* isolate from sheep identified XIIa. To date six subtype families (XIIa to XIIf) have been identified in *C. ubiquitum* (Li et al., 2014). Of these, XIIa, XIIb, XIIc, and XIId have been found in humans and therefore XIIa is a potentially zoonotic subtype (Li et al., 2014). The *C. cuniculus* subtype identified in the present study was VbA23. Two distinct *gp60* subtype families, designated Va and Vb have been identified in *C. cuniculus* (Chalmers et al., 2009). Most cases described in humans relate to clade Va and the first waterborne outbreak was typed as VaA22 (Robinson et al., 2008; Chalmers et al., 2009). Previous studies in Australia have identified subtype VbA26 from an eastern grey kangaroo (Koehler et al., 2014b), subtypes VbA23R3 and VbA26R4 (Nolan et al., 2010, 2013), VbA22R4, VbA24R3 and VbA25R4 (Nolan et al., 2013) in rabbits and subtype VbA25 (Koehler et al., 2014b) and VbA27 (Sari et al., 2013 unpublished-KF279538) in a human patient.

Accurate quantification of *Cryptosporidium* oocysts in animal faecal deposits on land is important for estimating catchment *Cryptosporidium* loads. In the present study, oocyst concentration (numbers per gram of faeces-g^{-1}) was also determined for 18 *C. hominis* and four *C. parvum* positives using qPCR and for a subset of samples (n = 8) by microscopy. qPCR quantitation was conducted at the *Clec* locus rather than the 18S rRNA locus as the former is unique to *Cryptosporidium* and therefore more specific than the available 18S rRNA qPCR assays. There was poor correlation between qPCR and microscopy for the eight samples for which data from both methods were available, with qPCR detecting higher numbers of oocysts than microscopy with the exception of one sample (eastern grey kangaroo 3). Increased sensitivity of qPCR and the estimation of much higher numbers of oocysts in faecal samples by qPCR versus microscopy has been previously reported (Operario et al., 2015). A major limitation of qPCR is that the quantitative data generated are only as accurate as the standards used. A study which compared droplet digital PCR (ddPCR) (which provides absolute quantitation without the need for calibration curves) with qPCR, reported that qPCR overestimated the oocysts counts compared to ddPCR (Yang et al., 2014b). In the present study, the discrepancy between qPCR and microscopy could be due to a number of different factors; (1) IMS for microscopy and direct DNA extraction from faeces were conducted on different subsamples of each faecal sample and therefore the numbers of oocysts present in the subsamples may differ, (2) microscopy counts intact oocysts whereas qPCR will detect not only oocysts but also sporozoites that have been released from oocysts, other lifecycle stages and any free DNA, therefore qPCR may produce higher counts than microscopy. In the present study, the mean oocysts g^{-1} for kangaroos and cattle that were positive for *C. hominis* was 6,041 (range 26-16,890) and for cattle that were positive for *C. parvum* was 1,535 (range 936-2,197) as determined by qPCR. By microscopy, oocysts counts were available for kangaroo samples only and the mean was 5,643 (range <0.5-11,076). A previous study in WaterNSW catchments,

reported mean *Cryptosporidium* oocysts g^{-1} of 40 (range 1-5,988) for adult cattle, 25 for juvenile cattle (range <1-17,467), 23 for adult sheep (range <1-152,474), 49 for juvenile sheep (range <1-641) and 54 for adult kangaroos (range <1-39,423) (Davies et al., 2003). The age of the kangaroos and cattle sampled in the present study are unknown, but qPCR quantitation suggests that these were actual infections and not mechanical transmission. However, future studies should include oocyst purification via IMS prior to qPCR for more accurate quantitation. In addition, homogenisation of samples is important when comparing microscopy and qPCR i.e faecal slurries should be made, mixed well and aliquots of that mixture used for both microscopy and qPCR to ensure better consistency between techniques.

It is important to note that of the numbers of oocysts detected in animal faeces in catchments, only a fraction of oocysts may be infectious. For example, a recent study has shown that the infectivity fraction of oocysts within source water samples in South Australian catchments was low (~3.1%) (Swaffer et al., 2014). While it would be expected that oocysts in faecal samples would have much higher infectivity than oocysts in source water, reports suggest that only 50% of oocysts in fresh faeces are infectious, and that temperature and desiccation can rapidly inactivate oocysts in faeces while solar inactivation, predation and temperature will all impact oocyst survival in water (King and Monis, 2007).

The identification of mostly zoonotic *Cryptosporidium* species in animals inhabiting Sydney catchments indicates that there is a need to diligently monitor *Cryptosporidium* in source waters. Such monitoring is also critical, given the resistance of *Cryptosporidium* oocysts to chlorine (Yoder and Beach, 2010). Further studies are essential to confirm the nature of the *C. hominis* variant detected in this study and to determine if it represents an infection risk for humans.

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2.10 Chapter summary

For the purpose of this thesis, it is hypothesised that animals inhabiting drinking water catchments can potentially contribute a large volume of manure, and therefore *Cryptosporidium* oocysts to drinking water sources. This indicates the need for continued monitoring of animals in catchment areas to identify sources and carriers of zoonotic *Cryptosporidium* species in animal populations. Currently, in the case of *Cryptosporidium*, standard monitoring data are based on fluorescence microscopy, which does not include species and subtype identification. This results in inaccurate risk assessments that either under or overestimate the public health risk. This chapter successfully explored and characterised *Cryptosporidium* species in 952 faecal samples collected from both livestock and wildlife in Sydney's largest watersheds. All three of the aims outlined within this chapter's preface (Section 2.1) were satisfied; the prevalence of *Cryptosporidium* in faecal samples collected from various hosts was determined by qPCR at the 18S locus, samples positive for *Cryptosporidium* were characterised at three loci including 18S, *gp60* and *clec*, and the results were independently confirmed by the Australian Water Quality Centre (AWQC). Oocyst numbers per gram of faeces for all PCR positive samples were also determined using qPCR and for a subset of samples (n = 8) using microscopy. Of the five *Cryptosporidium* species identified in this study, four species are of public health significance; *C. hominis* (detected in both cattle and kangaroos) was the most prevalent species detected in total of 952 faecal samples, followed by *C. cuniculus* (from rabbits), *C. parvum* (from cattle), *C. macropodum* (from kangaroos), *C. ubiquitum* (from sheep). One of the major outcomes of this chapter was successful subtyping of *C. hominis* IbA10G2 in twelve kangaroo and four cattle, and *C. hominis* IdA15G1 in two kangaroo faecal samples at the *gp60* locus. Subtype IbA10G2 is a dominant subtype responsible for *C. hominis* associated outbreaks of cryptosporidiosis worldwide and

has also been previously reported in a range of animals, including cattle. However, this is the first report of this subtype of *C. hominis* in kangaroos. Other *Cryptosporidium gp60* subtypes identified in this chapter are also of public health significance (*C. hominis* IdA15G1, *C. parvum* IIaA18G3R1, *C. cuniculus* VbA23, and *C. ubiquitum* XIIa). The presence of zoonotic *Cryptosporidium* species in both livestock and wildlife inhabiting drinking water catchments may have implications for management of drinking water sources. Therefore, continued identification of the sources/carriers of human pathogenic strains would be useful to more accurately assess risk. Although the approach taken in this chapter provides a wealth of information on the species of *Cryptosporidium* present in faecal samples, it was unable to further investigate intra-isolate diversity of low-abundance intra-isolate variants of *Cryptosporidium* at the *gp60* locus which is crucial to better understand the epidemiology and transmission dynamics of cryptosporidiosis. In order to address this knowledge gap, in Chapter three compared Sanger and Next Generation Sequencing of *gp60* amplicons from a subset of samples from this chapter and additional samples from China were compared to examine the extent of within-host genetic diversity of *Cryptosporidium gp60* subtypes from different geographic areas.

Chapter Three - Next Generation Sequencing uncovers within-host differences in the genetic diversity of *Cryptosporidium gp60* subtypes

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3.1 Preface

The finding of zoonotic *Cryptosporidium* species in both livestock and wildlife inhabiting Sydney's drinking water catchments indicates a need to investigate the nature of the *C. hominis, C. parvum* and *C. cuniculus* variants identified in these animals to better estimate the potential risk for humans. Therefore, in this chapter the molecular findings described in Chapter Two are expanded upon by further investigating the composition of *Cryptosporidium gp60* subtypes in samples positive for zoonotic *Cryptosporidium* species. It consists of a published paper in the International Journal for Parasitology, entitled "Next Generation Sequencing uncovers within-host differences in the genetic diversity of *Cryptosporidium gp60* subtypes". The text of this manuscript is the same as the published version of the paper found in the Appendices (*see* Appendix 4) except for modifications, such as in-thesis referencing. This chapter particularly aims to use high throughput NGS to better identify levels of intra-isolate diversity of zoonotic *Cryptosporidium* species compared to conventional Sanger sequencing, in three different *Cryptosporidium* species (*C. hominis, C. parvum* and *C. cuniculus*) sourced from two distinct geographic regions (Australia and China).

3.2 Statement of contribution

As first author, **AZ** developed the concept and, conducted all the labwork and data analysis, and prepared the original and final draft of the manuscript. **FJ** provided assistance in labwork and **AG** helped with sample preparation for Next Generation Sequencing and data analysis. **AP** and **CO** contributed to manuscript preparation. **AB** provided logistic support for sample collection in Australia and revised the manuscript. **IR** assisted with statistical analysis, and

reviewed and edited the manuscript. **UR** supervised the project, was directly involved in every aspect of the research, contributed substantially to drafts and critically revised the manuscript.

AZ: 65%

3.3 Abstract

The extent of within-host genetic diversity of parasites has implications for our understanding of the epidemiology, disease severity and evolution of parasite virulence. As with many other species, our understanding of the within-host diversity of the enteric parasite *Cryptosporidium* is changing. The present study compared Sanger and Next Generation Sequencing (NGS) of glycoprotein 60 (*gp60*) amplicons from *Cryptosporidium hominis* (*n* = 11), *Cryptosporidium parvum* (*n* = 22) and *Cryptosporidium cuniculus* (*n* = 8) DNA samples from Australia and China. Sanger sequencing identified only one *gp60* subtype in each DNA sample: one *C. hominis* subtype (IbA10G2) (*n* = 11), four *C. parvum* subtypes belonging to IIa (*n* = 3) and IId (*n* = 19) and one *C. cuniculus* subtype (VbA23) (*n* = 8). NGS identified the same subtypes initially identified by Sanger sequencing, but also identified additional *gp60* subtypes in *C. parvum* and *C. cuniculus* but not in *C. hominis*, DNA samples. The number of *C. parvum* and *C. cuniculus* subtypes identified by NGS within individual DNA samples ranged from two to four, and both *C. parvum* IIa and IId subtype families were identified within the one host in two samples. The finding of the present study has important implications for *Cryptosporidium* transmission tracking as well as vaccine and drug studies.

3.4 Introduction

Humans and animals often become co-infected with different species and genotypes of the same parasite genus, resulting in within-host parasite interactions (Holmes and Price, 1986; Read and Taylor, 2001; Choisy and de Roode, 2010; Seppälä and Jokela, 2016). Importantly, the presence of co-infecting parasite species genotypes within a host can potentially modify parasite fitness by allowing them to adapt to different selection pressures and can drive the

evolution of parasite virulence and alter host susceptibility to other parasites, infection duration, disease severity, transmission risks, clinical symptoms and consequently treatment and prevention strategies (Vaumourin et al., 2015; Grinberg and Widmer, 2016; Seppälä and Jokela, 2016). Therefore, within-host parasite interactions have important repercussions for human or animal health. For instance, parasite co-infections within a single host can result in gene exchange via recombination. This can drive parasite evolution by making the parasites more resistant to drugs. Interactions among co-infecting parasite species, genotypes and subtypes of the same parasite genus can also modify co-evolutionary dynamics between the host and parasites. In addition, parasite interactions can help with maintaining genetic variation in parasite traits such as infectivity and virulence which are crucial components of pathogen fitness and are important to better understand disease dynamics and the changing epidemiology of parasitic diseases (Seppälä et al., 2012; Vaumourin et al., 2015; Seppälä and Jokela, 2016).

Cryptosporidium spp. are ubiquitous enteric parasites that infect a broad range of hosts including humans and animals (Xiao, 2010). They are a major contributor to moderate-to-severe diarrhoeal disease in developing countries and are second only to rotavirus as a cause of moderate-to-severe diarrhoea in children younger than 2 years (Kotloff et al., 2013). Of the 37 species currently recognised, *Cryptosporidium parvum* and *Cryptosporidium hominis* account for the majority of human infections and typed outbreaks (Xiao, 2010; Ryan et al., 2017; Zahedi et al., 2017a; Čondlová et al., 2018; Kváč et al., 2018), with the exception of *Cryptosporidium cuniculus* which was responsible for a waterborne outbreak in the UK (Puleston et al., 2014). The parasite is transmitted via the faecal-oral route through human to human, animal to human and animal to animal contact, and via contaminated water; therefore, hosts are exposed to multiple sources of potentially genetically diverse oocysts (Xiao, 2010; Grinberg and Widmer, 2016). Once ingested, sporozoites excyst from the oocyst, invade the host cells and undergo subsequent rounds of asexual and sexual reproduction.

Currently, the only available drug for human infections (nitazoxanide - Romark Laboratories, Florida, USA), has variable efficacy (Abubakar et al., 2007; Amadi et al., 2009) and an effective vaccine has yet to be developed (Mead, 2014; Ryan et al., 2016). Halofuginone lactate (Halocur; Intervet, New Zealand) is commercially available against cryptosporidiosis in dairy calves, with variable efficacy (Trotz-Williams et al., 2011; Almawly et al., 2013). Therefore, *Cryptosporidium* control currently relies mainly on improved sanitation and understanding its transmission dynamics.

Analysis of the extent of within-host genetic diversity in *Cryptosporidium* has been hampered due to the difficulties in culturing this parasite, with clonal lineages derived from individual sporozoites unavailable (Grinberg and Widmer, 2016). Of the multilocus sequencing typing strategies employed to examine within-host genetic diversity, sequence analysis of the glycoprotein 60 (*gp60*) gene is the most common (Xiao, 2010), as it is the most polymorphic locus in the genome (Abrahamsen et al., 2004). Despite the importance of within-host genetic diversity for our understanding of cryptosporidiosis epidemiology, relatively little is known (Cama et al., 2006; Jeníková et al., 2011; Waldron and Power, 2011; Ramo et al., 2014, 2016; Shrestha et al., 2014). Most studies have relied on conventional PCR and Sanger-based genotyping methods, and automated fragment analysis, however a major limitation of these approaches is their inability to resolve complex DNA mixtures and detect low-abundance intra-isolate variants (Paparini et al., 2015; Grinberg and Widmer, 2016).

Next Generation Sequencing (NGS) of amplicons offers the advantage of massive parallelization of sequencing reactions to more effectively identify low-abundance genotypes in mixed infections. To date, only one study has examined the extent of intra-isolate diversity of *Cryptosporidium* at the *gp60* locus using NGS (Grinberg et al., 2013). In that study, NGS analysis of two *C. parvum* samples from one geographic location (New Zealand) revealed much higher levels of intra-isolate diversity compared with Sanger sequencing. In the present

study, we examined intra-host genetic diversity of a much larger number of *Cryptosporidium* samples (*n* = 41) from three different species (*C. hominis*, *C. parvum* and *C. cuniculus*) and from two distinct geographic regions (Australia and China), using both NGS and conventional Sanger sequencing at the *gp60* locus to better understand the epidemiology of this important parasite. Animal faecal samples in Australia were collected from watersheds within the WaterNSW (New South Wales) area of operations and included two dairy farms and faecal samples collected from the surrounding bushland. Faecal samples from China were collected from a cattle breeding centre and two dairy farms, all located in Henan province.

3.5 Materials and methods

3.5.1 Sample collection and processing

A total of 41 DNA samples positive for *Cryptosporidium*, belonging to three *Cryptosporidium* spp. (as determined by Sanger sequencing - *see* Section 3.5.3), were analysed in the present study; *C. parvum* (*n* = 22) from cattle (*Bos taurus*), *C. hominis* (*n* = 11) from eastern grey kangaroos (*Macropus giganteus*), and *C. cuniculus* (*n* = 8) from rabbits (*Oryctolagus cuniculus*).

3.5.2 DNA isolation

Upon collection, faecal samples were stored at 4°C until analysed. Following five cycles of freeze–thaw, genomic DNA was extracted from 250 mg of each faecal sample using a Power Soil DNA Kit (MO BIO, Carlsbad, California, USA). Extraction blanks (no faecal sample) were used in each extraction group. Purified DNA was stored at -20°C prior to PCR. DNA

extraction and post-DNA extraction procedures were performed in separate dedicated laboratories.

3.5.3 Sanger sequencing

All samples were initially identified to species level at the 18S locus using nested PCR amplification and Sanger sequencing of a fragment of the 18S locus as previously described (Silva et al., 2013). Samples were then subtyped at the *gp60* locus using a nested PCR to amplify an approximately 400 bp product using the primers AL3531 (5′ ATAGTCTCCGCTGTATTC 3′) and AL3533 (5′ GAGATATATCTTGGTGCG 3′) for the primary PCR, and AL3532 (5′ TCCGCTGTATTCTCAGCC 3′) and LX0029 (5′ CGAACCACATTACAAATGAAGT 3′) for the secondary PCR (Sulaiman et al., 2005). Each 25 µl PCR mixture contained 1 µl of genomic DNA, 1x KAPA Taq buffer (KAPA Biosystems, South Africa), 3.75 mM $MgCl_2$, 400 µM of each dNTP, 0.4 µM of forward and reverse primers and 1 U of Kapa Taq DNA polymerase (KAPA Biosystems, South Africa). The PCR cycling conditions were modified and consisted of an initial denaturation at 94°C for 3 min and then 40 cycles of 94°C for 45 sec, 54°C for 45 sec and 72°C for 1 min, followed by a final extension step at 72°C for 7 min. PCR contamination controls were used including negative controls. PCR setup and DNA handling procedures were performed in separate and dedicated exclusion hoods; PCR and post-PCR procedures were performed in separate dedicated laboratories.

Gel electrophoresis was used to separate the amplified DNA fragments from the secondary PCR products at the *gp60* locus, which were subsequently purified for sequencing using an in-house filter tip method as previously described (Yang et al., 2013). Purified PCR products were sequenced independently in both directions using an ABI Prism™ Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, California, USA)

according to the manufacturer's instructions and with a 54°C annealing temperature. Sanger sequencing chromatogram files were imported into Geneious Pro 8.1.6 (Kearse et al., 2012), and the nucleotide sequences of each gene were analysed and aligned with reference sequences from GenBank using Clustal W (www.clustalw.genome.jp).

3.5.4 Next Generation Sequencing (NGS)

Partial *Cryptosporidium gp60* gene sequences were amplified for NGS on the MiSeq (Illumina) platform using the same assay described for Sanger sequencing (Sulaiman et al., 2005), with the exception that secondary PCR primers were modified to contain MiSeq adapter sequences on the 5′ end, as per standard protocols for the MiSeq platform (Illumina Demonstrated Protocol: Metagenomic Sequencing Library Preparation). PCRs were performed in 25 µl volumes containing PCR buffer (KAPA Biosystems), 2 mM $MgCl_2$, 0.01 mg of BSA (Fisher Biotech, Australia), 1 mM dNTPs (Fisher Biotech), 0.4 µM of each primer and 0.5 U of KAPA Taq DNA Polymerase (KAPA Biosystems, South Africa). Primary PCRs used 2 µl of DNA as a template and secondary reactions contained 1-2 µl of the primary product as a template. All PCRs contained no-template controls and extraction reagent blank controls. All PCRs were performed with an initial denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 30 sec, 50°C for 30 sec and 72°C for1 min, and a final extension period at 72°C for 5 min.

From the resulting *Cryptosporidium gp60* amplicons, sequencing libraries for the MiSeq sequencing platform were produced according to Illumina recommended protocols (Illumina Demonstrated Protocol: Metagenomic Sequencing Library Preparation), with the following amendments. Briefly, purified, uniquely indexed libraries from individual DNA samples were pooled for sequencing in equimolar quantities based on the fluorescent intensity of amplicon

libraries after electrophoresis through a 2% agarose gel stained with GelRed (Fisher Biotech) and visualised under UV light. Sequencing was performed on an Illumina MiSeq using 500-cycle V2 chemistry (250 paired-end reads) following the manufacturer's recommendations.

3.5.5 Bioinformatics analysis

Sequences were processed to retain only reads with perfect AL3532 and LX0029 primer sequences (no mismatches allowed). Primer sequences and distal bases were removed in Geneious Pro 8.1.6 (Kearse et al., 2012) and remaining reads were quality filtered using USEARCH v9.1.13 (Edgar, 2010), retaining only sequences with a <1% expected error rate (96.4% of sequences retained). Singletons, doubletons, and sequences with replicate copies less than 0.01% of the total number of unique sequences per sample (14.8% of quality filtered sequences) were discarded due to their high probability of being generated by sequencing and/or PCR error. Chimeric sequences (<0.08%) were identified and removed using USEARCH v9.1.13 (Edgar, 2010).

All remaining high-quality sequences were compared, using BLAST (Altschul et al., 1990), with an in-house reference database containing 131 *Cryptosporidium gp60* sequences from all characterised *C. parvum*, *C. hominis* and *C. cuniculus* subtypes available in GenBank (Supplementary Table 3-S1 - Digital appendix 1). For *C. hominis* and *C. parvum*, *gp60* subtypes were only assigned if NGS reads matched identically (100% pairwise identify and query coverage) to only one *gp60* subtype reference sequence. For *C. cuniculus*, all available reference GenBank sequences were 1-3 bp shorter at the 5′ end compared with the NGS reads obtained in the present study. To accommodate this incongruity between query and reference sequences, *C. cuniculus* subtypes were assigned only if NGS reads matched to only one *C.*

cuniculus gp60 subtype reference sequence with 100% pairwise identify and 99% query coverage.

3.6 Results

3.6.1 Sanger sequencing

Sanger sequencing identified one subtype per amplicon with only one *C. hominis* subtype (IbA10G2) identified in all 11 *C. hominis* DNA samples from eastern grey kangaroos. Four *C. parvum* subtypes were identified in ruminant-derived DNA samples from Australia and China: IIaA16G2R1 (*n* = 3), IIdA15G1 (*n* = 2), IIdA18G3R1 (*n* = 2), IIdA19G1 (*n* = 15); and only one *C. cuniculus* subtype (VbA23) was identified in all eight DNA samples positive for *C. cuniculus* from rabbits (Table 3.1). Both IIa and IId *C. parvum* subtype families were identified in Australian samples positive for *C. parvum*, but only the IId *C. parvum* subtype family was identified in samples from China.

3.6.2 NGS

From the 41 DNA samples, a total of 566,719 high quality NGS reads were obtained after initial quality filtering procedures. For the 11 *C. hominis* DNA samples, there was 100% agreement between Sanger and NGS sequencing with *C. hominis* IbA10G2, the only subtype identified in assigned reads (Table 3.1). For the 22 *C. parvum* samples, however, although the subtype identified by Sanger was also the main subtype identified by NGS, multiple additional subtypes, ranging from 0.4% to 31% of the total assigned reads, were identified. A total of 11 *C. parvum* subtypes were identified by NGS; IIaA14G2R1 (*n* = 3), IIaA15G2R1 (*n* = 3),

IIaA16G2R1 (*n* = 3), IIaA16G3R1 (*n* = 2), IIdA14G1 (*n* = 2), IIdA15G1 (*n* = 2), IIdA17G1 (*n* = 7), IIdA18G1 (*n* = 15), IIdA18G3R1 (*n* = 2), IIdA19G1 (*n* = 15), IIdA20G1 (*n* = 12). The number of *C. parvum* subtypes identified by NGS within individual samples ranged from two to four, with both IIa and IId subtype families identified within the one host in two samples (i.e. AUSC9 and AUSC20) (Table 3.1).

For *C. cuniculus*, as with *C. parvum*, the subtype identified by Sanger was also identified by NGS, with multiple additional subtypes ranging from 0.4% to 6.7% of the total assigned reads identified. A total of three *C. cuniculus* subtypes were identified by NGS: VbA22 (*n* = 8); VbA23 (*n* = 8); VbA25 (*n* = 8); with all three subtypes identified within individual DNA samples.

The extremely high level of stringency used in identifying the *C. parvum*, *C. hominis*, and *C. cuniculus* subtypes resulted in a high number of unassigned reads that failed to match known reference sequences with 100% pairwise identity and 100% query cover (or 99% for *C. cuniculus*) (Table 3.1).

Table 3.1. Comparison of glycoprotein 60 (*gp60*) subtypes identified in *Cryptosporidium* spp. from Australia and China using Sanger and Next Generation Sequencing (NGS). Genotypes in bold are the main subtypes identified by NGS, based on fraction of assigned reads. EGK, Eastern Grey kangaroo (Macropus giganteus).

Sample code	**Host**	**Country of origin**	**Sanger *gp60* subtype**	**Number of NGS sequences**	**Number and (%) of NGS sequences assigned**	**NGS *gp60* subtypes**
EGK 1	EGK	Australia	*C. hominis* IbA10G2	15,609	7,896 (50.6)	***C. hominis* IbA10G2**
EGK 2	EGK	Australia	*C. hominis* IbA10G2	13,335	6,651(49.9)	***C. hominis* IbA10G2**
EGK 3	EGK	Australia	*C. hominis* IbA10G2	7,845	4,019 (54.2)	***C. hominis* IbA10G2**
EGK 4	EGK	Australia	*C. hominis* IbA10G2	1,963	845 43.0	***C. hominis* IbA10G2**
EGK 5	EGK	Australia	*C. hominis* IbA10G2	1,840	858 (46.6)	***C. hominis* IbA10G2**
EGK 6	EGK	Australia	*C. hominis* IbA10G2	1,869	781 (41.8)	***C. hominis* IbA10G2**
EGK 8	EGK	Australia	*C. hominis* IbA10G2	2,024	900 (44.5)	***C. hominis* IbA10G2**
EGK 9	EGK	Australia	*C. hominis* IbA10G2	2,473	1,156 (46.7)	***C. hominis* IbA10G2**
EGK 10	EGK	Australia	*C. hominis* IbA10G2	12,760	6,291 (51.0)	***C. hominis* IbA10G2**
EGK 11	EGK	Australia	*C. hominis* IbA10G2	12,824	6,536 (51.5)	***C. hominis* IbA10G2**
EGK 12	EGK	Australia	*C. hominis* IbA10G2	12,927	6,663 (48.4)	***C. hominis* IbA10G2**
AUSC 9	Cattle	Australia	*C. parvum* IIdA18G3R1	10,524	4,871 (46.2)	***C. parvum* IIdA18G3R1**
					228 (2.1)	*C. parvum* IIaA16G3R1
AUSC 20	Cattle	Australia	*C. parvum* IIdA18G3R1	17,161	8,223 (47.9)	***C. parvum* IIdA18G3R1**
					399 (2.3)	*C. parvum* IIaA16G3R1
AUSC 21	Cattle	Australia	*C. parvum* IIaA16G2R1	24,600	2,670 (10.8)	***C. parvum* IIaA16G2R1**
					531 (2.1)	*C. parvum* IIaA15G2R1
					102 (0.4)	*C. parvum* IIaA14G2R1
AUSC 22	Cattle	Australia	*C. parvum* IIaA16G2R1	21,155	2,313 (10.9)	***C. parvum* IIaA16G2R1**
					512 (2.4)	*C. parvum* IIaA15G2R1
					91 (0.4)	*C. parvum* IIaA14G2R1
AUSC 24	Cattle	Australia	*C. parvum* IIaA16G2R1	21,838	2,458 (11.2)	***C. parvum* IIaA16G2R1**
					485 (2.2)	*C. parvum* IIaA15G2R1
					95 (0.4)	*C. parvum* IIaA14G2R1
AUSC 25	Rabbit	Australia	*C. cuniculus* VbA23	14,597	4,941 (33.8)	***C. cuniculus* VbA23**

					437 (2.9)	*C. cuniculus* VbA25
					61 (0.4)	*C. cuniculus* VbA22
AUSC 26	Rabbit	Australia	*C. cuniculus* VbA23	9,177	3,654 (39.8)	***C. cuniculus* VbA23**
					349 (3.8)	*C. cuniculus* VbA25
					50 (0.5)	*C. cuniculus* VbA22
AUSC 27	Rabbit	Australia	*C. cuniculus* VbA23	7,340	2,984 (40.6)	***C. cuniculus* VbA23**
					492 (6.7)	*C. cuniculus* VbA25
					58 (0.7)	*C. cuniculus* VbA22
AUSC 28	Rabbit	Australia	*C. cuniculus* VbA23	11,031	4,226 (38.3)	***C. cuniculus* VbA23**
					487 (4.41)	*C. cuniculus* VbA25
					79 (0.7)	*C. cuniculus* VbA22
AUSC 29	Rabbit	Australia	*C. cuniculus* VbA23	10,548	3,863 (36.6)	***C. cuniculus* VbA23**
					401 (3.8)	*C. cuniculus* VbA25
					71 (0.6)	*C. cuniculus* VbA22
AUSC 30	Rabbit	Australia	*C. cuniculus* VbA23	12,066	4,794 (39.7)	***C. cuniculus* VbA23**
					387 (3.2)	*C. cuniculus* VbA25
					80 (0.6)	*C. cuniculus* VbA22
AUSC 31	Rabbit	Australia	*C. cuniculus* VbA23	9,006	3,609 (40)	***C. cuniculus* VbA23**
					312 (3.4)	*C. cuniculus* VbA25
					81 (0.8)	*C. cuniculus* VbA22
AUSC 32	Rabbit	Australia	*C. cuniculus* VbA23	14,987	5,743 (38.3)	***C. cuniculus* VbA23**
					581 (3.8)	*C. cuniculus* VbA25
					156 (1)	*C. cuniculus* VbA22
ChS 1	Sheep	China	*C. parvum* IIdA15G1	15,851	7,577 (47.8)	***C. parvum* IIdA15G1**
					932 (5.8)	*C. parvum* IIdA14G1
ChG 3	Goat	China	*C. parvum* IIdA15G1	23,637	7,346 (31)	***C. parvum* IIdA14G1**
					6,681 (28.2)	*C. parvum* IIdA15G1
ChC 6	Cattle	China	*C. parvum* IIdA19G1	22,857	11,169 (48.8)	***C. parvum* IIdA19G1**
					2,430 (10.7)	*C. parvum* IIdA18G1

					359 (1.5)	*C. parvum* IIdA17G1
					171 (0.7)	*C. parvum* IIdA20G1
ChC 7	Cattle	China	*C. parvum* IIdA19G1	17,491	8,103 (46.3)	***C. parvum* IIdA19G1**
					1,736 (1)	*C. parvum* IIdA18G1
					271 (1.5)	*C. parvum* IIdA17G1
					130 (0.7)	*C. parvum* IIdA20G1
ChC 8	Cattle	China	*C. parvum* IIdA19G1	22,137	10,857 (49)	***C. parvum* IIdA19G1**
					2,766 (12.4)	*C. parvum* IIdA18G1
					582 (2.6)	*C. parvum* IIdA17G1
					253 (1.1)	*C. parvum* IIdA20G1
ChC 9	Cattle	China	*C. parvum* IIdA19G1	13,843	6,891 (49.7)	***C. parvum* IIdA19G1**
					1,477 (10.6)	*C. parvum* IIdA18G1
					280 (2)	*C. parvum* IIdA17G1
					113 (0.8)	*C. parvum* IIdA20G1
ChC 10	Cattle	China	*C. parvum* IIdA19G1	9,854	3,859 (39.1)	***C. parvum* IIdA19G1**
					632 (6.4)	*C. parvum* IIdA18G1
ChC 11	Cattle	China	*C. parvum* IIdA19G1	16,988	8,543 (50.2)	***C. parvum* IIdA19G1**
					2,150 (12.6)	*C. parvum* IIdA18G1
					128 (0.7)	*C. parvum* IIdA20G1
ChC 12	Cattle	China	*C. parvum* IIdA19G1	15,253	7,856 (51.5)	***C. parvum* IIdA19G1**
					1,902 (12.4)	*C. parvum* IIdA18G1
					118 (0.7)	*C. parvum* IIdA20G1
ChC 13	Cattle	China	*C. parvum* IIdA19G1	19,493	8,945 (45.8)	***C. parvum* IIdA19G1**
					2,289 (11.7)	*C. parvum* IIdA18G1
					591 (3)	*C. parvum* IIdA17G1
					176 (0.9)	*C. parvum* IIdA20G1
ChC 14	Cattle	China	*C. parvum* IIdA19G1	11,222	5,873 (52.3)	***C. parvum* IIdA19G1**
					1,238 (11)	*C. parvum* IIdA18G1
					80 (0.7)	*C. parvum* IIdA20G1
ChC 15	Cattle	China	*C. parvum* IIdA19G1	16,251	8,125 (49.9)	***C. parvum* IIdA19G1**

					1,975 (12.1)	*C. parvum* IIdA18G1
					126 (0.7)	*C. parvum* IIdA20G1
ChC 17	Cattle	China	*C. parvum* IIdA19G1	21,536	11,598 (53.8)	***C. parvum* IIdA19G1**
					2,464 (11.4)	*C. parvum* IIdA18G1
					204 (0.9)	*C. parvum* IIdA20G1
ChC 18	Cattle	China	*C. parvum* IIdA19G1	13,933	6,801 (48.8)	***C. parvum* IIdA19G1**
					1,404 (10)	*C. parvum* IIdA18G1
ChC 20	Cattle	China	*C. parvum* IIdA19G1	17,406	7,152 (41.1)	***C. parvum* IIdA19G1**
					1,294 (7.4)	*C. parvum* IIdA18G1
ChC 24	Cattle	China	*C. parvum* IIdA19G1	15,193	8,117 (51.3)	***C. parvum* IIdA19G1**
					1,885 (12.4)	*C. parvum* IIdA18G1
					347 (2.2)	*C. parvum* IIdA17G1
					125 (0.8)	*C. parvum* IIdA20G1
ChC 25	Cattle	China	*C. parvum* IIdA19G1	24,275	11,985 (49.3)	***C. parvum* IIdA19G1**
					2,694 (11.1)	*C. parvum* IIdA18G1
					598 (2.4)	*C. parvum* IIdA17G1
					213 (0.8)	*C. parvum* IIdA20G1

3.7 Discussion

In the present study, the extent of within-host diversity of *gp60* subtypes in three *Cryptosporidium* spp. (*C. hominis*, *C. parvum* and *C. cuniculus*) from two geographic locations (Australia and China) was analysed using Sanger and NGS. Sanger sequencing identified only one *gp60* subtype in each DNA sample (positive for *Cryptosporidium*); NGS identified the same subtype, but also identified additional within-host subtypes for samples positive for *C. parvum* and *C. cuniculus*, but not for *C. hominis*. The direct Sanger sequencing of PCR amplicons employed in the present study is not necessarily the best-suited method for uncovering within-host diversity. Other tools such as sequencing clones and Single Strand Conformational Polymorphism (SSCP) can also be employed to identify minor sequence variants or to determine whether products of a PCR are homogeneous or heterogeneous. All the Australian samples were sourced from water catchments in Sydney (New South Wales), and Sanger-based typing of these samples have been previously discussed (Chapter 2). Typing of the samples from China (collected from dairy cattle farms) has also been previously reported (Wang et al., 2011; 2014).

Two *C. parvum* subtype families (IIa and IId) were identified by both Sanger and NGS in ruminant-derived DNA samples from both Australia and China, with four and 11 subtypes identified, respectively. The IIa and IId subtypes are found in both humans and ruminants and are responsible for zoonotic cryptosporidiosis (Xiao, 2010). One *C. cuniculus* subtype family (Vb) was identified by both Sanger and NGS, with one and three subtypes identified in individual samples, respectively. Two *gp60* subtype families (Va and Vb) have been previously identified in *C. cuniculus*, with most human cases of cryptosporidiosis caused by the Va subtype family, including the first waterborne outbreak of cryptosporidiosis with *C. cuniculus* in the UK which was typed as VaA22 (Puleston et al., 2014). Both Sanger and NGS identified

only one *C. hominis* subtype (IbA10G2) in all 11 DNA samples from kangaroos. This is a dominant subtype responsible for the majority of *C. hominis*-associated outbreaks worldwide (Xiao, 2010) and the identification of this subtype in kangaroos has previously been independently confirmed (Zahedi et al., 2016).

The finding of multiple *gp60* subtypes (2-4) in individual hosts in the present study is consistent with the study by Grinberg et al. (2013) which identified 10 unique subtypes within a single *C. parvum* sample through NGS techniques. However, in that study, the number of identified subtypes for a single sample using NGS is much greater than that observed in our study. However, in the study by Grinberg et al. (2013), two of the four least abundant subtypes were only observed once (singletons) out of a total of 1,589 sequence types and the remaining two subtypes fell outside the percentage cut-off used in this present study. Therefore, it is likely that a more stringent analysis would have resulted in a reduced number of subtypes being identified.

There are several limitations of the present study including the possibility that assignment of multiple subtypes may have been due to NGS sequence artefacts. Error rates for Illumina Miseq sequencing have been estimated at an average of 0.9 errors per 100 bases (Loman et al., 2012; Salipante et al., 2014), which is considerably less than for other benchtop sequencers, such as the Ion Torrent (~1.5 errors per 100 bases) (Loman et al., 2012; Salipante et al., 2014). To accommodate for this potential source of error, we employed a very high stringency for assignment to subtype: 100% pairwise identity and 100% query cover (or 99% for *C. cuniculus*). This resulted in a large number of unassigned reads (Table 3.1). However, at the high stringency level used to assign subtypes, no sequences were equivocally assigned to multiple subtypes, which lends confidence to the data.

It is possible that PCR polymerase slippage artefacts contributed to the number of subtypes detected. However, it does not account for all the diversity. For example, co-

occurrence of *C. parvum* IIa and IId in two samples cannot be explained by PCR slippage, as this would have required the occurrence of slippage by multiple trinucleotides in the TCA repeat region repeatedly across samples, which is unlikely, given the stability of imperfect repeat regions (Bacon et al., 2000; Klintschar and Wiegand, 2003). In addition, if PCR polymerase slippage was the main cause of subtype diversity identified, then it would be expected that subtype diversity would also have been seen in *C. hominis*, which was not the case.

It is possible that the lower PCR annealing temperature required to amplify *gp60* sequences for NGS (50°C) compared with Sanger (54°C) resulted in non-target sequences being amplified and sequenced alongside *Cryptosporidium gp60* sequences, which would contribute greatly to the number of unassigned sequences in this analysis.

Another limitation of the present study is that both Sanger and NGS were conducted using nested and not single round PCR. Nested PCR approaches have an inherent risk of contamination and have previously been shown to exhibit strong amplification biases (Park and Crowley, 2010). By involving two sequential rounds of amplification, nested PCR may not accurately represent the extent of genetic diversity initially present in the sample, because it introduces a bottleneck in the genetic variation between the first and second round. However, attempts to produce amplicons from single round PCRs were unsuccessful for most of the samples and therefore a nested PCR approach was necessary. This is a well-recognised but inherent problem of *Cryptosporidium* epidemiological analysis, as frequently *Cryptosporidium*-positive faecal samples contain very low numbers of oocysts and high levels of PCR inhibitors, which necessitates a nested PCR approach (Paparini et al., 2015). However, while nested PCR bias may reduce the number of variants detected, in the present study, multiple subtypes were successfully identified in individual samples.

While multiple *gp60* subtypes were identified within *C. parvum* and *C. cuniculus*, only one subtype was identified in DNA samples positive for *C. hominis*, which may reflect their local population structures. Little information is available for *C. cuniculus*, but available data for *C. parvum* and *C. hominis* indicates a flexible reproductive strategy with panmictic (where genetic exchange occurs at random with limited or no sub-structuring), clonal and epidemic population structures (Mallon et al., 2003; Morrison et al., 2008; Tanriverdi et al., 2008; Widmer and Sullivan, 2010; Drumo et al., 2012; De Waele et al., 2013; Widmer et al., 2015; Ramo et al., 2015, 2016). The relative contribution of each type of population structure appears to vary between regions and hosts, and may reflect the prevailing ecological transmission dynamics (Mallon et al., 2003; Tanriverdi et al., 2008; Widmer and Sullivan, 2010; Herges et al., 2012; Widmer et al., 2015). The finding of only one *C. hominis* subtype by both Sanger and NGS in the kangaroo-derived DNA samples may reflect a clonal population structure operating locally in kangaroo populations from the main Sydney drinking water catchment. Analysis of the population structure, however, requires analysis of multiple loci which was not conducted in the present study.

Importantly, the identification of only one *C. hominis* subtype by both Sanger and NGS in the kangaroo-derived DNA samples suggests a single, recent introduction of *C. hominis* into kangaroos, which may spill over to infect other hosts in catchments, providing a reservoir for human infection. However, further research is required on a much larger number of samples belonging to different subtypes. In addition, inferences regarding the population structure are complicated by the fact that the rate of mutation of the *gp60* gene remains unknown, multi-locus analysis is required and *Cryptosporidium* within-host genetic diversity may occur both within and between the oocysts (Grinberg et al., 2013; Grinberg and Widmer, 2016).

The extent of within-host genetic diversity at the *gp60* locus, as demonstrated by the present study, may have implications for *Cryptosporidium* vaccine and drug development. For

example, vaccine research for *Cryptosporidium* has focused on proteins involved in attachment to, and invasion of, host cells (Mead, 2014; Ludington and Ward, 2015). Therefore, immunisation with predominant antigens could result in vaccine failures in some regions where heterogeneous parasite populations dominate (Grinberg and Widmer, 2016). As with malaria, undetected low-level drug-resistant co-infecting species and genotypes of *Cryptosporidium* within the same host could also impact anti-*Cryptosporidium* drug discovery studies and result in unexplained chemotherapy failure (Tyagi et al., 2013; Grinberg and Widmer, 2016). These findings also have implications for our understanding of the epidemiology and transmission dynamics of *Cryptosporidium*, as previous studies have relied on Sanger sequencing, which may not reflect the extent of within-host diversity and result in incorrect assumptions regarding transmission of the parasite. More extensive studies employing NGS approaches on a wider range of samples are important to determine the extent of *Cryptosporidium* within-host genetic diversity and should be an essential prerequisite for vaccine, drug and epidemiological studies.

3.8 Acknowledgments

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3.10 Summary

Co-infection with different species and genotypes of *Cryptosporidium* can have important repercussions on disease dynamics and the changing epidemiology of cryptosporidiosis. One of the most common tools used in tracking transmission of zoonotic *Cryptosporidium* species is Sanger sequencing of the glycoprotein 60 (*gp60*) (the most hypervariable locus in the genome). Despite this, little is known about the extent of within host genetic diversity of *Cryptosporidium*. In this chapter, the intra-host genetic diversity of *Cryptosporidium* using Sanger sequencing and NGS of *gp60* amplicons from the main species infecting humans including *C. hominis*, *C. parvum* and *C. cuniculus* isolates from Australia and China were compared. Sanger sequencing identified only one *gp60* subtype in each isolate, whereas NGS identified the same subtypes initially identified by Sanger sequencing but also identified additional *gp60* subtypes. These findings have important implications for *Cryptosporidium* transmission tracking as well as vaccine and drug studies. Building on methodology validated in the last two chapters, in the following chapter the screening, enumeration and characterisation of *Cryptosporidium* species and genotypes in faecal samples collected from animals in water catchment areas across three states in Australia using both Sanger sequencing and NGS are described.

Chapter Four - *Cryptosporidium* species and subtypes in animals inhabiting drinking water catchments in three states across Australia

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4.1 Preface

This chapter builds upon the methodological advances described in chapters two and three, and consists of a manuscript published in Water Research, entitled "*Cryptosporidium* species and subtypes in animals inhabiting drinking water catchments in three states across Australia" (*see* Appendix 5). In Chapter two, the species of *Cryptosporidium* detected in a subset of faecal samples collected from animals inhabiting watersheds within the WaterNSW area of operations, and the presence of zoonotic *Cryptosporidium* species in both livestock and wildlife inhabiting drinking water catchments are described. These findings highlighted the potential role these animals may play in the dissemination of zoonotic *Cryptosporidium* species to drinking water sources and the associated human health risks and therefore, necessitated continued monitoring and identification of the sources/carriers of human infectious *Cryptosporidium* spp. in water catchments. In the present chapter, a more comprehensive longitudinal analysis of *Cryptosporidium* spp. in faecal samples from cattle, marsupials, sheep, rabbits, birds and wildlife (n = 5,574) across various drinking water catchments in three states in Australia (NSW, QLD and WA) over a three-year period, was conducted using a combination of Sanger sequencing and NGS.

4.2 Statement of contribution

As first author in this paper, **AZ** developed the concept, collected the samples (except samples from QLD and NSW which were collected by Seqwater and WaterNSW staff members, respectively), conducted all the labwork, analysed the data, interpreted the findings and wrote the first and final draft of the manuscript. **PM**, **AG** and **CO** contributed to data analysis and manuscript preparation. **AB**, **AB** and **MB** provided logistic support to the project, organised

samples collection from other states (QLD and NSW) and reviewed and edited the manuscript. **IR** assisted with statistical analysis and sample collection methodology and design. **UR** managed and supervised the project, was directly involved in project administration, conceptualisation, funding acquisition, methodology, data analysis and validation, manuscript writing and critically revised drafts of the manuscript. All authors reviewed and approved the final version of the manuscript.

AZ: 70%

4.3 Abstract

As part of long-term monitoring of *Cryptosporidium* in water catchments serving Western Australia, New South Wales (Sydney) and Queensland, Australia, we characterised *Cryptosporidium* in a total of 5,774 faecal samples from 17 known host species and 7 unknown bird samples, in 11 water catchment areas over a period of 30 months (July 2013 to December 2015). All samples were initially screened for *Cryptosporidium* spp. at the 18S rRNA locus using a quantitative PCR (qPCR). Positives samples were then typed by sequence analysis of an 825 bp fragment of the 18S gene and subtyped at the glycoprotein 60 (*gp60*) locus (832 bp). The overall prevalence of *Cryptosporidium* across the various hosts sampled was 18.3% (1,054/5,774; 95% CI, 17.3-19.3). Of these, 873 samples produced clean Sanger sequencing chromatograms, and the remaining 181 samples, which initially produced chromatograms suggesting the presence of multiple different sequences, were re-analysed by Next- Generation Sequencing (NGS) to resolve the presence of *Cryptosporidium* and the species composition of potential mixed infections. The overall prevalence of confirmed mixed infection was 1.7% (98/5,774), and in the remaining 83 samples, NGS only detected one species of *Cryptosporidium*. Of the 17 *Cryptosporidium* species and four genotypes detected (Sanger sequencing combined with NGS), 13 are capable of infecting humans; *C. parvum, C. hominis, C. ubiquitum, C. cuniculus, C. meleagridis, C. canis*, *C. felis*, *C. muris*, *C. suis, C. scrofarum, C. bovis, C. erinacei* and *C. fayeri.* Oocyst numbers per gram of faeces (g^{-1}) were also determined using qPCR, with medians varying from 6,021 - 61,064 across the three states. The significant findings were the detection of *C. hominis* in cattle and kangaroo faeces and the high prevalence of *C. parvum* in cattle. In addition, two novel *C. fayeri* subtypes (IVaA11G3T1 and IVgA10G1T1R1) and one novel *C. meleagridis* subtype (IIIeA18G2R1) were identified. This

is also the first report of *C. erinacei* in Australia. Future work to monitor the prevalence of *Cryptosporidium* species and subtypes in animals in these catchments is warranted.

4.4 Introduction

Globally, it is estimated that there are between 1.7- 4.6 billion episodes of diarrhoea every year with 2.2 million associated deaths (Keusch et al., 2016; WHO, 2017). The transmission of many gastrointestinal diseases is closely linked to water, yet the true disease burden attributable to water-associated pathogens is currently unknown, largely due to lack of adequate detection and surveillance systems (Ryan et al., 2017). This is particularly the case in countries such as Australia, that have a relatively low level of endemic diarrhoeal disease, which means that even specially designed high-quality epidemiological trials have a limited ability to detect cases of diarrhoea attributable to drinking water (Sinclair et al., 2015). Routine disease surveillance systems are even less sensitive and detect only a small fraction of the pathogen infections that occur in the community (O'Toole et al., 2015). For example, a national survey of gastroenteritis in Australia in 2002, suggested a ratio of about 500 community cases to one notified case (Hall et al., 2006).

Cryptosporidium is one of the most prevalent waterborne parasitic infections. From the start of the last century to 2016, there were a total of 905 reported waterborne outbreaks caused by protozoan parasites and of these *Cryptosporidium* accounted for almost 60% (524 outbreaks) (Karanis et al., 2007; Baldursson and Karanis, 2011; Efstratiou et al., 2017). The largest *Cryptosporidium* outbreak occurred in Milwaukee in 1993, which affected 403,000 individuals via contaminated drinking water (MacKenzie et al., 1995), with an estimated illness-associated cost of US$ 96.2 M and 100 deaths (Corso et al., 2003). Rates of waterborne parasitic protozoan outbreaks have been increasing due to increased and improved surveillance.

Between 2010-2016, 381 outbreaks were reported, nearly half of which (49% -188 outbreaks), were reported in Australia and New Zealand (Efstratiou et al., 2017). However, the true level of waterborne disease in Australia is unknown.

Currently, 37 *Cryptosporidium* species are recognised (Jezkova et al., 2016; Zahedi et al., 2017; Čondlová et al., 2018; Kváč et al., 2018), of which 17 have been reported in humans worldwide. In Australia, seven *Cryptosporidium* species (*C. hominis, C. parvum, C. meleagridis, C. cuniculus, C. fayeri, C. andersoni* and *C. bovis*) have been reported in humans (Koehler at al., 2014a, 2014b; Zahedi et al., 2016a). However, *C. hominis* and *C. parvum* have been responsible for the majority of human infections throughout the world (Xiao, 2010; Ryan and Power, 2012) and for all waterborne outbreaks typed to date, with the exception of a single outbreak in the UK caused by *C. cuniculus* (Xiao, 2010; Puleston et al., 2014; Efstratiou et al., 2017).

The 2011 Australian Drinking Water Guidelines 2011 (ADWG) contains mostly qualitative information on treatment requirements (https://www.nhmrc.gov.au/guidelines-publications/eh52), but will soon move to a health-based target of 10^{-6} disability adjusted life years (DALYs) per person per year for *Cryptosporidium* (O'Toole et al., 2015). This allows for up to approximately 6% of diarrhoeal disease caused by *Cryptosporidium* to be associated with consumption of drinking water (O'Toole et al., 2015). To meet this target, data about the prevalence of human-infectious *Cryptosporidium* species in source waters and catchments is important for quantitative microbial risk assessment (QMRA) processes. However, relatively few large-scale longitudinal studies have been undertaken in Australia (e.g. Ryan et al., 2005; Ng et al., 2011a; Nolan et al., 2013; Koehler et al., 2016; Zahedi et al., 2016b) and no studies have compared catchments in different states in Australia. The aim of the present study therefore was to use molecular tools to more accurately determine the prevalence, species and

oocyst load of *Cryptosporidium* in Australian water catchments across three states; New South Wales (NSW), Queensland (QLD) and Western Australia (WA).

4.5 Materials and methods

4.5.1 Catchment and sample collection in each state

To comply with the ADWG, water utilities employ a risk-based multiple barrier approach with water source/catchment management and protection, being the first barrier, and other barriers at the treatment, storage, and distribution stages of water supply systems. Some drinking water catchments have a relatively low density of development, and little significant anthropogenic activity; however, this is not always the case. With the exception of rodents, which are seldom infected with human-infectious *Cryptosporidium* species (Feng et al., 2007; Foo et al., 2007), the predominant animals in Australian catchments are marsupials (mainly kangaroos), rabbits, sheep and cattle (Ryan and Power, 2012). However, the importance of these host species varies between states and individual catchments. The most abundant hosts were selected on a per catchment basis after consultation with water utility staff from each state. The population size of livestock examined in individual catchments was known and appropriate sample sizes were estimated using Epitools (http://epitools.ausvet.com.au/content.php?page=home). Unfortunately, the size of wildlife populations in these catchments is unknown, which precluded sample size analysis.

Greater Sydney's drinking water catchments cover 16,000 km^2 of land and are managed by WaterNSW. About 30% of catchment land is national park and bushland, but over 60% of the catchments are privately owned. Two catchments were chosen in NSW (Catchment A and Catchment B), for which cattle and sheep grazing is the largest single land use, but horse studs,

piggeries, dairies and poultry production are also present. Eastern grey kangaroos (*Macropus giganteus*) and rabbits (*Oryctolagus cuniculus*) are the dominant non-domestic species inhabiting these catchments. More than one-quarter of catchment B (2,600 km^2) has been protected from most human activities for over 70 years.

Seqwater is one of Australia's largest water businesses with the most geographically spread and diverse asset base of any capital city water authority. South-east QLD's catchments cover more than 12,000 km^2 of land but only 650 km^2 hectares of this land is owned by Seqwater. Three catchment areas were analysed; Catchment A covers an area of 67 km^2 and supplies a large portion of the Sunshine Coast's drinking water. Catchment B is located in the Gold Coast hinterland in South-east QLD and supplies bulk raw water to local irrigators and Seqwater. Catchment C is situated between Brisbane and the Sunshine Coast. Cattle and sheep are the main livestock present in these catchments.

Water Corporation (WC) in Western Australia, is one of the world's largest water utilities servicing an area of over 2.5 million km^2. Six catchments were analysed; Catchment A is used for agriculture and urban development; catchment B is located in the South of WA; catchment C supplies approximately 20 percent of Perth's fresh water; catchment D is located in the southwest of WA; catchment E located ~70 km from metropolitan Perth; and catchment F is located approximately 100 km south of Perth.

4.5.2 Sample collection and processing

In NSW, animal faecal samples were collected by WaterNSW staff within the WaterNSW area of operations. Samples were collected at monthly intervals over a 30-month period (July, 2013 to December, 2015). A total of 1,521 faecal samples were collected from

Eastern grey kangaroos (*Macropus giganteus*) (*n* = 835), beef cattle (*n* = 243), sheep (*n* = 217), rabbits (*n* = 217), horses (*n* = 5) and pigs (*n* = 4) (Table 4.1).

In QLD, animal faecal samples were collected by Seqwater staff over a 14-month period (September, 2014 to November, 2015). A total of 653 faecal samples were collected from cattle (*n* = 568, of which 216 were dairy cattle and 352 were beef cattle), sheep (*n* = 9), horses (*n* = 38), birds (*n* = 9), pigs (*n* = 4), rabbits (*n* = 5), flying foxes (*Pteropus* sp.) (*n* = 9), feral red deer (*Cervus elaphus*) (*n* = 6), wild dogs (*n* = 3), a single goat, and a single wallaby (species unknown) (Table 4.1).

In WA, a total of 3,600 faecal samples were collected from beef cattle (*n* = 300), sheep (*n* = 150), Western grey kangaroos (*Macropus fuliginosus*) (*n* = 2,393), rabbits (*n* = 450), birds (*n* = 7) and feral pigs (*n* = 300) (Table 4.1).

The animal sources of the faecal samples were determined by visually sighting the animals defaecating and with the aid of a scat and tracking manual published for Australian animals (Triggs, 2004). All faecal samples were collected off the ground into individual 75 ml faecal collection pots and stored at 4°C until required, with samples collected in NSW and QLD transported at 4°C to Murdoch University for analysis.

4.5.3 DNA isolation and qPCR

Genomic DNA (gDNA) was extracted from 250mg of each faecal sample using a Power Soil DNA Kit (MO BIO, Carlsbad, California, USA). An extraction reagent blank (no faecal sample) was used in each extraction group. Purified gDNA was stored at -20°C prior to molecular analyses. All samples were screened for the presence of *Cryptosporidium* at the 18S rRNA locus using a quantitative PCR (qPCR) as previously described (King et al., 2005; Yang et al., 2014) using a *Ct* threshold of <35 cycles. At the 18S locus, *C. macropodum,* a marsupial

adapted species of *Cryptosporidium,* was used as a positive control for PCR amplifications of non-marsupial-derived samples. For samples collected from marsupials (kangaroos), *C. parvum* was used as a positive control.

Quantitation was conducted using standards consisting of recombinant plasmids containing partial fragments of the *Cryptosporidium* 18S rRNA, calibrated by droplet digital PCR (ddPCR) as described by Yang et al. (2014). Target copy numbers detected were converted to numbers of oocysts based on the fact that the 18S gene in *Cryptosporidium* has five copies (Le Blancq et al., 1997), and there are four haploid sporozoites per oocyst. Therefore, every 20 copies of 18S detected by qPCR were equivalent to one oocyst.

Table 4.1. Prevalence of *Cryptosporidium* in different hosts and catchments across three states of Australia; NSW, QLD and WA, as determined by Sanger sequencing.

Catchment	Cattle No +/total no (% prevalence + 95% CI)	Sheep No +/total no (% prevalence + 95% CI)	Kangaroos No +/total no (% prevalence + 95% CI)	Rabbits No +/total no (% prevalence + 95% CI)	Horses No +/total no (% prevalence + 95% CI)	Birds No +/total no (% prevalence + 95% CI)	Pigs No +/total no (% prevalence + 95% CI)	Other No +/total no (% prevalence + 95% CI)
QLD								
Catchment A	48/179 (26.8%- 20.5-33.9)	1/9 (11.1%- 0.3-48.2)	NC	NC	0/15 (0%- 0-21.8)	2/2 (100%- 15.8-100)	2/3 (66.7%- 9.4-99.2)	0/1* (0%- 0-97.5)
Catchment B	56/194 (28.9%- 22.6-35.8)	NC	NC	NC	0/16 (0%- 0-26)	2/4 (50%- 6.8-93.2)	N/C	NC
Catchment C	25/195 (12.8- 8.5-18.3)	NC	NC	0/5 (0%- 0-52.2)	0/7 (0%- 0-41)	2/3 (66.7%- 9.4-99.2)	0/1 (0%- 0-97.5)	11/19** (57.9%- 33.5-79.7)
Total	**129/568 (22.7%- 19.3-26.4)**	**1/9 (11.1%- 0.3-48.2)**	**NC**	**0/5 (0%- 0-52.2)**	**0/38 (0%- 0-9.3)**	**6/9 (66.7%- 29.9-92.5)**	**2/4 (50%- 6.8-93.2)**	**11/20 (55%- 31.5-76.9)**
NSW								
Catchment A	45/243 (18.5%- 13.8-24)	12/217 (5.5%- 2.9-9.5)	35/261 (13.4%- 9.5-18.2)	60/217 (27.6%- 21.8-34.1)	NC	NC	NC	NC
Catchment B	NC	NC	37/574 (6.4%- 4.6-8.8)	NC	0/5 (0%- 0-52.2)	NC	0/4 (0%- 0-60.2)	NC
Total	**45/243 (18.5%- 13.8-24)**	**12/217 (5.5%- 2.9-9.5)**	**72/835 (8.6%- 6.8-10.7)**	**60/217 (27.6%- 21.8-34.1)**	**0/5 (0%- 0-52.2)**	**NC**	**0/4 (0%- 0-60.2)**	**NC**
WA								
Catchment A	NC	NC	14/443 (3.2%- 1.7-5.2)	4/150 (2.7%- 0.7-6.7)	NC	5/7 (71.4%- 29-96.3)	NC	NC
Catchment B	NC	NC	56/600 (9.3%- 7.1-11.9)	NC	NC	NC	NC	NC
Catchment C	NC	NC	84/450 (18.7%- 15.2-22.6)	23/150 (15.3%- 10-22.1)	NC	NC	NC	NC
Catchment D	65/300 (21.7%- 17.1-26.8)	17/150 (11.3%- 6.7-17.5)	29/150 (19.3%- 13.3-26.6)	NC	NC	NC	NC	NC
Catchment E	NC	NC	85/450 (18.9%- 15.4-22.8)	9/150 (6%- 2.8-11.1)	NC	NC	NC	NC

Catchment F	NC	NC	96/300 (32%- 26.8-37.6)	NC	NC	NC	48/300 (16%- 12-20.6)	NC
Total	**65/300 (21.7%- 17.1-26.8)**	**17/150 (11.3%- 6.7-17.5)**	**364/2393 (15.2%- 13.8-16.7)**	**36/450 (8%- 5.7-10.9)**	**NC**	**5/7 (71.4%- 29-96.3)**	**48/300 (16%- 12-20.6)**	**NC**
Overall Prevalence	**239/1111 (21.5%- 19.1-24)**	**30/376 (8%- 5.4-11.2)**	**436/3228 (13.5%- 12.3-14.7)**	**96/672 (14.3%- 11.7-17.2)**	**0/43 (0%- 0-8.2)**	**11/16 (68.7%- 41.3-89)**	**50/308 (16.2%- 12.3-20.8)**	**11/20 (55%- 31.5-76.9)**

NC = Not Collected.

* Goat sample

** Samples collected opportunistically from wildlife (7/9 flying fox, 0/1 wallaby, 2/6 feral deer, 2/3 wild dog)

4.5.4 PCR amplification at the 18S and *gp60* loci by nested PCR

Samples that were positive by qPCR were amplified at the 18S locus using nested primers which produced an approximately 825 bp product as previously described (Xiao et al., 1999b). PCR contamination controls were used, including negative controls to detect contamination and separate laboratory areas were used for DNA and PCR mastermix preparation and post-PCR handling.

Samples that were typed as *C. hominis, C. parvum*, *C. cuniculus, C. meleagridis, C. ubiquitum* and *C. fayeri* by Sanger sequencing at the 18S locus were subtyped at the 60 kDa glycoprotein (*gp60*) locus using nested PCRs as previously described (Strong et al., 2000; Glaberman et al., 2001; Peng et al., 2003; Zhou et al., 2003; Power et al., 2009; Li et al., 2014). At the *gp60* locus, for samples which were initially identified as *C. hominis* and *C. parvum* at the 18S locus, *C. cuniculus* was used as a positive control. For samples which were previously identified as *C. cuniculus* at the 18S locus, *C. parvum* was used as a positive control.

4.5.5 Sanger sequence analysis

The amplified DNA from secondary PCRs were separated by gel electrophoresis and purified for sequencing using an in-house filter tip method (Yang et al., 2013). Purified PCR products from both loci were sequenced independently on an ABI Prism™ Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, California) according to the manufacturer's instructions, at 57°C annealing temperature for the 18S rRNA and a range of different annealing temperatures for different species at *gp60* (56°C for *C. fayeri,* 58°C for *C. meleagridis* and 54°C for the remaining species). Sanger sequences were assigned taxonomy by aligning chromatograms to curated reference sequences from GenBank using 99% sequence

identity. Alignments were produced with Clustal W (http://www.clustalw.genome.jp), utilised as a plugin within Geneious Pro 8.1.6 (Kearse et al., 2012) and phylogenetic analysis was conducted using MEGA6 (after selection of the best nucleotide substitution models) (Tamura et al., 2013) (data not shown).

4.5.6 Next Generation Sequencing (NGS)

Samples that produced mixed chromatograms by Sanger sequencing (n = 251) were analysed by NGS on the MiSeq (Illumina) platform at the 18S locus using the 18S iF/iR primers (Morgan et al., 1997) as previously described (Paparini et al., 2015). These primers were selected over the longer Xiao et al. (1999) primers used for Sanger sequencing, due to length limitation imposed by the 250 bp paired-end sequencing technology utilised. Briefly, PCR primers were modified to contain MiSeq adapter sequences on the 5′ end, as per standard protocols for the MiSeq platform (Illumina Demonstrated Protocol: Metagenomic Sequencing Library Preparation). All PCR amplicons were double purified using the Agencourt AMPure XP Bead PCR purification protocol (Beckman Coulter Genomics, USA) and pooled in approximate equimolar ratios. To minimize laboratory and cross-contamination all DNA handling and PCR-setup procedures were performed within dedicated and physically separated PCR containment hoods that are UV-sterilized between each use. Post-PCR procedures were all performed in a physically separate dedicated laboratory.

Sequencing was performed on an Illumina MiSeq using 500-cycle V2 chemistry (250 paired-end reads) following the manufacturer's recommendations. Two no-template controls and two DNA extraction reagent blank controls were included in the library preparation, and distributed between samples in the PCR plate layout. All no-template and DNA extraction reagent blank controls produced no detectable amplification of *Cryptosporidium* DNA after

initial amplicon-generation PCRs or indexing PCRs. This indicated that level of cross contamination between samples, or from the laboratory environment, was below the detection limit of the library preparation procedure and for this reason were not sequenced.

4.5.7 Statistical Analysis

The prevalence of *Cryptosporidium* in each host species was expressed as the percentage of samples positive by qPCR, which were also confirmed by Sanger and/or NGS analysis of the 18S rRNA locus, with 95% confidence intervals calculated assuming a binomial distribution, using the software Quantitative Parasitology 3.0 (Rózsa et al., 2000). DNA extraction efficiency was estimated for each extraction, based on the number of the gene copies/oocysts equivalents measured by ddPCR. Chi-square and non-parametric analyses were performed using IBM SPSS 21.0 (statistical package for the social sciences) for Windows (SPSS inc. Chicago, USA) to determine if there were any associations between the prevalence and concentration of *Cryptosporidium* oocysts at different sampling times and across states.

4.5.8 Bioinformatics analysis

Paired-end reads (73.42% of basecalls >Q30) were merged and quality filtered with USEARCH v10 (Edgar, 2010), retaining reads with >50 bp merged overlap, <0.1% expected error, no mismatches in the primer sequences, a minimum length of 200 bp, and a minimum of 100 identical replicate copies. Primer sequences and any distal bases were also removed from all reads. Reads were then denoised and chimera filtered with the UNOISE3 algorithm (Edgar, 2016), to generate 107 zero-radius operational taxonomic units (ZOTUs), that represent unique biologically correct sequences (Edgar, 2016). Remaining high quality *Cryptosporidium* spp.

18S ZOTU sequences were assigned to taxonomic groups by comparing ZOTUs to a curated custom in-house database using BLAST 2.6.0 (Altschul et al., 1990). The reference database contained 63 reliable 18S reference sequences from 63 *Cryptosporidium* species and genotypes extracted from GenBank (Benson et al., 2005). Taxonomy was only assigned if there was a single unambiguous best BLAST hit with >99% pairwise identity over >98% of the query sequence length. This high stringency threshold was based on the minimum pairwise percentage dissimilarity between any two *Cryptosporidium* species/genotypes in the database to unsure unambiguous taxonomic classifications. Of the 107 ZOTUs generated, 41 ZOTUs were not *Cryptosporidium* 18S sequences when compared to GenBank using BLAST. Where possible genus-level taxonomy was assigned when queries hit reference sequences with >99% identity over >98% of the reads, and assigned to no other taxa at the same level. Although non-*Cryptosporidium* ZOTUs were abundant (38.3% of total ZOTUs), they represented a very small proportion of the total reads, with only a median of 580 sequences each.

4.6 Results

4.6.1 Overall prevalence of *Cryptosporidium* as determined by Sanger sequencing and NGS

The overall PCR prevalence of *Cryptosporidium* species in the 5,774 faecal samples collected systematically from seven main host species and opportunistically from 10 host species, in addition to seven unknown bird samples, was 18.3% (1,054/5,774; 95% CI, 17.3-19.3), based on PCR positive samples that were confirmed by Sanger or NGS sequencing.

The overall prevalence in each state based on qPCR positive samples that have been confirmed by Sanger sequencing or NGS was 25.9% (169/653; 95% CI, 22.6-29.4) in QLD,

17.5% (632/3,600; 95% CI, 16.3-18.8) in WA and 16.6% (253/1,521; 95% CI, 14.8-18.6) in NSW. For ease of analysis and reporting, the prevalence and species detected by Sanger and NGS are discussed separately.

4.6.2 Prevalence of *Cryptosporidium* as determined by Sanger sequencing in various hosts and catchments

Of the 873 samples which produced clean Sanger chromatograms, the highest prevalence was detected in birds (68.7%), followed by 21.5% in cattle, 16.2% in pigs, 14.3% in rabbit samples, 13.5% in kangaroos and 8% in sheep. No *Cryptosporidium* was detected in the 43 horse faecal samples screened (Table 4.1). A high prevalence was also detected in the small numbers of wildlife sampled (Table 4.1). The overall prevalence of *Cryptosporidium* in each state was 22.8%; 95% CI, 19.7-26.2 (149/653) in QLD, 14.9%; 95% CI, 13.7-16.1 (535/3,600) in WA, and 12.4%; 95% CI, 10.8-14.2 (189/1,521) in NSW.

The prevalence of *Cryptosporidium* in cattle was not significantly different across the three states; 18.5% in NSW, 21.7% in WA and 22.7% in QLD (29.6% in dairy cattle and 18.5% in beef cattle). The prevalence of *Cryptosporidium* was highest in sheep from WA (11.3%), compared to 11.1% in QLD and 5.5% in NSW. Kangaroos were only sampled in NSW (8.6%) and WA (15.2%). *Cryptosporidium* was not detected in the small numbers of rabbits sampled in QLD (n = 5), but was more prevalent in rabbits in NSW (27.6%) than WA (8%). *Cryptosporidium* was detected in two (out of 4) pigs in QLD and in 16% of feral pigs in WA (Table 4.1).

4.6.3 Oocyst load

Oocyst numbers per gram of faeces (g^{-1}) were also determined using qPCR (Table 4.2; mean, median and range for samples identified as *Cryptosporidium* species by Sanger sequencing and NGS). The highest median concentration of *Cryptosporidium* oocysts was identified in cattle; (31,072 oocysts/g^{-1}), followed by rabbits (27,919 oocysts/g^{-1}), while the lowest median concentration of oocysts was observed among samples collected opportunistically from wildlife in QLD (9,063 oocysts/g^{-1}). Overall, there was no significant difference between the median *Cryptosporidium* oocysts per gram of faeces in samples collected from sheep, kangaroos, pigs and birds, which ranged from 10,032 to 26,756 oocysts/g^{-1} (Table 4.2).

Table 4.2. Numbers of *Cryptosporidium* oocysts in positive samples per gram of faeces (g^{-1}) (mean, median with range in parenthesis (determined by qPCR) per host species per catchment across three states of Australia; NSW, QLD and WA.

Catchment	Cattle	Sheep	Kangaroos	Rabbits	Horses	Birds	Pigs	Other
QLD								
Catchment A	36,093, 28,290 (928-122,080)	24,500, NA (only one sample)	NC	NC	ND	11,518, 11,518 (9,982-13,054)	16,135, 16,135 (1,2450-19,820)	ND
Catchment B	53,508, 42,803 (1,920-182,664)	NC	NC	NC	NC	6,201, 6,201 (2,622-9,780)	NC	NC
Catchment C	44,914, 33,581 (680-228,548)	NC	NC	ND	ND	7,376, 7,376 (4,720-10,032)	NC	8,832, 9,063 (622-19,047)
Total	**45,002, 33,585 (680-228,548)**	**24,500, NA (only one sample)**	**NC**	**ND**	**ND**	**8,365, 9,882 (2,622-13,054)**	**16,135, 16,135 (19,820-32,270)**	**8,832, 9,063 (622-19,047)**
NSW								
Catchment A	24,685, 22,568 (836-88,014)	29,455, 33,522 (6,068-58,892)	9,766, 8,044 (26-44,738)	27,510, 27,155 (498-67,233)	NC	NC	NC	NC
Catchment B	NC	NC	11,615, 10,265 (131-41,088)	NC	ND	NC	ND	NC
Total	**24,685, 22,568 (836-88,014)**	**29,454, 33,522 (6,068-58,892)**	**10,549, 8,735 (26-44,738)**	**27,510, 27,155 (498-67,233)**	**ND**	**NC**	**ND**	**NC**
WA								
Catchment A	NC	NC	15,907, 13,728 (2,076-40,210)	64,572, 61,034 (1,004-108,706)	NC	38,841, 44,812 (5,270-78,433)	NC	NC
Catchment B	NC	NC	19,978, 17,738 (440-63,002)	NC	NC	NC	NC	NC
Catchment C	NC	NC	21,892, 19,507 (698- 71,194)	38,336, 38,297 (1,004-82,633)	NC	NC	NC	NC
Catchment D	43,418, 37,322 (6,014-144,328)	27,281, 19,701 (4,492-51,178)	14,420, 12,529 (522-45,490)	NC	NC	NC	NC	NC
Catchment E	NC	NC	19,747, 17,820 (11-66,904)	30,412, 18,595 (13,090-89,160)	NC	NC	NC	NC
Catchment F	NC	NC	23,482, 18,085 (714-275,402)	NC	NC	NC	22,861, 22,062 (873-88,026)	NC
Total	**43,418, 37,322 (6,014-144,328)**	**27,281, 19,701 (4,492-51,178)**	**24,680, 19,098 (11-275,042)**	**39,175, 36,064 (1,004-108,706)**	**NC**	**38,841, 44,812 (5,270-78,433)**	**22,861, 22,062 (873-88,026)**	**NC**

Overall	**39,834, 31,072 (680-228,548)**	**28,058, 26,756 (4,492-58,892)**	**18,442, 16,018 (11-275,042)**	**32,380, 27,919 (498-108,706)**	**ND**	**22,217, 10,032 (2,622-78,433)**	**22,625, 21,164 (873-88,026)**	**8,832, 9,063 (622-19,047)**

NA = Not Available.
NC = Not Collected.
ND = Not Detected.

4.6.4 *Cryptosporidium* species detected in various hosts at the 18S locus by Sanger sequencing

Clean 18S Sanger sequences were obtained from 873 positives. Of these, a total of 14 species and two genotypes were detected; *C. macropodum* (*n* = 260), *C. fayeri* (*n* = 150), *C. parvum* (*n* = 106), *C. cuniculus* (*n* = 96), *C. bovis* (*n* = 60), *C. hominis* (*n* = 42), *C. ryanae* (*n* = 41), *C. ubiquitum* (*n* = 36), *C. scrofarum* (*n* = 35), *C. suis* (*n* = 15), *C. muris* (*n* = 15), *C. galli* (*n* = 8), *C. meleagridis* (*n* = 3), *C. canis* (*n* = 2), *Cryptosporidium* rat genotype I (*n* = 3) and *C. molnari*-like genotype (*n* = 1) (Table 4.3).

In cattle, of the 239 positives, a total of seven species and one genotype were detected; *C. parvum* (*n* = 106), *C. bovis* (*n* = 60), *C. ryanae* (*n* = 41), *C. hominis* (*n* = 16), *C. muris* (*n* = 8), *C. ubiquitum* (*n* = 4), *C. galli* (*n* = 1) and rat genotype I (*n* = 3), with *C. ubiquitum*, *C. galli* and rat genotype I only detected in beef cattle. All the sheep were infected with *C. ubiquitum* (30/30) and all the rabbits were infected with *C. cuniculus* (96/96). In kangaroos, *C. hominis* (26/436), *C. macropodum* (260/436) and *C. fayeri* (150/436) were detected. In pigs, *C. suis* (15/50) and *C. scrofarum* (35/50) were detected. Three species were detected in the 11 positives from birds, a *C. molnari-like* genotype in a single shag, *C. galli* (*n* = 7) and *C. meleagridis* (*n* = 3). In wildlife, of the 11 positives typed, *C. ubiquitum* was identified in feral deer (*n* = 2), *C. muris* in flying foxes (*n* = 7) and *C. canis* from wild dogs (*n* = 2) (Table 4.3).

Table 4.3. *Cryptosporidium* species detected by Sanger sequencing in different hosts and catchments across three states of Australia; NSW, QLD and WA.

Host	*C. parvum* No +/total no (% proportion+ 95 CI)	*C. hominis* No +/total no (% proportion + 95 CI)	*C. bovis* No +/total no (% proportion + 95 CI)	*C. ubiquitum* No +/total no (% proportion + 95 CI)	*C. ryanae* No +/total no (% proportion + 95 CI)	*C. suis* No +/total no (% proportion + 95 CI)	*C. scrofarum* No +/total no (% proportion + 95 CI)	*C. cuniculus* No +/total no (% proportion + 95 CI)	*C. macropodum* No +/total no (% proportion + 95 CI)	Other No +/total no (% proportion + 95 CI)
QLD										
Cattle	55/129 (42.6%-34-51.6)	7/129 (5.4%-2.2-10.9)	26/129 (20.2%-13.6-28.1)	4/129 (3.1%-0.9-7.7)	29/129 (22.5%-15.6-30.7)	ND	ND	ND	ND	8/129[a] (6.2%- 2.7-11.9)
Sheep	ND	ND	ND	1/1 (100%- 2.5-100)	ND	ND	ND	ND	ND	ND
Horses	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Birds	ND	ND	ND	ND	ND	ND	ND	ND	ND	6/6[b] (100%-54.1-100)
Pigs	ND	ND	ND	ND	ND	ND	2/2 (100%-15.8-100)	ND	ND	ND
Wildlife	ND	ND	ND	2/11[c] (18.2%-2.3-51.8)	ND	ND	ND	ND	ND	9/11[d] (81.8%-48.2-97.7)
Total	**55/129 (42.6%- 34-51.6)**	**7/129 (5.4%- 2.2-10.9)**	**26/129 (20.2%-13.6-28.1)**	**7/141 (5%- 2-10)**	**29/129 (22.5%-15.6-30.7)**	**ND**	**2/2 (100%-15.8-100)**	**ND**	**ND**	**23/146 (15.7%-10.3-22.7)**
NSW										
Cattle	19/45 (42.2%-27.7-57.8)	6/45 (13.3%-(5.1-26.8)	18/45 (40%-25.7-55.7)	N/D	1/45 (2.2%-0.1-11.8)	ND	ND	ND	ND	1/45[e] (2.2%- 0.1-11.8)
Sheep	N/D	ND	ND	12/12 (100%-73.5-100)	ND	ND	ND	ND	ND	ND
Kangaroos	N/D	26/72 (36.1%-25.1-48.3)	ND	ND	ND	ND	ND	ND	44/72 (61.1%-48.9-72.4)	2/72[f] (2.8%-0.3-9.7)
Rabbits	N/D	ND	ND	ND	ND	ND	ND	60/60 (100%- 94-100)	ND	ND

Total	**19/45 (42.2%- 27.7-57.8)**	**32/117 (27.5%- 19.5-36.4)**	**18/45 (40%- 25.7-55.7)**	**12/12 (100%- 73.5-100)**	**1/45 (2.2%- 0.1-11.8)**	**ND**	**ND**	**60/60 (100%- 94-100)**	**44/72 (61.1%- 48.9-72.4)**	**3/117 (2.6%- 0.5-7.3)**
WA										
Cattle	32/65 (49.2%- 36.6-61.9)	3/65 (4.62%- 1-12.9)	16/65 (24.6%- 14.8-36.9)	ND	11/65 (24.6%- 14.8-36.9)	ND	ND	ND	ND	3/65[g] (4.6%- 1-12.9)
Sheep	ND	ND	ND	17/17 (100%- 80.5-100)	ND	ND	ND	ND	ND	ND
Kangaroos	ND	ND	ND	ND	ND	ND	ND	ND	216/364 (59.3%- 54.1-64.4)	148/364[f] (40.7%- 35.6-45.9)
Rabbits	ND	ND	ND	ND	ND	ND	ND	36/36 (100%- 90.3-100)	ND	ND
Pigs	ND	ND	ND	ND	ND	15/48 (31.2%- 18.7-46.3)	33/48 (75%- 60.4-86.4)	ND	ND	ND
Birds	ND	ND	ND	ND	ND	ND	ND	ND	ND	5/5[h] (100%- 47.8-100)
Total	**32/65 (49.2%- 36.6-61.9)**	**3/65 (4.62%- 1-12.9)**	**16/65 (24.6%- 14.8-36.9)**	**17/17 (100%- 80.5-100)**	**11/65 (24.6%- 14.8-36.9)**	**15/48 (31.2%- 18.7-46.3)**	**33/48 (75%- 60.4-86.4)**	**36/36 (100%- 90.3-100)**	**216/364 (59.3%- 54.1-64.4)**	**156/434 (35.9%- 31.4-40.7)**

ND = Not Detected

[a]*Cryptosporidium muris* (*n* = 8)

[b]*Cryptosporidium molnari* (*n* = 1, Shag), *Cryptosporidium gall*i (*n* = 5; 2 from an ibis, 1 from a goose, 1 from a boiler chicken, 1 from a swallow)

[c]*Cryptosporidium ubiquitum* from feral deer (*n* = 2)

[d]*Cryptosporidium muris* from flying fox (*n* = 7), *Cryptosporidium canis* from wild dog (*n* = 2)

[e]*Cryptosporidium galli*

[f]*Cryptosporidium fayeri*

[g]*Cryptosporidium* rat genotype I (*n* = 3)

[h]*Cryptosporidium galli* (*n* = 2), *Cryptosporidium meleagridis* (*n* = 3)

4.6.5 *Cryptosporidium gp60* subtypes detected in various hosts by Sanger sequencing

Of the 89 *C. parvum* cattle isolates subtyped at the *gp60* locus, a total of seven subtypes were identified; IIaA15G2R1 (*n* = 5), IIaA16G2R1 (*n* = 5), IIaA17G2R1 (*n* = 27), IIaA18G3R1 (*n* = 40), IIaA19G2R1 (*n* = 1), IIaA19G3R1 (*n* = 11), and a variant of the IIaA13G1 subtype in five cattle, which exhibited one single nucleotide polymorphism from the only other previous record of this subtype (JX471005). Two *C. hominis* subtypes were identified; IbA10G2 (of which 11 were identified in cattle and 23 in kangaroos) and IdA15G1 (two in kangaroos and three in cattle). All the *C. ubiquitum* typed (*n* = 28) belonged to subtype family XIIa and all the *C. meleagridis* (*n* = 3) were identified as a novel subtype; IIIeA18G2R1. Six *C. cuniculus* subtypes were identified; VbA18 (*n* = 12), VbA23 (*n* = 46), VbA25 (*n* = 16), VbA26 (*n* = 8), VbA28 (*n* = 2) and VbA29 (*n* = 5). Three *C. fayeri* subtypes were identified; IVfA12G1T1 (*n* = 23) and two novel subtypes; IVaA11G3T1 (*n* = 16) and IVgA10G1T1R1 (*n* = 81) (Table 4.4). The novel subtypes (IIaA13G1, IIIeA18G2R1, IVaA11G3T1 and IVgA10G1T1R1) identified in the present study have been submitted to GenBank under accession numbers MG516789, MG516778, MG516791 and MG516790. All other nucleotide sequences reported in this paper are available in the GenBank database under accession numbers MG516739 to MG516774 (18S) and MG516775 to MG516798 (*gp60*).

Table 4.4. *gp60* subtypes detected in different hosts and catchments across three states of Australia; NSW, QLD and WA.

	QLD						NSW					WA							
Host	Cattle	Sheep	Birds	Pigs	Wildlife	**Total**	Cattle	Sheep	Kangaroo	Rabbits	**Total**	Cattle	Sheep	Kangaroo	Rabbits	Pigs	Birds	**Total**	**Overall number**
***C. parvum* IIaA13G1**	ND	ND	ND	ND	ND	**ND**	ND	ND	ND	ND	**ND**	5	ND	ND	ND	ND	ND	**5**	**5**
***C. parvum* IIaA15G2R1**	ND	ND	ND	ND	ND	**ND**	ND	ND	ND	ND	**ND**	5	ND	ND	ND	ND	ND	**5**	**5**
***C. parvum* IIaA16G2R1**	ND	ND	ND	ND	ND	**ND**	5	ND	ND	ND	**5**	ND	ND	ND	ND	ND	ND	**ND**	**5**
***C. parvum* IIaA17G2R1**	13	ND	ND	ND	ND	**13**	ND	ND	ND	ND	**ND**	14	ND	ND	ND	ND	ND	**14**	**27**
***C. parvum* IIaA18G3R1**	24	ND	ND	ND	ND	**24**	9	ND	ND	ND	**9**	7	ND	ND	ND	ND	ND	**7**	**40**
***C. parvum* IIaA19G2R1**	ND	ND	ND	ND	ND	**ND**	1	ND	ND	ND	**1**	ND	ND	ND	ND	ND	ND	**ND**	**1**
***C. parvum* IIaA19G3R1**	8	ND	ND	ND	ND	**8**	3	ND	ND	ND	**3**	ND	ND	ND	ND	ND	ND	**ND**	**11**
***C. hominis* IbA10G2**	7	ND	ND	ND	ND	**7**	4	ND	23	ND	**27**	ND	ND	ND	ND	ND	ND	**ND**	**34**
***C. hominis* IdA15G1**	ND	ND	ND	ND	ND	**ND**	ND	ND	2	ND	**2**	3	ND	ND	ND	ND	ND	**3**	**5**

***C. ubiquitum* XIIa**	4	1	ND	ND	2*	**7**	ND	10	ND	ND	**10**	ND	11	ND	ND	ND	ND	**11**	**28**
***C. cuniculus* VbA18**	ND	ND	ND	ND	ND	**ND**	ND	ND	ND	ND	**ND**	ND	ND	ND	12	ND	ND	**12**	**12**
***C. cuniculus* VbA23**	ND	ND	ND	ND	ND	**ND**	ND	ND	ND	37	**37**	ND	ND	ND	9	ND	ND	**9**	**46**
***C. cuniculus* VbA25**	ND	ND	ND	ND	ND	**ND**	ND	ND	ND	16	**16**	ND	ND	ND	ND	ND	ND	**ND**	**16**
***C. cuniculus* VbA26**	ND	ND	ND	ND	ND	**ND**	ND	ND	ND	ND	**ND**	ND	ND	ND	8	ND	ND	**8**	**8**
***C. cuniculus* VbA28**	ND	ND	ND	ND	ND	**ND**	ND	ND	ND	2	**2**	ND	ND	ND	ND	ND	ND	**ND**	**2**
***C. cuniculus* VbA29**	ND	ND	ND	ND	ND	**ND**	ND	ND	ND	5	**5**	ND	ND	ND	ND	ND	ND	**ND**	**5**
***C. meleagridis* IIIeA18G2R1**	ND	ND	ND	ND	ND	**ND**	ND	ND	ND	ND	**ND**	ND	ND	ND	ND	ND	3	**3**	**3**
***C. fayeri* IVgA10G1T1R1**	ND	ND	ND	ND	ND	**ND**	ND	ND	ND	2	**2**	ND	ND	79	ND	ND	ND	**79**	**81**
***C. fayeri* IVaA11G3T1**	ND	ND	ND	ND	ND	**ND**	ND	ND	ND	ND	**ND**	ND	ND	16	ND	ND	ND	**16**	**16**

***C. fayeri* IVfA12G1T1**	ND	ND	ND	ND	ND	**ND**	ND	ND	ND	ND	**ND**	ND	ND	23	ND	ND	ND	**23**	**23**

ND = Not Detected
*Feral deer

4.6.6 Prevalence and species of *Cryptosporidium* identified by NGS

A total of 251 samples produced mixed Sanger chromatograms and were re-analysed by NGS. However, of these, only 181 were assigned to *Cryptosporidium* species and the remaining 70 samples were discarded due to unassigned reads or failure to pass quality filtering. Of these 181 samples, which produced mixed chromatograms by Sanger, 83 samples did not exhibit mixed infections via NGS, i.e. only one species/genotype was detected (Supplementary Table 4-S1 - Digital appendix 2). This suggests that some of the mixed chromatograms identified by Sanger, were due to co-amplification of *Cryptosporidium* with non-specific contaminants. Therefore, for the scope of the present study, the prevalence of mixed infections is reported based on only those samples which were assigned to multiple *Cryptosporidium* species by NGS (n = 98).

Therefore, the overall prevalence of mixed infections was 1.7% (98/5,774; 95% CI, 1.4-2.1) (Table 4.5 and Supplementary Table 4-S1 - Digital appendix 2). These mixed infections were detected in faecal samples collected from cattle, kangaroos, rabbits, wild pigs and two feral deer. Apart from 10% overall prevalence among samples collected opportunistically from wildlife, the highest prevalence of mixed infections was detected in kangaroos (2.8%), followed by 1.7% in cattle, 0.9% in rabbits and 0.3% in pigs (Table 4.5). Overall, a total of 15 species and four genotypes of *Cryptosporidium* were detected by NGS; *C. bovis*, *C. cuniculus*, *C. fayeri*, *C. felis*, *C. galli*, *C. hominis*, *C. macropodum*, *C. meleagridis*, *C. muris*, *C. parvum*, *C. rayane*, *C. scrofarum*, *C. suis*, *C. erinacei, C. ubiquitum*, kangaroo genotype I, rat genotype I, rat genotype II and rat genotype III (Table 4.6). The number of species identified in individual hosts ranged from one to six species. However, *C. parvum*, *C. hominis*, *C. cuniculus*, *C. muris*, *C. fayeri*, *C. macropodum*, *C. suis*, *C. galli,* kangaroo genotype I and rat genotype III were the most abundant species identified in samples with mixed infections and therefore, were

considered for prevalence and statistical analysis in this study. A total of 39 cattle samples, of which 32 were from beef cattle (11 from QLD, 19 from NSW and two from WA), and seven from dairy cattle from QLD), produced mixed chromatograms by Sanger sequencing. NGS analysis identified mixed infections and the four most abundant species and one most abundant *Cryptosporidium* genotype in individual samples were; *C. parvum* (*n* = 16), *C. hominis* (*n* = 3), *C. muris* (*n* = 14), *C. galli* (*n* = 5) and rat genotype III (*n* = 1). In kangaroos, *C. parvum* (*n* = 52), *C. hominis* (*n* = 48), *C. macropodum* (*n* = 17), *C. fayeri* (*n* = 5), *C. galli* (*n* = 3) and kangaroo genotype I (*n* = 1) were identified. Re-analysis of all seven faecal samples from wild pigs, all seven rabbit samples and two faecal samples collected from feral deer by NGS, identified *C. suis*, *C. cuniculus* and *C. muris* as the most abundant species, respectively (Table 4.6).

Table 4.5. Prevalence of mixed infection with different species of *Cryptosporidium* identified by NGS in samples which produced mixed chromatograms by Sanger-sequencing across three states of Australia; NSW, QLD and WA.

Catchment	Cattle No +/total no (% prevalence + 95 CI)	Sheep No +/total no (% prevalence + 95 CI)	Kangaroos No +/total no (% prevalence + 95 CI)	Rabbits No +/total no (% prevalence + 95 CI)	Horses No +/total no (% prevalence + 95 CI)	Birds No +/total no (% prevalence + 95 CI)	Pigs No +/total no (% prevalence + 95 CI)	Other No +/total no (% prevalence + 95 CI)
QLD								
Catchment A	5/179 (2.8%- 0.9-6.4)	0/9 (0%- 0-33.6)	NC	NC	0/15 (0%- 0-21.8)	0/2 (0%- 0-84.2)	0/3 (0%- 0-70.8)	0/1 (0%- 0-97.5)
Catchment B	2/194 (1%- 0.1-3.7)	NC	NC	NC	0/16 (0%- 0-26)	0/4 (0%- 0-60.2)	N/C	NC
Catchment C	5/195 (2.6- 0.8-5.9)	NC	NC	0/5 (0%- 0-52.2)	0/7 (0%- 0-41)	0/3 (0%- 0-70.8)	0/1 (0%- 0-97.5)	2*/19 (10.5%- 1.3-33.1)
Total	**12/568 (2.1%- 1.1-3.7)**	**0/9 (0%- 0-33.6)**	**NC**	**0/5 (0%- 0-52.2)**	**0/38 (0%- 0-9.3)**	**0/9 (0%- 0-33.6)**	**0/4 (0%- 0-60.2)**	**2/20 (10%- 1.2-31.7)**
NSW								
Catchment A	5/243 (2.1%- 0.7-4.7)	0/217 (0%- 0-1.7)	23/261 (8.8%- 5.7-12.9)	0/217 (0%- 0-1.7)	NC	NC	NC	NC
Catchment B	NC	NC	6/574 (1%- 0.4-2.3)	NC	0/5 (0%- 0-52.2)	NC	0/4 (0%- 0-60.2)	NC
Total	**5/243 (2.1%- 0.7-4.7)**	**0/217 (0%- 0-1.7)**	**29/835 (3.5%- 2.3-4.9)**	**0/217 (0%- 0-1.7)**	**0/5 (0%- 0-52.2)**	**NC**	**0/4 (0%- 0-60.2)**	**NC**
WA								
Catchment A	NC	NC	3/443 (0.7%- 0.1-2)	1/150 (0.7%- 0-3.7)	NC	0/7 (0%- 0-41)	NC	NC
Catchment B	NC	NC	12/600 (2%- 1-3.5)	NC	NC	NC	NC	NC
Catchment C	NC	NC	11/450 (2.4%- 1.2-4.3)	3/150 (2%- 0.4-5.7)	NC	NC	NC	NC
Catchment D	2/300 (0.6%- 0.1-2.4)	0/150 (0%- 0-2.4)	3/150 (2%- 0.4-5.7)	NC	NC	NC	NC	NC
Catchment E	NC	NC	7/450 (1.6%- 0.6-3.2)	2/150 (1.3%- 0.2-4.7)	NC	NC	NC	NC
Catchment F	NC	NC	5/300 (1.7%- 0.5-3.8)	NC	NC	NC	1/300 (0.3%- 0-1.8)	NC

Total	**2/300 (0.6%- 0.1-2.4)**	**0/150 (0%- 0- 2.4)**	**41/2,393 (1.7%- 1.2-2.3)**	**6/450 (1.3%- 0.5-2.9)**	**NC**	**0/7 (0%- 0-41)**	**1/300 (0.3%- 0 -1.8)**	**NC**
Overall Prevalence	**19/1,111 (1.7%- 1-2.7)**	**0/376 (0%- 0-1)**	**70/3,228 (2.8%- 1.7-2.7)**	**6/672 (0.9%- 0.3-1.9)**	**0/43 (0%- 0- 8.2)**	**0/16 (0%- 0- 20.6)**	**1/308 (0.3%- 0-1.8)**	**2/20 (10%- 1.2-31.7)**

*Feral deer

Table 4.6. Most abundant *Cryptosporidium* species detected by NGS in different hosts and catchments across three states of Australia; NSW, QLD and WA.

Host	***C. parvum*** **No +/total no (% proportion+ 95 CI)**	***C. hominis*** **No +/total no (% proportion + 95 CI)**	***C. bovis*** **No +/total no (% proportion + 95 CI)**	***C. ubiquitum*** **No +/total no (% proportion + 95 CI)**	***C. ryanae*** **No +/total no (% proportion + 95 CI)**	***C. suis*** **No +/total no (% proportion + 95 CI)**	***C. scrofarum*** **No +/total no (% proportion + 95 CI)**	***C. cuniculus*** **No +/total no (% proportion + 95 CI)**	***C. macropodum*** **No +/total no (% proportion + 95 CI)**	**Other No +/total no (% proportion + 95 CI)**
QLD										
Cattle	8/18 (44.4%-21.5-69.2)	ND	ND	ND	ND	ND	ND	ND	ND	10/18[a] (55.6%-30.8-78.5)
Sheep	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Horses	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Birds	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Pigs	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Wildlife	ND	ND	ND	ND	ND	ND	ND	ND	ND	2/2[b] (100%-15.8-100)
Total	**8/18 (44.4%-21.5-69.2)**	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	**12/20 (60%-36.1-80.9)**
NSW										
Cattle	6/19 (31.6%-12.6- 56.6)	3/19 (15.8%- 3.4-39.6)	ND	N/D	ND	ND	ND	ND	ND	10/19[c] (52.6%-28.9-75.6)
Sheep	N/D	ND	ND	ND	ND	ND	ND	ND	ND	ND
Kangaroos	24/45 (53.3%-37.9-68.3)	17/45 (37.8%-23.8-53.5)	ND	ND	ND	ND	ND	ND	2/45 (4.4%-0.5-15.1)	2/45[d] (4.4%-0.5-15.1)
Rabbits	N/D	ND	ND	ND	ND	ND	ND	ND	ND	ND
Total	**30/64 (46.9%-34.3-60)**	**20/64 (31.2%-20.2-44.1)**	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	**2/45 (4.4%-0.5-15.1)**	**12/64 (18.7%-10.1-30.5)**
WA										
Cattle	2/2 (100%-15.8-100)	ND	ND	ND	ND	ND	ND	ND	ND	ND
Sheep	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

Kangaroos	28/81 (34.6%-24.3-46)	31/81 (38.3%-27.7-29.7)	ND	ND	ND	ND	ND	ND	15/81 (18.5%-10.8-28.7)	7/81[e] (5.6%-3.5-17)
Rabbits	ND	ND	ND	ND	ND	ND	ND	7/7 (100%-59-100)	ND	ND
Pigs	ND	ND	ND	ND	ND	7/7 (100%-59-100)	ND	ND	ND	ND
Birds	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Total	**30/83 (30.1%-25.9-47.4)**	**31/81 (38.3%-27.7-29.7)**	**ND**	**ND**	**ND**	**7/7 (100%-59-100)**	**ND**	**7/7 (100%-59-100)**	**15/81 (18.5%-10.8-28.7)**	**7/81 (5.6%-3.5-17)**

ND = Not Detected

[a]*Cryptosporidium muris* (*n* = 4), *Cryptosporidium galli* (*n* = 5), rat genotype III (*n* = 1)

[b]*Cryptosporidium muris* (*n* = 2, feral deer)

[c]*Cryptosporidium muris* (*n* = 10)

[d]*Cryptosporidium galli*

[e]*Cryptosporidium fayeri* (*n* = 5), *Cryptosporidium galli* (*n* = 1), kangaroo genotype I (*n* = 1)

4.7 Discussion

Relatively few large-scale studies of *Cryptosporidium* species in animals inhabiting drinking water catchments have been conducted. The present study is the largest single published study conducted to date globally and analysed *Cryptosporidium* species and subtypes in 5,774 animal faecal samples from catchments across three states of Australia; NSW, QLD and WA. As such, it provides a unique perspective on the epidemiology of *Cryptosporidium* in animals inhabiting water catchments across Australia. In the present study, the overall prevalence of *Cryptosporidium* was 18.3% (1,054/5,774; 95% CI, 17.3-19.3), with a prevalence of 25.9% (169/653, 95% CI; 22.6-29.4) in QLD, 17.6% (632/3,600, 95% CI; 16.3-18.8) in WA and 16.6% (253/1,521, 95% CI; 14.8-18.6) in NSW. Previous studies in Australia have reported *Cryptosporidium* in animal faecal samples from catchments across Australia at varying prevalence; 5-25.8% in NSW (Power et al., 2004, 2005; Cox et al., 2005; Ryan et al., 2005; Ng et al., 2011b; Zahedi et al., 2016b), 1.6-2.8% in Victoria (Cinque et al., 2008; Nolan et al., 2013; Koehler et al., 2014a, 2016) and 6.7-16% in WA (McCarthy et al., 2008; Ferguson, 2010). All of these, with the exception of one study (Cox et al., 2005), have conducted genetic characterisation. The largest study was conducted in Melbourne drinking water catchments and screened 4,256 wildlife faecal samples between 2011-2015 (Koehler et al., 2016) and overall between 2009-2015, analysed 6,265 samples (Nolan et al., 2013; Koehler et al., 2016) and reported a prevalence of 2.8% (56/2,009) and 1.6% (69/4,256), respectively (Nolan et al., 2013; Koehler et al., 2016). The reason for the much lower prevalence of *Cryptosporidium* in Melbourne catchments is unclear, but it has been suggested that the low prevalence could be due to animal culls (resulting in lower density of animals), changing water levels of the reservoirs and the end of a nine-year drought (Koehler et al., 2016).

Several studies have examined the transport of oocysts from the site of deposition in catchments into surface waters used for producing drinking water (Davies et al., 2004; Atwill et al., 2006b; Ferguson et al., 2007; Curriero et al., 2011; Khaldi et al., 2011). However, in Australia, little published information is available on the prevalence of *Cryptosporidium* in source water. Analysis of *Cryptosporidium* monitoring data across Australia in source water over a period of 11-18 years by Water Research Australia (WaterRA), revealed that detection of total (i.e. presumptive) *Cryptosporidium* ranged from ~ 20% of samples from South Australia and WA, to ~ 15% in Melbourne and Canberra, and 2-7% in NSW (Deere et al., 2014), suggesting that significant numbers of oocysts in faecal samples deposited in catchments are transported into source waters due to rainfall run-off. Another study in South Australia, reported a significant increase in oocyst concentrations after a rainfall event (Swaffer et al., 2014). Transport of oocysts into drinking water will be affected by climate change, as it is expected to result in less winter rainfall but more extreme precipitation events during summer (Sterk et al., 2016). Initial modelling of the impact of climate change on runoff of *Cryptosporidium* from land to surface water suggests a complex relationship that will require site-specific analysis (Sterk et al., 2016), and highlights the importance of continued monitoring of *Cryptosporidium* in catchments.

In the present study, the prevalence of mixed infection assigned to *Cryptosporidium* spp. in faecal samples collected from animals inhabiting 11 catchments across three states in Australia was 1.7% (98/5,774; 95% CI, 1.4-2.1). This is the first study to apply NGS in a large-scale study to determine the prevalence of *Cryptosporidium* species in animal faecal samples with mixed *Cryptosporidium* infections, with up to six species identified in a single host. Interestingly *C. parvum* was not detected by Sanger sequencing in kangaroos, yet *C. parvum* and *C. hominis* were among the most abundant species detected in co-infections in cattle and kangaroos by NGS (Table 4.6 and Supplementary Table 4-S1 - Digital appendix 2). This has

important implications for catchment management, as animals may be shedding human-infectious species intermittently or in lesser abundance than a dominant species, but if analysed by Sanger sequencing, these species may not be detected. In the present study, NGS was used only on samples that produced mixed chromatograms via Sanger sequencing due to cost constraints. Future studies should apply NGS to type all *Cryptosporidium* positives from catchments.

Because NGS technologies are highly sensitive, allowing for the detection of low abundance sequences in complex DNA mixtures, the presence of cross-contamination between samples, or contamination from the laboratory environment has an increased potential to influence the taxonomic composition of samples. In addition, sequences that occur in very low abundances are likely to be caused by sequencing error, as errors are introduced randomly and are not reproduced, and are likely to be unique. Therefore, there is an intrinsic distrust of sequences or taxa that are present in low abundances in samples, such as some of the mixed *Cryptosporidium* infections detected in the present study.

However, no amplified *Cryptosporidium* DNA was detected in no-template or extraction reagent blank PCR controls after both initial amplicon-generation PCR or the indexing PCR, indicating that any cross contamination, or contamination from the laboratory, was below the detectable limit for the library preparation process. In addition, sequences with less than 100 identical replicate copies were excluded from the dataset in an attempt to mitigate sequencing error. Many taxa in the present study, such as *C. erinacei, C. ubiquitum*, *C. scrofarum, C. ryanae*, *C. meleagridis*, and *C. bovis* are only present in low abundances, and therefore their presence cannot be attributed to cross contamination from highly infected samples.

Analysis of the oocyst load per gram of faeces (g^{-1}) revealed that the highest median concentration of *Cryptosporidium* oocysts was shed by cattle; 31,072 oocyst/g^{-1}, followed by rabbits (27,919 oocyst/g^{-1}), sheep (26,756 oocyst/g^{-1}), pigs (21,164 oocyst/g^{-1}), kangaroos

(16,018 oocyst/g^{-1}) and birds (10,032 oocyst/g^{-1}). These values are much higher than a previous study, which examined a range of animal faeces in Sydney catchments and reported that the range of oocyst shedding concentration for cattle was <1-17,467 g^{-1}, with medians of 0.5-23 oocysts g^{-1} for adult and juvenile cattle respectively, for sheep, a range of 1-152,474 g^{-1} with medians of 148 and 275 g^{-1} for adults and juveniles respectively, <1-770 g^{-1} for pigs with a median of 0.5 g^{-1} and 1-39,423 g^{-1} for kangaroos with a median of 0.5 g^{-1} (Davies et al., 2003). Studies in the US reported that feedlot cattle shed 7.7×10^4 to 2.3×10^5 and 1.3 to 3.6 oocysts g^{-1} respectively (Hoar et al., 2000; Atwill et al., 2006a). This translates to 1.7×10^5 and 1.4-2.8 $\times 10^4$ oocysts/animal per day (Hoar et al., 2000; Atwill et al., 2006a). A limitation of these studies, is that oocyst shedding is intermittent (Xiao and Herd, 1994) and recovery rates from faecal samples and across animal types can be highly variable. For example, recovery rates ranging from 14-70% for adult cattle faeces, 0-83% for calf faeces, 4-48% for sheep faeces, 40-73% for kangaroo faeces, and 3-24% for pig faeces have been reported (Davies et al., 2003). A more recent study based on qPCR, reported a range of 63 - 7.9×10^6 and a median of 3.2×10^4 g^{-1} for oocysts in sheep faeces across three states (WA, NSW and South Australia) (Yang et al., 2014). Other studies have reported that neonatal calves can excrete up to 30 billion oocysts or more over a 1-2 week period (Kuczynska and Shelton, 1999) and that even apparently healthy animals can shed high numbers of oocysts (>5×10^6 oocysts g^{-1}) (Chalmers and Giles, 2010). This coupled with the very low infectious dose (10-100 oocysts) of *Cryptosporidium* (DuPont et al., 1995), has resulted in very significant numbers of oocysts entering drinking water supplies resulting in outbreaks e.g. the waterborne outbreak of cryptosporidiosis in humans in England in 2008 caused by *C. cuniculus* (Puleston et al., 2014).

In the present study, oocyst numbers (g^{-1}) were determined directly by qPCR using droplet digital PCR (ddPCR) calibrated standards, which obviates the need for recovery rate calculations and has the advantage of providing more accurate quantitation (Yang et al., 2014).

Unfortunately, as the population size of animal hosts are unknown in all the catchments, it is not possible to calculate catchment loading of oocysts. This is clearly a knowledge gap that needs to be addressed.

In the present study, a total of 17 *Cryptosporidium* species and four genotypes were detected (Sanger sequencing combined with NGS). Of these, 13 are infectious to humans; *C. parvum, C. hominis* and *C. meleagridis* are the most common species in humans in Australia (Ryan and Power, 2012), *C. ubiquitum* and *C. cuniculus* are considered emerging human pathogens (Puleston et al., 2014; Koehler et al., 2014a; Li et al., 2015), there have been numerous reports of *C. canis*, *C. felis*, *C. muris*, *C. suis, C. erinacei* and *C. scrofarum* in humans (cf. Ryan et al., 2017), two reports of *C. bovis* (Khan et al., 2010; Ng et al., 2012) and one report of *C. fayeri* in humans (Waldron et al., 2010). This is the first report of *C. erinacei* in Australia. It was detected in cattle and kangaroo faecal samples by NGS only, where it accounted for 2-32% of reads (Supplementary Table 4-S1 - Digital appendix 2). As with the prevalence of oocysts in source water, very little is known about the species of *Cryptosporidium* in source water in Australia, but a study in South Australia identified *C. parvum, C. muris, C. ubiquitum, C. ryanae, C. bovis, C. cuniculus* (subtypes Va and Vb), *C. fayeri, C. canis*, rat genotype and mouse genotype II (Swaffer et al., 2014; King et al., 2015). Little is known however about the prevalence of *Cryptosporidium* species in source water in other states and future studies in this area are needed.

In cattle, the prevalence of *Cryptosporidium* was high (22.3%-26.3%) across three states and *C. parvum* was the dominant species ranging from 39.1%-50.7% of samples positive for *Cryptosporidium* in cattle in each state, followed by *C. bovis* (17.6%-28.1%), *C. muris* (8.1%-15.6%) *C. hominis* (4.7-14.1%), *C. ubiquitum* (2.7%) and *C. ryanae* (1.6%-19.7%). *Cryptosporidium andersoni* was not detected. Most of the cattle sampled were adult cattle and therefore the high prevalence of *C. parvum* is surprising, as other studies have suggested that

C. parvum dominates in pre-weaned calves but that *C. bovis, C. ryanae* and *C. andersoni* dominate in older cattle (Santín et al., 2008). This highlights the importance of site-specific analysis for accurate QMRA analysis. The *C. parvum gp60* subtypes identified (IIaA15G2R1, IIaA16G2R1, IIaA17G2R1, IIaA18G3R1, IIaA19G2R1, IIaA19G3R1 and IIaA13G1) are commonly identified subtypes in humans and animals worldwide (Xiao, 2010; Feng et al., 2013), with the exception of subtype IIaA13G1, which has previously only been detected in a single human patient from WA (Ng-Hublin et al., 2013).

Cryptosporidium hominis was detected in cattle faecal samples across all three states at a prevalence ranging from 4.5-14.1%. Although *C. hominis* predominately infects humans, it has been previously reported in cattle in Australia (Zahedi et al., 2016b), China (Chen and Huang, 2012; Zhang et al., 2018), Kenya (Kang'ethe et al., 2012), Korea (Park et al., 2006), Malawi (Banda et al., 2009), New Zealand (Abeywardena et al., 2012), and Scotland (Smith et al., 2005). However, there is no molecular evidence confirming transmission of *C. hominis* between cattle and humans, and therefore more studies should be conducted to fully elucidate the transmission dynamics of *C. hominis* in cattle. In the present study, two *C. hominis* subtypes were detected in cattle; IbA10G2 and IdA15G1. Subtype IbA10G2 is a dominant subtype responsible for *C. hominis*-associated outbreaks of cryptosporidiosis worldwide (Xiao, 2010). Subtype IdA15G1 was identified in three cattle isolates from WA and has been detected in humans from Victoria with a history of gastrointestinal disorders (Koehler et al., 2013). It is also the dominant subtype infecting Aboriginal people in WA (Ng-Hublin et al., 2017).

Cryptosporidium macropodum, which is currently considered non-zoonotic, was the dominant species in kangaroos (49.2% of samples positive for *Cryptosporidium* in kangaroos), followed by *C. fayeri* (27.5%). Three *C. fayeri gp60* subtypes were identified; IVfA12G1T1 and two novel subtypes; IVaA11G3T1 and IVgA10G1T1R1. The former subtype has previously been reported in kangaroos (Power et al., 2009), but this is the first report of

IVaA11G3T1 and IVgA10G1T1R1 in marsupials. The subtype identified in the first human patient infected with *C. fayeri* was IVaA9G4T1R1 (Waldron et al., 2010) and has previously been identified in eastern grey kangaroos from NSW catchments (Power et al., 2009).

In addition to *C. macropodum* and *C. fayeri, C. hominis, C. parvum*, *C. galli* and kangaroo genotype I were also detected in kangaroos from NSW and WA in 13.2%, 9.2%, 0.5% and 0.2% of positives, respectively. Unfortunately, no faecal samples from kangaroos were collected from QLD catchments and given the identification of *C. hominis* in kangaroos from NSW, future studies in catchment areas of QLD should include kangaroos. In NSW catchments, a previous study reported an overall prevalence of 5% for *Cryptosporidium* from 952 animal faecal samples and 3.6% (21/576) in kangaroos (Zahedi et al., 2016b). The present study includes these samples and extends the analysis to include a total of 1,521 samples from NSW with an overall prevalence of 16.6% (95% CI, 14.8-18.6) and a prevalence of 14% (95% CI, 11.7-16.6) in kangaroos in NSW. The prevalence of *Cryptosporidium* species in Eastern grey kangaroo faecal samples in Sydney catchments has been analysed in several studies. The first genetic study (2000-2002), reported a prevalence of 6.7% (239/3,557) (Power et al., 2005) and identified only *C. macropodum* and *C. fayeri* in the populations (Power et al., 2004). The second major study (2006-2008), identified a prevalence of 16.9% (27/160) and identified *C. macropodum* (*n* = 2), *C. parvum* (*n* = 6), *C. hominis* (*n* = 18) and a *C. parvum*-like isolate (*n* = 1) (Ng et al., 2011b). However, the finding of *C. hominis* and *C. parvum* in the kangaroo faecal samples in that study could not be confirmed at additional loci, presumably due to the low levels of oocysts in the samples. In the present study, *C. hominis* was identified by Sanger analysis and NGS in 5.2% (43/835; 3.8-6.9) of kangaroos screened in NSW and in the previous related study by Zahedi et al. (2016b), the median numbers of *C. hominis*/g^{-1} was 4,831 with a range of 26-16,890 g^{-1} (Zahedi et al., 2016b), indicating that significant numbers of oocysts were present in some samples. Another recent study also analysed these kangaroo-derived *C.*

hominis isolates using both Sanger and NGS (Zahedi et al., 2017). In that study, unlike *C. parvum* isolates, in which additional within-host *gp60* subtype diversity was identified by NGS, only one *C. hominis* subtype was identified by both Sanger and NGS in the kangaroo-derived DNA samples, suggesting a single, recent introduction of *C. hominis* into kangaroos (Zahedi et al., 2017). The *C. hominis* in the kangaroos may have come from spill-back from humans in the catchments, which may have also have spilled-over to infect cattle in the catchments. The lack of identification of *C. hominis* in kangaroos in NSW catchments prior to 2011 tends to support this. However, only a small fraction of samples were typed in those studies and it is not possible to determine if even the same kangaroo populations were analysed in the previous studies and therefore it is impossible to draw any real inferences. Collection site coordinates of *C. hominis* positive kangaroo and cattle samples in NSW indicated that there was a geographical overlap between areas from which six cattle and nine kangaroo *C. hominis* positives (including both subtypes IbA10G2 and IdA15G1) were collected (S-34.61278, E150.58498). *Cryptosporidium galli* (a common bird parasite) and kangaroo genotype I, previously only reported in western grey kangaroos in WA (Yang et al., 2011) were also detected in kangaroos, but neither are considered zoonotic.

Despite being the third most common *Cryptosporidium* species detected in humans in Australia (Ryan and Power, 2012), *C. meleagridis* was only detected in three bird isolates from WA, however very low numbers of faecal samples were collected from birds from WA ($n = 7$) and QLD ($n = 9$) and no samples were collected from NSW. The *C. meleagridis gp60* subtype detected was IIIeA18G2R1, which has not been previously reported.

Cryptosporidium cuniculus was not detected in the five samples that were collected from QLD, but was detected at an overall prevalence of 27.6% (60/217) in rabbits in NSW and 9.6% (43/450; 95% CI, 7-12.7%) in rabbits in WA. This species has been previously identified in rabbits, humans and a kangaroo in Australia (Nolan et al., 2010, 2013; Sari et al., 2013

unpublished - KF279538; Koehler et al., 2014a). It has also been linked to a number of sporadic human cases across the UK (Chalmers et al., 2011; Elwin et al., 2012), Nigeria (Molloy et al., 2010) and France (ANOFEL, 2010) and was implicated in a waterborne outbreak of cryptosporidiosis in humans in England in 2008 (Chalmers et al., 2009; Puleston et al., 2014). Two distinct *gp60* subtype families, designated Va and Vb have been identified in *C. cuniculus* (Chalmers et al., 2009). In the present study, all six subtypes belonged to the Vb subtype family (VbA18, VbA23, VbA25, VbA26, VbA28 and VbA29). Most cases described in humans relate to Va and the first waterborne outbreak was typed as VaA22 (Robinson et al., 2008; Chalmers et al., 2009), but a VbA25 variant and VbA27 have been reported in human patients in Australia (Sari et al., 2013 unpublished - KF279538; Koehler at al., 2014b).

Cryptosporidium ubiquitum was the only species detected in sheep by Sanger sequencing (5.5%, 11.1% and 11.3% prevalence across NSW, QLD and WA respectively). It was also detected 33.3% of feral deer faecal samples in QLD. *Cryptosporidium ubiquitum* has not been identified in Australia in the limited typing of Australian human *Cryptosporidium* isolates that has been conducted to date (Ryan and Power, 2012), however it has been identified in surface waters in Australia (Monis et al., unpublished). Subtyping identified all *C. ubiquitum* positives as subtype XIIa, which has been found in humans and therefore XIIa is a potentially zoonotic subtype (Li et al., 2014).

In North America, both wildlife and domestic animals contribute to contamination of *Cryptosporidium* spp. in drinking water catchments, with prevalence ranging from 6 to 20.5% (Feng et al., 2007; Starkey et al., 2007; Ziegler et al., 2007a, 2007b; Jellison et al., 2009; Szonyi et al., 2010). In those studies, the majority of *Cryptosporidium* spp. detected in wildlife species were host adapted (Feng et al., 2007; Ziegler et al., 2007a; Jellison et al., 2009), while some species such as *C. hominis*-like, *C. parvum*, *C. ubiquitum* and *C. meleagridis* occasionally detected in some hosts such as eastern gray squirrels, eastern chipmunks, beavers, woodchucks,

raccoons, red-backed voles, deer, geese, and deer mice (Feng et al., 2007; Ziegler et al., 2007a; Jellison et al., 2009). In the present study, the majority of samples collected from non-domestic species were from kangaroos and rabbits and the most significant difference between the findings of the present study and studies conducted in North America, is the identification of *C. hominis* and *C. parvum* in wildlife (kangaroo) populations. In North American catchments, *C. parvum, C. bovis, C. ryanae* and *C. andersoni* are the most commonly reported *Cryptosporidium* in cattle faeces (Starkey et al., 2007; Sznoyi et al., 2010). With the exception of *C. andersoni*, these species were also detected in cattle faeces in the present study, along with *C. hominis*. Further studies are required to understand the source and human health significance of *C. hominis* in both wildlife and livestock in drinking water catchments.

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4.10 Summary

Risk assessment and risk management of the drinking water supply in relation to *Cryptosporidium* is based, firstly, on identifying the sources of these pathogens, and secondly, whether they are human-infectious or not. Management priorities can then focus on these sites, especially those that input oocysts that may be infectious to humans. However, current methods to screen for protozoans generally rely on fluorescence microscopy (EPA method 1623), which is time-consuming and does not identify species. In this chapter advanced molecular tools were employed to conduct for the first time in Australia, a comprehensive quantitative survey of species and genotypes of *Cryptosporidium* in the dominant animals inhabiting water catchment areas across NSW, QLD and WA. This involved analysing a total of 5,774 animal faecal samples including 1,521 samples from two drinking water catchments in NSW, 653 samples from three catchments in QLD, and 3,600 samples from six catchments in WA. The overall prevalence of *Cryptosporidium* was higher than previously reported from Australia and the majority of species detected are of public health significance. The significant findings were the detection of *C. hominis* in cattle and kangaroos, and the high prevalence of *C. parvum* in cattle. In addition, two novel *C. fayeri* subtypes and one novel *C. meleagridis* subtype were identified, and *C. erinacei* was reported for the first time in Australia. Conclusive molecular evidence linking contamination of water supplies by animals in catchments with outbreaks of cryptosporidiosis in human populations is scant. However, several studies have strongly linked outbreaks of cryptosporidiosis with sheep and cattle grazing near the implicated reservoir, catchment or river. This intensifies during extreme ecological events such as rainstorms and snowmelt, which usually lead to run-off from the environment and can increase the pollution of surface water with animal excreta. Therefore, *Cryptosporidium* from animals may end up in public water supplies at levels that could pose a risk to human health and cause a public health

problem. This magnifies the importance of the identification and quantification of zoonotic *Cryptosporidium* species in animals contaminating catchment areas in the present study for optimal catchment management. Data from this study, will lead to a better understanding of epidemiology and transmission dynamics of *Cryptosporidium* species across catchments investigated in the present project. However, further studies are required to document loads of *Cryptosporidium* oocysts transported to raw water, oocyst viability and the effectiveness of treatment processes to inactivate or remove oocysts, so that more robust risk assessments can be undertaken.

Chapter Five - Profiling the diversity of *Cryptosporidium* species and genotypes in wastewater treatment plants in Australia using Next Generation Sequencing

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5.1 Preface

This chapter consists of a manuscript entitled "Profiling the diversity of *Cryptosporidium* species and genotypes in wastewater treatment plants in Australia using Next Generation Sequencing", which has been published in Science of the Total Environment for publication (*see* Appendix 6). In Chapter four, zoonotic *Cryptosporidium* species in animal faecal samples collected from drinking water catchments across three states in Australia were identified and analysed. In this Chapter, the methodology and molecular data described in Chapter four is built upon, however it focuses on the screening, quantification and characterisation of *Cryptosporidium* species in wastewater treatment plant (WWTP) influent samples. For the first time, NGS was utilised on a large scale, to more accurately determine the prevalence and composition of *Cryptosporidium* species in WWTPs (aims 1-4 of the present thesis).

5.2 Statement of contribution

As first author in this paper, **AZ** processed all the samples, conducted all the labwork, analysed the data, interpreted the findings and wrote the first and final draft of the manuscript. **AG** and **TG** assisted with preparation of samples for NGS and data analysis. **PM** and **CO** contributed to data analysis and manuscript preparation. **AB**, **AB** and **AW** provided logistic support to the project, organised samples collection from other states (NSW, QLD and WA) and reviewed and edited the manuscript. **IR** validated statistical analysis. **UR** managed and supervised the project, was directly involved in project administration, conceptualisation, funding acquisition, methodology, data analysis and validation, and critically revised the manuscript. All authors reviewed and approved the final version of the manuscript.

AZ: 70%

5.3 Abstract

Wastewater recycling is an increasingly popular option in worldwide to reduce pressure on water supplies due to population growth and climate change. *Cryptosporidium* spp. are among the most common parasites found in wastewater and understanding the prevalence of human-infectious species is essential for accurate quantitative microbial risk assessment (QMRA) and cost-effective management of wastewater. The present study conducted next generation sequencing (NGS) to determine the prevalence and diversity of *Cryptosporidium* species in 730 raw influent samples from 25 Australian wastewater treatment plants (WWTPs) across three states: New South Wales (NSW), Queensland (QLD) and Western Australia (WA), between 2014 and 2015. All samples were screened for the presence of *Cryptosporidium* at the 18S rRNA (18S) locus using quantitative PCR (qPCR), oocyst numbers were determined directly from the qPCR data using DNA standards calibrated by droplet digital PCR, and positives were characterized using NGS of 18S amplicons. Positives were also screened using *C. parvum* and *C. hominis* specific qPCRs. The overall *Cryptosporidium* prevalence was 11.4% (83/730): 14.3% (3/21) in NSW; 10.8% (51/470) in QLD; and 12.1% (29/239) in WA. A total of 17 *Cryptosporidium* species and six genotypes were detected by NGS. In NSW, *C. hominis* and *Cryptosporidium* rat genotype III were the most prevalent species (9.5% each). In QLD, *C. galli*, *C. muris and C. parvum* were the three most prevalent species (7.7%, 5.7%, and 4.5%, respectively), while in WA, *C. meleagridis* was the most prevalent species (6.3%). The oocyst load/Litre ranged from 70 to 18,055 oocysts/L (overall mean of 3,426 oocysts/L: 4,746 oocysts/L in NSW; 3,578 oocysts/L in QLD; and 3,292 oocysts/L in WA). NGS-based profiling demonstrated that *Cryptosporidium* is prevalent in the raw influent across Australia and revealed a large diversity of *Cryptosporidium* species and genotypes, which indicates the potential contribution of livestock, wildlife and birds to wastewater contamination.

5.4 Introduction

Australia is the driest of the world's inhabited continents, with the lowest percentage of rainfall as run-off and the lowest amount of water in rivers (Anonymous, 2004). Drinking water resources are under considerable strain as a result of major shifts in long-term climate change, and climate predictions for all Australian States and Territories suggest increasing temperatures, a decline in average rainfall, but increasing severity and frequency of storm events (Garnaut Review, 2008). Consequently, there is increasing pressure for more efficient use of water resources, both in urban and rural environments (Toze, 2006a). Recycling wastewater will help address these challenges and is a prominent option among the various alternative sources of water in both developing and developed countries (Miller, 2006; Mekala and Davidson, 2016). However, infection with pathogenic microorganisms is a major risk factor (Rodriguez-Manzano et al., 2012) and therefore water destined for reuse must be fit for purpose (Toze, 2006b).

The waterborne parasite *Cryptosporidium* represents an important public health concern for water utilities, as it is a major cause of diarrhoea and there is neither a vaccine nor an effective treatment (Ryan et al., 2016; Zahedi et al., 2016a). *Cryptosporidium* is particularly suited to waterborne transmission as the oocyst stage is highly resistant to chlorine disinfection and can penetrate and survive routine water and wastewater treatment systems (King and Monis, 2007; King et al., 2017; Ryan et al., 2017a). The parasite has been responsible for numerous large-scale waterborne outbreaks worldwide (Efstratiou et al., 2017) and is highly prevalent in wastewater (Amorós et al., 2016; Ma et al., 2016).

Cryptosporidium species are currently monitored in wastewater using standard detection methodologies (i.e. fluorescence microscopy using EPA method 1623 - USEPA, 2012), however, this method cannot discriminate between different *Cryptosporidium* species. Of the

37 recognised *Cryptosporidium* species, *C. hominis* and *C. parvum* are the dominant species that infect humans (Ryan et al., 2016; Zahedi et al., 2017a; Čondlová et al., 2018; Kváč et al., 2018). As not all species of *Cryptosporidium* are infectious to humans (Ryan et al., 2016), understanding the diversity of *Cryptosporidium* in wastewater is crucial for more accurate quantitative microbial risk assessment (QMRA), for proper management of wastewater and its recycling. Due to the complex composition, abundance, and distribution patterns of *Cryptosporidium* species present in wastewater samples, molecular techniques such as conventional PCR and Sanger sequencing-based genotyping methods are unable to resolve complex DNA mixtures due to mixed sequencing chromatograms and are also unable to detect low abundance species or variants of *Cryptosporidium* (which typically appear as a "bumpy baseline" in Sanger chromatograms) (Murray et al., 2015; Paparini et al., 2015; Grinberg and Widmer, 2016).

Next-generation sequencing (NGS) technologies have allowed the comprehensive characterization and deep coverage of microbial community structure and diversity in environmental samples such as soil, water, the atmosphere and other environments (Cruaud et al., 2014). NGS is also more sensitive for the detection of less abundant species within microbial communities (Salipante et al., 2013). Recently, NGS approaches have been described that examine the composition and diversity of microbial communities (Shanks et al., 2013; Ma et al., 2015; Newton et al., 2015; Xu et al., 2017), adenovirus (Ogorzaly et al., 2015), norovirus (Prevost et al., 2015), astrovirus (Brinkman et al., 2013) and protists (Maritz et al., 2017) in sewage. However, to the best of the authors' knowledge, to date no large scale longitudinal studies have been undertaken to investigate the composition and diversity of *Cryptosporidium* species in wastewater using high-throughput amplicon NGS. As the costs of NGS continue to decrease and the bioinformatics analysis of data continues to improve, NGS screening of wastewater samples has become more feasible (Muir et al., 2016).

Therefore, the aim of the present study was to use NGS, for the first time on a large scale, to more accurately determine the prevalence and composition of *Cryptosporidium* species in Australian WWTPs across three states: New South Wales (NSW), Queensland (QLD) and Western Australia (WA).

5.5 Materials and methods

5.5.1 Study sites and sample collection

In NSW, WWTP samples (250 mL raw influent) were collected on a monthly interval over five months (April 2015 to August 2015). A total of 21 WWTP samples were collected from four wastewater plants within the WaterNSW area of operations (greater Sydney) (Table 5.1). In QLD, a total of 470 WWTP samples (250 mL raw influent) were collected on fortnightly intervals from WWTP sites ($n = 19$) across south east Queensland (Table 5.1) over a year (January 2014 to January 2015). In WA, a total of 239 WWTP samples (250 mL raw influent) were collected from two treatment plants on weekly intervals from December 2014 to December 2015 (Table 5.1). All raw influent WWTP samples were collected into individual 250 mL collection pots and stored at 4 °C until required and samples collected in NSW and QLD were shipped to Murdoch University for analysis.

Table 5.1. Wastewater treatment plants (WWTPs) included in the present study.

	Type of plant	Rural/urban plant	Source of sewage	Size of community served	Trade waste received/ not received?	Nature of trade waste	Any storm water intrusion	Animal presence/activity around the plant
QLD								
Plant A	Trickle filter	Rural	Domestic/some commercial	9,000	Not received	NA	Yes	Yes (Cattle in neighbouring properties)
Plant B	Lagoon	Rural	Domestic/some commercial	500	Not received	NA	Yes	Yes (Cattle in neighbouring field/ wildlife, kangaroo/koalas)
Plant C	Extended Activated sludge	Rural	Domestic/some commercial	1,000	Not received	NA	Yes	Yes (Bats)
Plant D	Activated sludge	Rural	Principally domestic	36,000	Small portion is trade waste	Industrial trade waste	Yes	No
Plant E	Activated sludge	Urban	Principally domestic	45,000	Significant portion is trade waste	Chemical trade waste/ Tannery waste	Yes	WWTP adjoined to scrub area with possible animal activity, i.e. kangaroos etc. but no linkage to plant inflow.
Plant F	Activated sludge	Rural	Principally domestic	1,000	Small portion is trade waste	Restaurant waste	Yes	WWTP in rural area with cattle grazing adjacent but no linkage to plant inflow.
Plant G	Activated sludge	Rural	Principally domestic	105,000	Small portion is trade waste	Industrial trade waste	Yes	WWTP adjoined to scrub area with possible animal activity, i.e. kangaroos etc. but no linkage to plant inflow.
Plant H	Activated sludge	Rural	Principally domestic	12,500	Insignificant portion is trade waste	Rendering plant that pre-treats waste before sending waste to WWTP	Yes	WWTP adjoined to scrub area with possible animal activity, i.e. kangaroos etc. but no linkage to plant inflow.
Plant I	Activated sludge	Rural	Principally domestic	2,400	Insignificant portion is trade waste	Industrial waste	Yes	WWTP in rural area with cattle grazing adjacent but no linkage to plant inflow.
Plant J	Activated sludge	Urban	Principally domestic	118,000	Significant portion is trade waste	Restaurant waste	Yes	No

Plant K	Activated sludge	Urban	Principally domestic	60,000	Small portion is trade waste	Seafood waste	Yes	No
Plant L	Activated sludge	Rural	Principally domestic	126,000	Significant portion is trade waste	Industrial trade waste	Yes	No
Plant M	Activated sludge	Rural	Decommissioned (August 2014)	NA	NA	NA	Yes	N/A
Plant N	Activated sludge	Rural	Principally domestic	22,000	Small portion is trade waste	Industrial trade waste	Yes	WWTP adjoined to scrub area with possible animal activity, i.e. kangaroos etc. but no linkage to plant inflow.
Plant O	Facultative lagoons	Rural	Principally domestic	300	Small portion is trade waste	Restaurant waste	Yes	WWTP adjoined to scrub area with possible animal activity, i.e. kangaroos etc. but no linkage to plant inflow.
Plant P	Activated sludge	Rural	Principally domestic	43,000	Significant portion is trade waste	Food manufacturing waste	Yes	No
Plant Q	Activated sludge	Rural	Principally domestic	47,000	Small portion is trade waste	Restaurant waste	Yes	WWTP adjoined to scrub area with possible animal activity, i.e. kangaroos etc. but no linkage to plant inflow.
Plant R	Activated sludge	Urban	Principally domestic	5,000	Small portion is trade waste	Restaurant waste	Yes	No
Plant S	Activated sludge	Rural	Principally domestic	26,000	Small portion is trade waste	Restaurant waste	Yes	WWTP adjoined to scrub area with possible animal activity, i.e. kangaroos etc. but no linkage to plant inflow.
NSW								
Plant A	Oxidation ditch (Pasveer), sludge lagoons	Urban	Domestic and industrial	2,000	Received	Septic tank waste, network waste (food prep, accommodation,	Yes	Yes (Native wildlife (wombats, kangaroos and birds))

						vehicle workshop)		
Plant B	IDEA tank, oxidation ditch (Pasveer) (not in operation), sludge drying / lagoons	Urban	Domestic and industrial	5,400	Received	Septic tank waste, network waste (food prep, accommodation, vehicle workshop)	Yes	Yes (Native wildlife (wombats, kangaroos and birds))
Plant C	IDEA tank, sludge drying/ lagoons	Urban	Domestic and industrial	9,000	Received	Septic tank waste, network waste (food prep, accommodation, vehicle workshop)	Yes	Yes (Native wildlife (wombats, kangaroos and birds))
Plant D	IDAL aeration, oxidation ditch (Pasveer), sludge drying/ lagoons	Urban	Domestic and industrial	14,600	Received	Septic tank waste, network waste (food prep, accommodation, vehicle workshop)	Yes	Yes (Native wildlife (wombats, kangaroos and birds))
WA								
Plant A	Activated sludge	Urban	Principally domestic (but also receives industrial waste through the sewer network)	75,000	Received	Septage and grease trap waste, abattoir and farm waste (through third party tankers)	Yes	Limited (foxes, feral cats, birds and snakes
Plant B	Pond system	Urban	Domestic	5,000	Not received	NA	Yes	Cattle, kangaroos and birds (turtles snakes and birds live in the ponds)

NA = Not Available.
IDEA = Intermittently Decanted Extended Aeration.
IDAL = Intermittently Decanted Aerated Lagoons.

5.5.2 Sample proceeding and DNA isolation

All 250 mL WWTP samples were transferred to 50 mL centrifuge tubes, and evenly weighed tubes ($n = 5$) were prepared from the same samples. These samples were centrifuged at 10,000 ×*g* for 20 min and pellets from the same samples were mixed together again. DNA was extracted from aseptically separated 250 mg aliquots of each sample (pellet), using a Power Soil DNA Kit (MO BIO, Carlsbad, California, USA) (Walden et al., 2017). An extraction blank (no WWTP sample) and a positive extraction control (a *Cryptosporidium* positive faecal sample from a kangaroo), was included in each extraction batch, as a process control for extraction efficiency. Purified DNA was stored at −20 °C prior to molecular analyses.

5.5.3 qPCR and oocyst enumeration

All WWTP sample extracts were screened for the presence of *Cryptosporidium* at the 18S rRNA (18S) locus using a quantitative PCR (qPCR) as previously described (King et al., 2005; Yang et al., 2014). A spike analysis of the 18S qPCR assay (addition of 0.5 µL of positive control DNA into test samples) was conducted on randomly selected negative samples from each group of DNA extractions, to determine if negative results were due to PCR inhibition by comparing the cycle threshold (Ct) values of the spike and the positive control (both with same concentration of DNA). In addition, *Cryptosporidium* oocyst concentrations in each sample (oocyst numbers per litre) were determined directly from the qPCR data using DNA standards calibrated by droplet digital PCR (ddPCR) (QX100™ droplet digital PCR system, Bio-Rad), which has the advantage of providing more accurate quantitation (Yang et al., 2014). Briefly, target copy numbers of the 18S gene detected in individual samples were converted to estimates of oocyst numbers based on the fact that the 18S gene in *Cryptosporidium* has five copies per haploid sporozoite (Le Blancq et al., 1997; Abrahamsen et al., 2004), and there are four haploid

sporozoites per oocyst. Therefore, every 20 copies of 18S detected by qPCR were equivalent to one oocyst. To estimate oocyst density per litre, oocyst numbers detected per 250 mg aliquots of each sample (pellet) were extrapolated to the corresponding total pellet weight extracted from each 250 mL wastewater sample, and then multiplied by four.

5.5.4 Next Generation Sequencing (NGS)

Samples that were positive by qPCR were analysed by NGS on the MiSeq (Illumina) platform at the 18S locus using the 18S iF/iR primers (Morgan et al., 1997) that were modified to contain MiSeq adapter sequences on the 5′ and 3′ end as previously described (Paparini et al., 2015). The library was prepared as per standard protocols for the MiSeq platform (Illumina Demonstrated Protocol: 16S Metagenomic Sequencing Library Preparation) with the following modifications: all PCR amplicons (uniquely indexed per sample) were double purified using the Agencourt AMPure XP Bead PCR purification protocol (Beckman Coulter Genomics, USA) and pooled in approximate equimolar ratios (based on gel electrophoresis). Sequencing was performed on an Illumina MiSeq using 500-cycle V2 chemistry (250 bp paired-end reads) following the manufacturer's recommendations. Two no-template controls and two DNA extraction reagent blank controls were included in the library preparation and distributed between samples in the PCR plate layout. All no-template and extraction reagent blank controls produced no detectable amplification of *Cryptosporidium* DNA throughout the library preparation. This indicated that level of cross contamination between samples, or from the laboratory environment, was below the detection limit of the library preparation procedure and for this reason were not sequenced. We have also previously sequenced extraction blanks and no-template controls from other NGS studies in our laboratory, and after quality filtering, <10 reads were detected in those samples.

5.5.5 Species-specific PCR for detection and enumeration of *C. hominis* and *C. parvum*

All WWTP samples positive for *Cryptosporidium* spp. by qPCR at the 18S locus were also screened and enumerated independently using primers and species-specific minor groove binder (MGB) probes to a unique *Cryptosporidium* specific gene (Clec) that codes for a novel mucin-like glycoprotein that contains a C-type lectin domain to confirm the presence/absence of *C. hominis* and *C. parvum* as described by Yang et al. (2013).

5.5.6 Statistical analysis

The overall prevalence of *Cryptosporidium* in samples collected from each WWTP was expressed as the percentage of samples positive by combined qPCR and NGS, with 95% confidence intervals calculated assuming a binomial distribution, using the software Quantitative Parasitology 3.0 (Rózsa et al., 2000). DNA extraction efficiency was estimated for each extraction, based on the number of the gene copies/oocyst equivalents measured by ddPCR. Odds ratios (OR) and their 95% confidence intervals were used to measure the strength of association of season (risk factor) with the occurrence of the *Cryptosporidium* species in WWTP samples. Chi-square and non-parametric analyses were performed using IBM SPSS 21.0 (statistical package for the social sciences) for Windows (SPSS Inc. Chicago, USA) to determine if there were any associations between the prevalence and concentration of *Cryptosporidium* oocysts at different sampling seasons and across states.

5.5.7 Bioinformatics analysis

Illumina MiSeq sequencing resulted in 1,068,270,250 bp paired-end reads with 78% of the basecalls >Q30. Paired-end reads were merged and quality filtered with USEARCH v9.2

(Edgar, 2010), retaining reads with >50 bp merged overlap, <0.1% expected error, no mismatches in the primer sequences, a minimum length of 200 bp, and a minimum of 100 identical replicate copies as previously described (Zahedi et al., 2017b). Primer sequences and any distal bases were also removed from all reads. Reads were then denoised and chimera filtered with the UNOISE3 algorithm (Edgar, 2016) to generate 169 zero-radius operational taxonomic units (ZOTUs) that represent unique biologically correct sequences (Edgar, 2016). *Cryptosporidium* 18S ZOTU sequences were assigned taxonomy by comparing ZOTUs to a curated custom database containing 63 reliable 18S reference sequences from 35 *Cryptosporidium* species and 28 genotypes extracted from GenBank using the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990). Taxonomy was only assigned if there was a single unambiguous BLAST hit with >99% pairwise identity over >98% of the query ZOTU sequence. Of the 169 ZOTUs generated, 62 did not match any known *Cryptosporidium* species or genotypes. These non-specific ZOTUs were compared to GenBank using BLAST, and where possible, taxonomy was assigned when queries hit reference sequences with >99% identity over >98% of the query reads and matched to no other taxa at the same level. Many of these non-specific ZOTUs were assigned to uncultured eukaryotes or benign waterborne fungi, algae and dinoflagellates (Supplementary Table 5-S1 - Digital appendix 3). Although abundant at the ZOTU level, these non-specific sequences represented a very small proportion of the total reads per sample (mean 0.71%).

5.6 Results

5.6.1 Overall prevalence of *Cryptosporidium* in WWTP samples

In the present study, a total of 730 WWTP samples from 25 WWTPs across three states in Australia (NSW, QLD and WA) were screened using qPCR, and the composition of

Cryptosporidium species in positive samples was determined by NGS. Results were tabulated as the prevalence of the most abundant single species (determined by NGS), detected per sample (Table 5.2) and the prevalence of all *Cryptosporidium* species detected across all samples regardless of their abundance (Table 5.3). Overall, *Cryptosporidium* was detected in 11.4% (83/730; 95% CI, 9.2–13.9) of WWTP samples collected across three states. (Table 5.4 and Supplementary Table 5-S1 - Digital appendix 3). This comprises a prevalence of 14.3% (3/21; 95% CI, 3–36.3) in NSW, 10.8% (51/470; 95% CI, 8.2–14) in QLD and 12.1% (29/239; 95% CI, 8.3–17) in WA. However, there was no significant difference between the prevalence in different states ($p > 0.05$). In general, across the three states, samples collected in summer were 1.9 times more likely to have *Cryptosporidium* than samples collected during winter months (Odds ratio = 1.9; 95% CI, 1.2–3.4), but there was no statistical difference between samples collected in spring, winter and autumn ($p > 0.05$). For NSW, samples were only collected for autumn and winter.

In QLD, the prevalence of *Cryptosporidium* peaked at 17.5% (18/103; 95% CI, 10.7–26.2) during summer months (averaged over two partial summers; 2014 and 2015), when the samples were 2.3 times more likely to have *Cryptosporidium* than samples collected during winter months (averaged over winter 2014 and 2015) (Odds ratio = 2.3; 95% CI, 1.2–5.2). There was no significant difference between the prevalence in spring, autumn and winter ($p > 0.05$). Unlike QLD, WA had the highest prevalence of *Cryptosporidium* in WWTP samples collected during spring (16.8%; 95% CI, 8.3–28.5), while there was no significant difference between the prevalence in summer, autumn and winter ($p > 0.05$). Although the prevalence of different species peaked at different times (Supplementary Table5-S1 - Digital appendix 3), in WA (Plant A), there was a winter peak in both *C. parvum* and rate genotype 1 and a spring peak for *C. felis*, and in plant B, there was a summer peak for *C. suis* (Supplementary Table5-S1 - Digital appendix 3).

Table 5.2. Prevalence of the most abundant *Cryptosporidium* species detected by NGS of individual wastewater treatment plant (WWTP) samples across three states of Australia; NSW, QLD and WA (based on a single species that was the most abundant species detected in each sample).

Host	*C. parvum* No +/total no (% proportion+ 95% CI)	*C. hominis* No +/total no (% proportion + 95% CI)	*C. bovis* No +/total no (% proportion + 95% CI)	*C. muris* No +/total no (% proportion + 95% CI)	*C. erinacei* No +/total no (% proportion + 95% CI)	*C. meleagridis* No +/total no (% proportion + 95% CI)	*C. galli* No +/total no (% proportion + 95% CI)	*C. canis* No +/total no (% proportion + 95% CI)	*C. felis* No +/total no (% proportion + 95% CI)	*C. suis* No +/total no (% proportion + 95% CI)	*C. macropodum* No +/total no (% proportion + 95% CI)	Other No +/total no (% proportion + 95% CI)
QLD												
Plant A	ND	ND	ND	1/25 (4%, 0.1-20.4)	ND	ND	1/25 (4%, 0.1-20.4)	ND	ND	ND	ND	ND
Plant B	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	2[a]/20 (10%, 1.2-31.7)
Plant C	ND	ND	ND	ND	ND	ND	3/26 (11.5%, 2.4-30.2)	1/26 (3.8%, 0.1-19.6)	ND	ND	ND	ND
Plant D	1/41 (2.4%, 0.1-12.9)	ND	ND	ND	ND	ND	ND	ND	1/41 (2.4%, 0.1-12.9)	ND	ND	ND
Plant E	ND	ND	ND	2/41 (4.9%, 0.6-16.5)	1/41 (2.4%, 0.1-12.9)	ND	7/41 (17%, 7.2-32.1)	ND	ND	ND	ND	ND
Plant F	ND	ND	1/40 (2.5%, 0.1-13.2)	1/40 (2.5%, 0.1-13.2)	ND	ND	4/40 (10%, 2.8-23.7)	ND	1/40 (2.5%, 0.1-13.2)	ND	ND	ND
Plant G	2/41 (4.9%, 0.6-16.5)	ND	ND	1/41 (2.4%, 0.1-12.9)	ND	ND	2/41 (4.9%, 0.6-16.5)	ND	ND	ND	ND	ND
Plant H	1/41 (2.4%-0.1-12.9)	ND	ND	ND	1/41 (2.4%, 0.1-12.9)	ND	2/41 (4.9%, 0.6-16.5)	ND	ND	ND	ND	ND
Plant I	ND	ND	ND	ND	ND	ND	1/41 (2.4%, 0.1-12.9)	ND	ND	ND	ND	ND
Plant J	ND	ND	1/41 (2.4%, 0.1-12.9)	ND	ND	ND	ND	ND	ND	ND	3/41 (7.3%, 1.5-19.9)	ND

Plant K	ND	ND	ND	ND	ND	ND	5/41 (12.2%, 4.1-26.2)	ND	ND	ND	ND	ND
Plant L	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Plant M	ND	ND	ND	1/7 (14.3%, 0.4-57.9)	ND	ND	ND	ND	ND	ND	ND	ND
Plant N	ND	ND	ND	ND	ND	ND	ND	ND	1/4 (25%, 0.6-80.6)	ND	ND	ND
Plant O	ND	ND	ND	ND	ND	ND	1/4 (25%, 0.6-80.6)	ND	ND	ND	ND	ND
Plant P	ND	ND	ND	ND	ND	ND	1/4 (25%, 0.6-80.6)	ND	ND	ND	ND	ND
Plant Q	ND	ND	ND	ND	ND	ND	1/4 (25%, 0.6-80.6)	ND	ND	ND	ND	ND
Plant R	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Plant S	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Total	**4/123 (3.2%, 0.9-8.1)**	**ND**	**2/81 (2.5%, 0.3-8.6)**	**6/154 (3.9, 1.4-8.3)**	**2/81 (2.5%, 0.3-8.6)**	**ND**	**28/308 (9.1%, 6.1-12.9)**	**1/26 (3.8%, 0.1-19.6)**	**3/85 (3.5%, 0.7-10)**	**ND**	**3/41 (7.3%, 1.5-19.9)**	**2/20 (10%, 1.2-31.7)**
NSW												
Plant A	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Plant B	1/5 (20%, 0.5-71.6)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Plant C	ND	1/5 (20%, 0.5-71.6)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Plant D	ND	1/6 (16.7%, 0.4-64.1)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Total	**1/5 (20%, 0.5-71.6)**	**2/11 (18.2%, 2.3-51.8)**	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**
WA												
Plant A	3/146 (2.1%, 0.4-5.9)	ND	ND	ND	ND	7/146 (4.8%, 1.9-9.6)	ND	ND	5/146 (3.4%, 1.1-7.8)	ND	ND	6[b]/146 (4.1%, 1.5-8.7)
Plant B	ND	ND	ND	ND	ND	6/93 (6.5%, 2.4-13.5)	ND	ND	ND	1/93 (1.1%, 0-5.8)	1/93 (1.1%, 0-5.8)	3[c]/93 (3.2%, 0.7-9.1)

Total	**3/146 (2.1%, 0.4-5.9)**	**ND**	**ND**	**ND**	**ND**	**13/239 (5.4%, 2.9-9.1)**	**ND**	**ND**	**5/146 (3.4%, 1.1-7.8)**	**1/93 (1.1%, 0-5.8)**	**1/93 (1.1%, 0-5.8)**	**9/239 (3.8%, 1.7-7)**

ND = Not Detected.

[a]rat genotype I (*n* = 1), rat genotype II (*n* = 1).

[b]rat genotype I (*n* = 2), rat genotype II (*n* = 2), rat genotype III (*n* = 1), kangaroo genotype I (*n* = 1).

[c]kangaroo genotype I (*n* = 3).

Table 5.3. Prevalence of all *Cryptosporidium* species/genotypes detected by NGS in wastewater treatment plant (WWTP) samples across three states of Australia; NSW, QLD and WA (regardless of abundance).

***Cryptosporidium* spp.**	**No +/total no (% proportion+ 95% CI)**		
	NSW	**QLD**	**WA**
C. hominis	**2/21 (9.5%; 95% CI, 1.2-30.4)**	**2/470 (0.4%; 95% CI, 0.1-1.5)**	**ND**
C. parvum	**1/21 (4.8%; 95% CI, 0.1-23.8)**	**21/470 (4.5%; 95% CI, 2.8-6.7)**	**3/239 (1.3%; 95% CI, 0.3-3.6)**
C. avium	ND	2/470 (0.4%; 95% CI, 0.1-1.5)	ND
C. bovis	ND	5/470 (1.1%; 95% CI, 0.3-2.5)	ND
C. canis	ND	1/470 (0.2%; 95% CI, 0-1.2)	ND
C. cuniculus	ND	1/470 (0.2%; 95% CI, 0-1.2)	ND
C. erinacei	1/21 (4.8%; 95% CI, 0.1-23.8)	14/470 (3.0%; 95% CI, 1.6-4.9)	3/239 (1.3%; 95% CI, 0.3-3.6)
C. fayeri	ND	1/470 (0.2%; 95% CI, 0-1.2)	ND
C. felis	ND	4/470 (0.8%; 95% CI, 0.2-2.2)	5/239 (2.1%; 95% CI, 0.7-4.8)
C. galli	1/21 (4.8%; 95% CI, 0.1-23.8)	36/470 (7.7%; 95% CI, 5.4-10.4)	ND
C. macropodum	ND	3/470 (0.6%; 95% CI, 0.1-1.9)	1/239 (0.4%; 95% CI, 0-2.3)
C. meleagridis	ND	ND	14/239 (5.9%; 95% CI, 3.2-9.6)
C. muris	1/21 (4.8%; 95% CI, 0.1-23.8)	27/470 (5.7%; 95% CI, 3.8-8.2)	1/239 (0.4%; 95% CI, 0-2.3)
C. ryanae	ND	3/470 (0.6%; 95% CI, 0.1-1.9)	ND
C. scrofarum	ND	3/470 (0.6%; 95% CI, 0.1-1.9)	2/239 (0.8%; 95% CI, 0.1-3)
C. suis	1/21 (4.8%; 95% CI, 0.1-23.8)	11/470 (2.3%; 95% CI, 1.2-4.1)	1/239 (0.4%; 95% CI, 0-2.3)
C. ubiquitum	ND	2/470 (0.4%; 95% CI, 0.1-1.5)	ND
bat genotype VI	ND	1/470 (0.2%; 95% CI, 0-1.2)	ND
kangaroo genotype I	ND	1/470 (0.2%; 95% CI, 0-1.2)	4/239 (1.7%; 95% CI, 0.5-4.2)
rat genotype I	ND	9/470 (1.9%; 95% CI, 09.1-3.6)	2/239 (0.8 %; 95% CI, 0.1-3)
rat genotype II	ND	5/470 (1.1%; 95% CI, 0.3-2.5)	2/239 (0.8%; 95% CI, 0.1-3)
rat genotype III	2/21 (9.5%; 95% CI, 1.2- 30.4)	4/470 (0.8%; 95% CI, 0.2-2.2)	2/239 (0.8%; 95% CI, 0.1-3)
rat genotype IV	ND	2/470 (0.4%; 95% CI, 0.1-1.5)	ND

ND = Not Detected.

Table 5.4. Seasonal prevalence, the mean and median *Cryptosporidium* oocyst concentration in positive samples per litre (mean, median with range in parenthesis (determined by qPCR) per season across three states of Australia; NSW, QLD and WA).

	Summer		**Autumn**		**Winter**		**Spring**		**Overall**	
State	No +/total no (% proportion + 95% CI)	Oocysts/L mean, median and range	No +/total no (% proportion + 95% CI)	Oocysts/L mean, median and range	No +/total no (% proportion + 95% CI)	Oocysts/L mean, median and range	No +/total no (% proportion + 95% CI)	Oocysts/L mean, median and range	No +/total no (% proportion + 95% CI)	Oocysts/L mean, median and range
QLD	18/103 (17.5%, 10.7-26.2)	5,966, 3,821 (192-18,055)	11/105 (10.5%, 5.3-18)	2,583, 1,974 (203-8,134)	10/131 (7.6%, 3.7-13.6)	2,323, 1,131 (172-14,602)	12/131 (9.2%, 4.8-15.5)	1,953, 1,107 (70-6,301)	**51/470 (10.8%, 8.2-14)**	**3,578, 1,619 (70-18,055)**
NSW	NC	NC	1/17 (5.9%, 0.1-28.7)	1,428, NA (only one sample)	2/4 (50%, 6.8-93.2)	6,405, 6,405 (two samples)	NC	NC	**3/21 (14.3%, 3-36.3)**	**4,746, 4,373 (1,428-8,438)**
WA	9/60 (15%, 7.1-26.6)	1,632, 1,590 (327-2,842)	4/60 (6.8%, 1.8-16.2)	1,217, 936 (599-2,398)	6/59 (10.2%, 3.8-20.8)	2,107, 1,928 (1,105-3,326)	10/60 (16.8%, 8.3-28.5)	6,326, 3,805 (2,267-16,812)	**29/239 (12.1%, 8.3-17)**	**3,292, 2,398 (327-16,812)**
Overall	**27/163 (16.6%, 11.2-23.2)**	**4,521, 2,191 (192-18,055)**	**16/174 (9.2%, 5.3-14.5)**	**2,170, 1,260 (203-8,134)**	**18/202 (8.9%, 5.4-13.7)**	**2,704, 1,627 (172-14,602)**	**22/191 (11.5%, 7.4-16.9)**	**3,941, 2,823 (70-16,812)**	**83/730 (11.4%, 9.2-13.9)**	**3,426, 1,828 (70-18,055)**

NC = Not Collected.

5.6.2 Prevalence of all *Cryptosporidium* species/genotypes as determined by NGS (regardless of abundance)

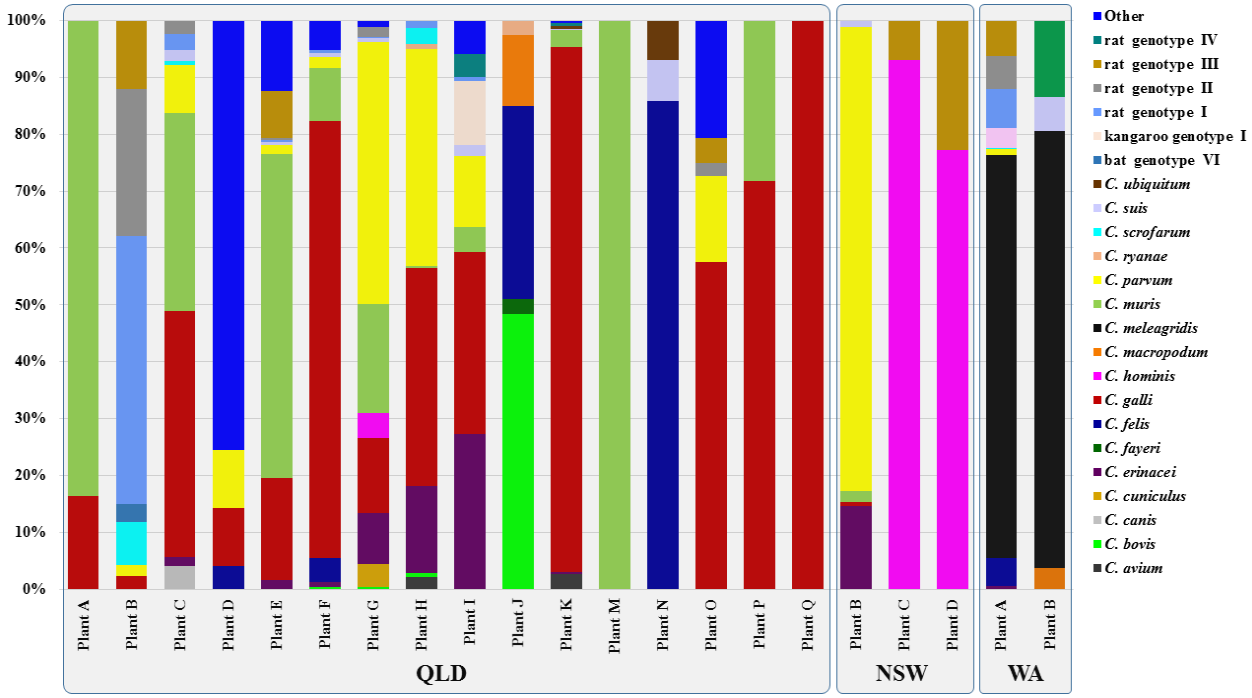
A total of 17 *Cryptosporidium* species and six genotypes were detected by NGS (Table 5.3, and Supplementary Table 5-S1 - Digital appendix 3). *Cryptosporidium hominis* and rat genotype III were the most prevalent species detected in wastewater samples collected from NSW (9.5% each, 2/21; 95% CI, 1.2–30.4). In addition to *C. hominis* and rat genotype III, *C. parvum* was detected in one NSW sample only (4.8%, 1/21; 95% CI; 0.1–23.8) and *C. erinacei*, *C. galli*, *C. muris* and *C. suis* were also detected in the same sample in low abundance (Table 5.3 and Supplementary Table 5-S1 - Digital appendix 3). In QLD, of the 51 WWTP samples positive for *Cryptosporidium*, NGS detected more than one *Cryptosporidium* species/genotype in 42 samples, ranging from two to eight species in individual samples, whereas in nine samples, only one *Cryptosporidium* species/genotype was identified. In general, the prevalence of different *Cryptosporidium* species/genotypes detected by NGS in WWTP samples across QLD ranged from 0.2% to 7.7% (Table 5.3). *Cryptosporidium galli* (7.7%), *C. muris* (5.7%) and *C. parvum* (4.5%) were the three most prevalent (and abundant) species detected in WWTP samples from QLD, followed by *C. erinacei* (3.0%), *C. suis* (2.3%) and rat genotype I (1.9%) (Table 5.3), and were significantly more prevalent than all other species detected in samples from QLD ($p < 0.05$) (Table 5.3). Unlike QLD, the majority of samples positive for *Cryptosporidium* in WA (22/29) contained only one species/genotype of *Cryptosporidium* (75.9%; 95% CI, 56.5–89.7), and only seven samples were identified with mixed *Cryptosporidium* species present (24.1%; 95% CI, 10.3–43.5). *Cryptosporidium meleagridis* was detected in 5.9% (15/239; 95% CI, 3.2–9.6) of wastewater samples collected from WA, and was significantly more prevalent than any other species detected ($p < 0.05$). However, there was no significant difference between the prevalence of other *Cryptosporidium* species

detected in WA samples ($p > 0.05$). *Cryptosporidium C. parvum* (1.3%), *C. erinacei* (1.3%), *C. scrofarum* (0.8%) and *C. muris* (0.4%) were detected at a low prevalence only in samples with mixed *Cryptosporidium* species/genotypes in WA (Table 5.3 and Supplementary Table 5-S1 - Digital appendix 3).

5.6.3 Abundance and diversity of all *Cryptosporidium* reads determined by NGS

Overall, the highest number of reads (sequences) across the 83 WWTP samples positive for *Cryptosporidium* was assigned to *C. galli* (22.8% of all sequences analysed). This was followed by *C. meleagridis* (15.7%), *C. muris* (11.9%), *C. felis* (8.7%), *C. parvum* (6.8%), kangaroo genotype I (JF316651) (4.9%), *C. macropodum* (4.5%), rat genotype I (3.9%), rat genotype II (2.9%), *C. hominis* (2.5%), *C. erinacei* (2.5%), rat genotype III (2.1%), *C. suis* (1.7%), *C. bovis* (0.8%), *C. scrofarum* (0.5%), *C. canis* (0.4%), *C. fayeri* (0.3%), *C. cuniculus* (0.2%), *C. avium* (0.2%), *C. ubiquitum* (0.1%), *C. ryanae* (0.1%), rat genotype IV (0.1%) and bat genotype VI (0.1%) (Supplementary Table 5-S1 - Digital appendix 3 and Fig 5.1). There were also a small proportion of NGS sequences (6.1%), across 11 samples, that were not assigned to any *Cryptosporidium* species or genotypes (Supplementary Table 5-S1 - Digital appendix 3 and Fig 5.1). In general, at the individual sample level across the three states, the number of species identified in individual wastewater samples ranged from one to eight species.

Fig 5.1. Percent composition of 18S sequences from *Cryptosporidium* species detected in wastewater treatment plant samples from NSW, QLD and WA.



5.6.4 Additional confirmation of presence/absence and enumeration of *C. hominis* and *C. parvum* in WWTP samples using a species-specific qPCR

Of 83 WWTP samples positive for *Cryptosporidium* spp. NGS detected *C. parvum* and *C. hominis* in 25 and four samples, respectively, including two samples that contained both species (QLD-E76 and QLD-G115) (Table 5.3, Table 5.5 and Supplementary Table 5-S1 - Digital appendix 3). A *C. parvum* species-specific qPCR assay confirmed the presence of *C. parvum* in 20/25 samples, but failed to amplify the remaining five samples, which were previously identified by NGS to contain *C. parvum* sequences in low abundance, ranging from 113 to 535 reads (Table 5.5). The occurrence of *C. hominis* in 3/4 WWTP samples was also confirmed a *C. hominis*-specific qPCR, with no *C. hominis* amplification in a single sample which was previously confirmed by NGS to contain *C. hominis* in low abundance (QLD-E76) (Table 5.5 and Supplementary Table 5-S1 - Digital appendix 3). The concentration of *C. hominis* and *C. parvum* oocysts per litre in these samples ranged from 386 to 3294 and from 14 to 6314, respectively (Table 5.5). The absence of *C. hominis* and *C. parvum* in the remaining samples ($n = 56$) was confirmed by the *C. hominis* and *C. parvum* species-specific qPCR assays (Table 5.5).

Table 5.5. *C. hominis* and *C. parvum* detected by NGS and species specific qPCR in individual wastewater treatment plant (WWTP) samples positive for *Cryptosporidium* spp. across three states of Australia; NSW, QLD and WA.

			NGS		***C. hominis* and *C. parvum* species oocysts numbers determined by specific qPCR (MGB probes)**	
State	**Plant**	**Sample**	**No of reads assigned to *C. hominis***	**No of reads assigned to *C. parvum***	***C. hominis***	***C. parvum***
QLD	Plant A	QLD-A7	0	0	ND	ND
		QLD-A24	0	0	ND	ND
	Plant B	QLD-B2	0	0	ND	ND
		QLD-B3	**0**	**109**	**ND**	**61**
	Plant C	**QLD-C2**	**0**	**779**	**ND**	**470**
		QLD-C3	**0**	**246**	**ND**	**117**
		QLD-C19	**0**	**910**	**ND**	**482**
		QLD-C25	**0**	**133**	**ND**	**50***
	Plant D	**QLD-D104**	**0**	**1,181**	**ND**	**448**
		QLD-D140	0	0	ND	ND
	Plant E	**QLD-E18**	**0**	**562**	**ND**	**82***
		QLD-E34	0	0	ND	ND
		QLD-E76	**109**	**918**	**76***	**361**
		QLD-E195	0	0	ND	ND
		QLD-E222	0	0	ND	ND
		QLD-E258	0	0	ND	ND
		QLD-E303	0	0	ND	ND
		QLD-E357	0	0	ND	ND
		QLD-E375	**0**	**265**	**ND**	**47**
		QLD-E393	0	0	ND	ND
	Plant F	QLD-F1	0	0	ND	ND
		QLD-F33	**0**	**140**	**ND**	**2,834**
		QLD-F84	**0**	**126**	**ND**	**51**
		QLD-F130	**0**	**189**	**ND**	**32**
		QLD-F157	0	0	ND	ND
		QLD-F319	0	0	ND	ND
		QLD-F382	**0**	**140**	**ND**	**38***
	Plant G	QLD-G53	0	113	**ND**	**292***
		QLD-G115	**2,382**	**4,336**	**386**	**812**
		QLD-G304	**0**	**20,571**	**ND**	**954**
		QLD-G331	0	0	ND	ND
		QLD-G340	0	0	ND	ND
	Plant H	**QLD-H8**	**0**	**18,529**	**ND**	**6,314**
		QLD-H179	**0**	**1,638**	**ND**	**922**
		QLD-H197	**0**	**4,674**	**ND**	**1,528**
		QLD-H386	**0**	**0**	ND	ND
	Plant I	**QLD-I41**	**0**	**1,873**	**ND**	**476**
	Plant J	QLD-J15	0	0	ND	ND
		QLD-J47	0	0	ND	ND
		QLD-J354	0	0	ND	ND
		QLD-J363	0	0	ND	ND
	Plant K	QLD-K71	0	0	ND	ND
		QLD-K119	0	0	ND	ND
		QLD-K281	0	0	ND	ND
		QLD-K380	0	0	ND	ND

		QLD-K389	0	0	ND	ND
	Plant M	QLD-M60	0	0	ND	ND
	Plant N	QLD-N35	0	0	ND	ND
	Plant O	**QLD-O54**	**0**	**991**	**ND**	**14**
	Plant P	QLD-P63	0	0	ND	ND
	Plant Q	QLD-Q11	0	0	ND	ND
NSW	Plant B	**NSW-B2**	**0**	**20,347**	**ND**	**1,380**
	Plant C	**NSW-C20**	**9,227**	**0**	**2,998**	**ND**
	Plant D	**NSW-D21**	**3,082**	**0**	**3,294**	**ND**
WA	Plant A	WA-A5	0	0	ND	ND
		WA-A8	0	0	ND	ND
		WA-A13	0	0	ND	ND
		WA-A16	0	0	ND	ND
		WA-A24	0	0	ND	ND
		WA-A37	0	0	ND	ND
		WA-A40	0	0	ND	ND
		WA-A55	0	0	ND	ND
		WA-A65	**0**	**884**	**ND**	**92**
		WA-A66	**0**	**2,563**	**ND**	**214**
		WA-A68	**0**	**535**	**ND**	**51***
		WA-A78	0	0	ND	ND
		WA-A79	0	0	ND	ND
		WA-A80	0	0	ND	ND
		WA-A81	0	0	ND	ND
		WA-A82	0	0	ND	ND
		WA-A88	0	0	ND	ND
		WA-A91	0	0	ND	ND
	Plant B	WA-B2	0	0	ND	ND
		WA-B4	0	0	ND	ND
		WA-B12	0	0	ND	ND
		WA-B13	0	0	ND	ND
		WA-B14	0	0	ND	ND
		WA-B19	0	0	ND	ND
		WA-B28	0	0	ND	ND
		WA-B30	0	0	ND	ND
		WA-B41	0	0	ND	ND
		WA-B42	0	0	ND	ND
		WA-B45	0	0	ND	ND

ND = Not Detected.

*For these samples, the *C. hominis* and *C. parvum* species-specific qPCR assay failed and and oocyst/L is reported based on 18S qPCR and the percentage of NGS reads attributed to *C. hominis* and *C. parvum*.

5.6.5 Enumeration of *Cryptosporidium* oocysts in wastewater samples using qPCR

Cryptosporidium oocyst concentration per litre was estimated using qPCR standards calibrated by ddPCR at the 18S locus (Table 5.4). Overall, the oocyst load per litre in samples collected across the three states ranged from 70 to 18,055 oocysts/L and the mean was 3,426 oocysts/L (Table 5.4). The mean *Cryptosporidium* oocyst concentration in samples collected from WWTPs in NSW was the highest among the states (4,746 oocysts/L). However, due to the low number of samples collected from NSW ($n = 21$), compared to 470 from QLD and 239 from WA, statistical analysis of oocyst load was only conducted for QLD and WA to avoid potential bias in the analysis.

The mean number of oocysts per litre in samples collected from the two WWTPs in WA over four seasons was 3,292 oocysts/L (ranging from 327 to 16,812), while the mean *Cryptosporidium* oocyst concentration in these samples peaked during spring 2015 at 6,326 oocysts/L (ranging from 2,267 to 16,812). This corresponded with a peak of prevalence at this time (spring 2015) (16.8%; 95% CI, 8.3–28.5) (Table 5.4).

Compared to WA, the overall mean *Cryptosporidium* oocyst concentration in WWTP samples from QLD was relatively higher (3,578 oocysts/L, ranging from 70 to 18,055). Seasonal mean concentrations (averaged over the two same seasons in 2014 and 2015) were 1,953 oocysts/L in spring, 2,323 oocysts/L in winter, 2,583 oocysts/L in autumn and 5,966 oocysts/L in summer. This also corresponded with a peak prevalence of 17.5% (95% CI, 10.7–26.2) during summer (averaged over summer 2014, 2015 and 2016) (Table 5.4).

5.7 Discussion

The present study has demonstrated the utility of NGS in detecting mixtures of *Cryptosporidium* species and genotypes in sewage and has shown that they are frequently present but variable and diverse in space, time and composition. The overall prevalence of *Cryptosporidium* in WWTP samples across Australia was 11.4% (83/730). Previous studies have reported prevalence ranging from 6.4% to 100% (Xiao et al., 2001; Ward et al., 2002; Zhou et al., 2003; Hanninen et al., 2005; Cantusio Neto et al., 2006; Hashimoto et al., 2006; Hirata and Hashimoto, 2006; Ottoson et al., 2006; Robertson et al., 2006; Castro-Hermida et al., 2008; Feng et al., 2009; Dungeni and Momba, 2010; Liu et al., 2011; Ajonina et al., 2012; Ben Ayed et al., 2012; Li et al., 2012; Gallas-Lindemann et al., 2013, Gallas-Lindemann et al., 2016; Hachich et al., 2013; Spanakos et al., 2015; Amorós et al., 2016; Hatam-Nahavandi et al., 2016; Ulloa-Stanojlović et al., 2016; ; Huang et al., 2017; Imre et al., 2017; Ramo et al., 2017; Santos and Daniel, 2017). However, to the best of the authors' knowledge, in Australia little published information is available on the prevalence and composition of *Cryptosporidium* species in wastewater (King et al., 2015, King et al., 2017).

In the present study, a total of 17 *Cryptosporidium* species and six genotypes were detected by NGS. This is higher than the diversity reported in previous studies due to the ability of NGS to detect mixtures of sequences in low abundance. Wastewater treatment networks however, rarely contain only domestic wastewater; they often also contain wastewater from industrial sources and can be influenced by environmental water sources, such as stormwater or groundwater (Pandey et al., 2014). In addition, wild animals may directly contribute to contamination of sewage, such as rodents in the sewer networks or birds present at wastewater treatment plants. Therefore, the presence of a variety of *Cryptosporidium* species from livestock, wildlife and birds in sewage samples may be attributed to other sources such as stormwater or industrial waste from animal processing.

In the present study, of 83 WWTP samples positive for *Cryptosporidium* spp., NGS detected *C. hominis* and *C. parvum* in only 27 samples (32.5%; 95% CI, 22.6–43.7), of which two samples contained both species. A *C. hominis* and *C. parvum* species-specific qPCR provided further support for the lack of *C. hominis* and *C. parvum* in the majority of samples, although it failed to detect *C. hominis* and *C. parvum* in one and five samples, respectively, which were mainly samples with low numbers of *C. parvum/C. hominis* reads (109–535 reads) by NGS. The qPCR assay has been tested extensively on human faecal samples and has been shown to have an analytical sensitivity of 1 oocyst/µL of DNA extract (Yang et al., 2013). This is the first time we have applied the qPCR to WWTP samples and no inhibition was observed.

In NSW, the dominant species detected were *C. hominis* and rat genotype III, whereas in QLD, *C. galli*, *C. muris* and *C. parvum* were the three most prevalent species, while in WA, *C. meleagridis* was the most prevalent species. Of these, *C. parvum* and *C. hominis* are the most common species reported in humans in Australia, accounting for >95% of human infections, with *C. meleagridis* the third most common species reported and usually accounting for 1–2% of notifications (Ryan and Power, 2012; Ng-Hublin et al., 2017). There have been numerous reports of *C. muris* in humans in other countries (cf. Ryan et al., 2017b). Other *Cryptosporidium* species with zoonotic potential, which were detected at a low prevalence in WWTP samples in the present study included *C. bovis*, *C. canis*, *C. cuniculus*, *C. erinacei*, *C. felis*, *C. scrofarum*, *C. suis* and *C. ubiquitum*. Nevertheless, caution is required when extrapolating any molecular data from WWTP samples to determine host sources of wastewater contamination by *Cryptosporidium*, as there are many potential input sources other than humans into wastewater networks. Unlike faecal material, there is no direct relationship between *Cryptosporidium* oocysts from wastewater samples and any potential host species, and an understanding of existing host-parasite interactions, parasite epidemiology and sources of faecal inputs into the wastewater network is required (Castro-Hermida et al., 2008).

A number of studies across the world have reported *C. hominis* (the predominant species in humans) among the most prevalent species detected in wastewater; Australia (King et al., 2015), Brazil and Peru (Ulloa-Stanojlović et al., 2016), China (Feng et al., 2009; Li et al., 2012; Huang et al., 2017), Japan (Hashimoto et al., 2006; Hirata and Hashimoto, 2006), Switzerland and Germany (Ward et al., 2002), the USA (Xiao et al., 2001; Zhou et al., 2003) and Tunisia (Ben Ayed et al., 2012). In addition to anthroponotic sources of *C. hominis*, several studies in Australia have previously identified *C. hominis* in Australian cattle and wildlife including bandicoots (*Isoodon obesulus*), brushtail possums (*Trichosurus vulpecula*), eastern grey kangaroos (*Macropus giganteus*) and brush-tailed rock-wallabies (*Petrogale penicillata*) (Hill et al., 2008; Ng et al., 2011; Dowle et al., 2013; Vermeulen et al., 2015; Zahedi et al., 2016b; Zahedi et al., 2018). To date there is no conclusive molecular or epidemiological evidence linking contamination of wastewater by animals with the occurrence of *C. hominis* in raw wastewater or in human populations in Australia and further research is required in this area. In the present study, *C. hominis* was detected in NSW in plants C and D which received mainly septic tank waste and accounted for 93.0% and 77.3% of all *Cryptosporidium* species detected in plants C and D respectively, suggesting humans were the source. In NSW, *C. hominis* was detected in plants E and G. Plant E received a significant portion of trade waste and the *C. hominis* detected accounted for only 4.4% of all *Cryptosporidium* species identified, while plant G received mostly human waste and the *C. hominis* detected accounted for 26.6% all *Cryptosporidium* species identified.

In Europe, several studies have reported that *C. parvum* is the dominant species in wastewater (Hanninen et al., 2005; Spanakos et al., 2015; Imre et al., 2017; Ramo et al., 2017), while some studies in China, Iran, Tunisia and the USA have reported that livestock associated species such as *C. andersoni* and *C. xiaoi* dominate (Xiao et al., 2001; Liu et al., 2011; Ben Ayed et al., 2012; Hatam-Nahavandi et al., 2016). In the present study, *C. andersoni* and *C.*

xiaoi were not detected in WWTPs across three states in Australia, however *C. parvum* was the third most prevalent species identified in QLD samples and was detected in a single sample and three samples from NSW and WA, respectively. *Cryptosporidium parvum* has been identified widely in both calves and humans in Australia (Ryan and Power, 2012) with reported prevalences for *C. parvum* in humans in Australia ranging from ~24% in Victoria (Jex et al., 2007; Koehler et al., 2013) to 17–19.8% in WA (Morgan et al., 1998; Ng et al., 2010) and 46.8% in NSW (Waldron et al., 2009). There are no published reports on the prevalence of *C. parvum* in the human population in QLD, which is a knowledge gap that needs to be addressed

Considering that most WWTPs in Australia are well fenced-off and protected, with minimal animal access, the predominance of *C. parvum* in wastewater in QLD may indicate that human sewage was the source of *C. parvum* or that it came from a combination of anthroponotic contributions and industry waste from abattoirs. In many of the QLD plants, a significant proportion was "trade waste" some of which may have come from abattoirs, however it was not possible to obtain further information on the sources of the trade waste. In WA, *C. parvum* was detected in plant A, which received both human and abattoir waste. In NSW, the single WWTP (Plant B) that was positive for *C. parvum* received waste predominately from septic tanks, suggesting an anthroponotic source. It is also important to remember that previous studies that reported Australian prevalence data for *Cryptosporidium* were from clinical samples, which in many cases were dominated by samples from the major metropolitan areas. Based on the population sizes for at least some of the WWTPs in the present study, most of the "urban sites" are more likely to be regional centres, so may have a different pattern of *Cryptosporidium* prevalence and species composition compared with major urban centres.

Cryptosporidium meleagridis is a common parasite of humans in Australia (Ryan and Power, 2012) and also infects a wide range of birds (Zahedi et al., 2016a), with many

overlapping *C. meleagridis* subtypes found in both birds and humans; suggesting both anthroponotic and zoonotic transmission (Silverlas et al., 2012). This is evidenced by the fact that *C. meleagridis* is commonly reported in wastewater worldwide (Hashimoto et al., 2006; Hirata and Hashimoto, 2006; Feng et al., 2009; Li et al., 2012; Huang et al., 2017). In the present study, *C. meleagridis* was the most prevalent species detected in WWTP samples collected from WA and in many cases was the only species detected (Supplementary Table 5-S1 - Digital appendix 3). However, it was not detected in NSW or QLD. Although a variety of bird species are commonly seen at WWTPs in Australia, particularly around lagoons and clarifiers (secondary and tertiary treatment), the raw sewage entries to most WWTPs are covered, and not exposed and accessible to birds and animals. Some of the *C. meleagridis* detected in WWTPs in WA could have been originated from humans, however, further investigation revealed that the raw influent samples were taken directly from the distribution chamber located just before the primary ponds, which was only covered with a layer of mesh, providing easy access to bird contamination. Alternatively, industrial sources of wastewater from poultry farms could also be a major contributor. The predominance of the bird-specific *C. galli* in WWTP samples from QLD also confirms the potential role birds may play in contamination of wastewater by *Cryptosporidium*, but currently data on the contribution of poultry farms to WWTP in both WA and QLD is lacking and is an important knowledge gap. To date, there has only been one report of *C. galli* in wastewater (Ramo et al., 2017), however, *C. baileyi*, another avian *Cryptosporidium* species, has been reported in several studies from China (Feng et al., 2009; Li et al., 2012; Huang et al., 2017). It is possible that the high levels of *C. meleagridis* and *C. galli* detected in WA and QLD respectively, were due to contamination in our laboratory. However, this is unlikely as neither species were included as controls on the same Illumina MiSeq run and quality filtering removed all reads <100. The high number of *C. meleagridis* reads in WA (107 to 58,246 reads/sample) and *C. galli* reads in

QLD (129 to 32,164 reads/sample) supports their validity. In addition, if it was due to gross contamination, then both species would be randomly distributed across all samples, with mixtures of both species in some samples.

Two emerging human-pathogenic *Cryptosporidium* species, including *C. ubiquitum* ($n = 2$) and *C. cuniculus* ($n = 1$), were also found in wastewater samples from QLD at a lower frequency and abundance than other major species. *Cryptosporidium cuniculus* is a common parasite of rabbits and has been reported in source water in South Australia (Swaffer et al., 2018) and linked to several sporadic human cases in Australia (Nolan et al., 2010, Nolan et al., 2013; Sari et al., 2013 unpublished - KF279538; Koehler et al., 2014), the UK (Chalmers et al., 2011; Elwin et al., 2012), Nigeria (Molloy et al., 2010) and France (ANOFEL, 2010). To date there are no published reports of *C. cuniculus* detected in WWTP samples in Australia, however, it has been previously reported from WWTPs in Brazil, Peru and China (Li et al., 2012; Ulloa-Stanojlović et al., 2016). Mainly infecting small ruminants, *C. ubiquitum* has been identified in a broad range of hosts including humans and wildlife (in particular rodents) with a wide geographic distribution across the world (Zahedi et al., 2016a). It has also been frequently reported from source water, stormwater runoff, stream sediment and wastewater across the world (Xiao et al., 2000; Nolan et al., 2013; Li et al., 2014). In Australia, *C. ubiquitum* has not been detected in the studies conducted to type *Cryptosporidium* isolates from humans (Ryan and Power, 2012); however, it has been identified in source water in Australia (Swaffer et al., 2018). More recently, the identification of similar *C. ubiquitum* subtypes in humans and in wastewater samples from China, Tunisia and the USA strengthens the hypothesis that sheep and wild rodents are a source of *C. ubiquitum* transmission to humans through contamination of untreated drinking water (Zhou et al., 2003; Liu et al., 2011; Ben Ayed et al., 2012; Li et al., 2014; Huang et al., 2017).

In the present study *C. muris*, a predominantly a rodent species of *Cryptosporidium*, was sporadically identified in wastewater samples from NSW and WA and was the second most prevalent species detected in QLD. There have been numerous reports of *C. muris* in humans and wastewater (Xiao et al., 2001; Ward et al., 2002; Zhou et al., 2003; Feng et al., 2009; Ben Ayed et al., 2012; Ryan and Power, 2012; Li et al., 2014; Spanakos et al., 2015; Huang et al., 2017), suggesting both human contribution as well as faecal contamination by rodents in wastewater distribution systems. However, as the frequency of detection of *C. muris* in humans is low (1–3%) (Wang et al., 2012), rodents are the more likely source. The identification of other rodent *Cryptosporidium* genotypes (rat genotypes I–IV) across all states in the present study, also supports this hypothesis.

Factors that influence oocyst density in wastewater are the incidence of cryptosporidiosis in the community (i.e. number of infected humans and animals in the community served by the WWTP), the intensity of infection (oocyst shedding), the size of the community (population), seasonality and dilution by other waste entering the WWTP (Domenech et al., 2017; King et al., 2017). In the present study, oocyst numbers per litre of sewage across the three states were estimated and ranged from 70 to 18,055 oocysts/L (mean = 3426 oocysts/L). This is similar to a previous study of WWTPs across South Australia and Victoria, with oocyst densities ranging from 3 to 21,335 oocysts/L with a mean density of 2355 oocysts/L (King et al., 2017). It is difficult, however, to compare across different studies using different methodologies. Worldwide, mean densities of between 10 and >700 oocysts/L have been commonly reported (Ajonina et al., 2012; Tonani et al., 2013; Nasser, 2016; Xiao et al., 2018) with a mean of 60,000 oocysts/L reported in one study (Cantusio Neto et al., 2006). The somewhat higher number of oocysts detected in the present study compared to other studies may be due to the fact that the oocyst concentrations were determined directly from total DNA extracted from WWTP samples by qPCR (using ddPCR calibrated standards), which may have overestimated

the oocyst concentration, as DNA from lysed (and therefore no longer viable) oocysts would also have been detected. Previous studies have purified oocysts from WWTP samples and counted intact oocysts using USEPA method 1623, however, recovery efficiencies from wastewater samples can be highly variable, ranging from 5.5 ± 1.3% to as high as 85% (Nasser, 2016). The DNA extraction efficiency in the present study is unknown.

Estimation of *Cryptosporidium* risk from wastewater requires an evaluation of the efficiency of oocyst removal and inactivation along the treatment process and the reduction in the levels of oocysts (and their infectivity) in final treated effluent compared with oocyst counts in raw sewage (Xiao et al., 2018). Guideline values have traditionally set $\log_{10}$ removal targets based on end-use application (King et al., 2017), but these guidelines still do not incorporate the potential for inactivation of oocysts throughout the treatment process. A limitation of the present study is that samples were only taken from influent raw wastewater, and oocyst numbers were not investigated across the treatment train including the final effluent. Another limitation is that the viability/infectivity of oocysts detected in WWTP samples was not analysed. A recent study developed an integrated assay to determine oocyst density and infectivity from a single-sample concentrate (King et al., 2017), which will allow for improved QMRA analysis, as only analysing total oocyst numbers in raw sewage could result in an overestimation or underestimation of the *Cryptosporidium* risk in treated water. Finally, in the present study, the weather on sampling days (and preceding days) was not taken into account in the study design and future studies should include this data to better understand the effects of storm water intrusion for all the plants studied.

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5.10 Summary

With the increasing importance of wastewater treatment and reuse, and considering that wastewater harbours an abundant and diverse range of pathogens, high-throughput sequencing is a highly efficient tool to explore the composition, abundance, and distribution patterns of microbial communities, including protozoans, present in engineered systems such as wastewater treatment facilities to ensure safer water supplies. In this Chapter, NGS was employed to explore snap shots of *Cryptosporidium* species WWTP influent samples, collected across three states (NSW, QLD and WA). The data generated demonstrated that *Cryptosporidium* is prevalent in the raw influent of wastewater treatment facilities across Australia, which suggests that the parasite may be more prevalent in human populations than diagnostic data indicates. However, it would appear that much of the *Cryptosporidium* may have come from industrial waste, livestock, wildlife and birds. NGS was central to unravelling the large diversity of *Cryptosporidium* species and genotypes in wastewater in Australia. These observations raise concerns regarding the environmental impact and public health risks associated with disposal and reuse of treated wastewater and highlight the need for updating legislation in wastewater reclamation regulations.

Chapter Six – General Discussion and Future Directions

6.1 Introduction

In this thesis, the benefits of utilising both conventional (qPCR and Sanger sequencing) and cutting-edge molecular technologies (ddPCR and NGS), in a large scale to address existing key knowledge gaps on the animal sources/carriers of human infectious species of *Cryptosporidium* in Australian drinking water catchments were demonstrated. The main focus of the present thesis was to detect, quantify and determine the *Cryptosporidium* spp. present in faecal samples from dominant animals inhabiting Australian drinking water catchments and WWTP samples over time and space. Analysis included screening 5,774 animal faecal samples collected from 11 drinking water catchment across NSW, QLD and WA, and 730 raw influent samples from 25 Australian WWTPs across these states for the presence of *Cryptosporidium* spp. at the 18S rRNA (18S) locus using qPCR, and further quantification of oocyst load in samples positive for *Cryptosporidium*. In addition, *Cryptosporidium* spp. detected in animal faecal samples were typed by sequence analysis of an 825 bp fragment of the 18S gene and subtyped at the glycoprotein 60 (*gp60*) locus (832 bp), and faecal samples which initially produced mixed chromatograms and WWTP influent samples positive for *Cryptosporidium* spp. by qPCR, were further analysed using NGS of 18S amplicons on the MiSeq (Illumina) platform.

The findings presented in this thesis have provided further evidence of the complex diversity and epidemiology of *Cryptosporidium* species in animals inhabiting Australian drinking water catchments and in WWTP influent, and suggest the potential role these animals play in dissemination of *Cryptosporidium* oocysts to drinking water sources and the associated zoonotic transmission and human health risks. The identification of zoonotic *Cryptosporidium*

species in both livestock and wildlife, particularly the detection of *C. hominis* in cattle and kangaroos and the high prevalence of *C. parvum* in cattle has implications for the management of drinking water sources, and warrants continued monitoring and identification of the sources/carriers of human pathogenic strains, which is essential for accurate risk assessment and optimal catchment management.

6.2 Epidemiology of zoonotic *Cryptosporidium* species in animals

Cryptosporidium spp. are among the most common enteric protozoan parasites of humans, domestic and wild animals. They are a major cause of severe diarrhoea and death in children (second to rotrovirus) and currently there is neither a vaccine nor an effective treatment (Kotloff et al., 2013; Ryan and Xiao., 2014; Ryan et al., 2016). There are multiple species and genotypes of *Cryptosporidium* with varying host-specificity and pathogenicity, only some of which are infectious to humans. To date, more than 20 species of *Cryptosporidium* have been reported in humans, and wildlife and domestic animals are believed to act as reservoirs for the majority of these species. Due to the ubiquitous nature of *Cryptosporidium,* it is very difficult to accurately establish sources and transmission dynamics of *Cryptosporidium* in the environment, however zoonotic waterborne transmission is considered a major transmission route in the epidemiology of human cryptosporidiosis, and therefore water contamination by animal-derived *Cryptosporidium* oocysts is of growing concern (Xiao, 2010). Several studies have linked outbreaks of cryptosporidiosis in human populations with sheep and cattle grazing near the implicated reservoir, catchment or river, but only a single recent waterborne outbreak of *C. cuniculus* in the UK has been linked to wildlife (rabbits) (Ruecker et al., 2007; Yang et al., 2008; Chalmers et al., 2009, 2011). Therefore, further molecular evidence is required to

more conclusively link contamination of water supplies by animals, in particular wildlife in catchments, with outbreaks of cryptosporidiosis in human populations.

To date, the majority of human cases of cryptosporidiosis worldwide are caused by two species; *C. parvum* and *C. hominis* (Putignani and Menichella, 2010; Xiao, 2010; Ryan and Xiao, 2014). Both species have been previousely reported in a wide range of domestic and wild animals (*cf* Zahedi et al., 2016), but a striking finding of the present study was the identification of *C. parvum* and *C. hominis* at a relatively high prevalence among cattle and kangaroo populations inhabiting Australian drinking water catchments (see Chapters 2 and 4). This provides supportive evidence for the central hypotheses of this thesis, which initially proposed that faecal contamination of water sources by animals could be a significant source of *Cryptosporidium* infection in humans. In particular, identification of the most commonly reported human-associated *gp60* subtypes of *C. hominis* and *C. parvum* (IbA10G2, IdA15G1, IIaA13G1, IIaA15G2R, IIaA16G2R1, IIaA17G2R1, IIaA18G3R1, IIaA19G2R1, and IIaA19G3R1) in cattle and kangaroos in the present study, strongly suggests the potential role these animals may play as sources/carriers of human pathogenic strains in the zoonotic transmission and epidemiology of cryptosporidiosis.

In addition to *C. hominis* and *C. parvum*, other species of *Cryptosporidium* including *C. andersoni*, *C. bovis*, *C. canis*, *C. cuniculus*, *C. ditrichi*, *C. erinacei*, *C. fayeri*, *C. felis*, *C. meleagridis*, *C. muris*, *C. scrofarum*, *C. suis*, *C. tyzzeri*, *C. ubiquitum*, *C. viatorum*, and *C. xiaoi* have been reported in both humans and a wide range of animals (*cf* Zahedi et al., 2016). In the present study, with the exception of *C. andersoni*, *C. ditrichi*, *C. tyzzeri*, and *C. viatorum*, all other species of *Cryptosporidium* had been previously detected in animals in catchment areas. This raises further questions about the complexity of transmission dynamics and epidemiology of this parasite, which need to be addressed in future studies.

It is, however, important to stress that swimming pools are likely to be a more important source of *Cryptosporidium* transmission in the human population compared to drinking water (Ryan et al., 2017). This is particularly important as drinking water-associated outbreaks of diarrhoeal illness due to *Cryptosporidium* and other pathogens are declining, particularly in high-income countries, but the number of outbreaks of cryptosporidiosis and other diarrhoeal diseases associated with swimming pools and water parks have increased dramatically (Hlavsa et al., 2015; Ryan et al., 2017).

6.3 *Cryptosporidium* and the Australian water industry

The quality and safety of drinking water in catchments is important to all members of the society, and drinking water must be fit for human consumption. This makes it essential for the water industry to ensure that they effectively manage risks to drinking water safety from catchments to taps, and deliver highly reliable drinking water to their customers. In addition, over the last few years, due to extremes in climatic conditions, management of water resources has become even more important globally. Most drinking water utilities in developed countries utilise holistic 'catchment to consumer' multi-barrier approaches to manage and assess risks that exist within public drinking water source areas used to supply drinking water. This approach is based on the premise that no single treatment mechanism is infallible and each barrier reduces risk to water quality incidents when it is applied in a robust manner. The most important barrier in water quality protection is the effective protection of the source or catchment, which is the first part of the multi barrier approach to providing safe drinking water. Human activities in catchment areas (e.g. farming, recreational activities), presence of livestock and wildlife, rainfall or storm events can all present a significant risk to water quality in catchments through building up and transporting pathogens (e.g. bacteria, viruses and

protozoa) into the main water bodies. Therefore, catchment management is an important concept in water regulations and policies, and is considered as a critical water quality control point to assess microbial contamination (Ferrier and Jenkins, 2010).

Recognition of the limited utility of traditional indicator organisms to assess risks from non-bacterial pathogens and the inability of epidemiological studies to detect small differences in illness rates led regulatory authorities to develop quantitative microbial risk assessment (QMRA) based approaches to assess drinking water safety and set regulatory targets (Sinclair et al., 2015). QMRA is a probabilistic modelling technique, and it is the main method used to estimate the microbiological risk of infection from exposure to a microorganism (Hamilton et al., 2006). In order to conduct basic QMRA, data that can be used to generate probability density functions of the occurrence/numbers of a particular pathogen are required. Thus, QMRA can estimate how safe the water is, how much the safety varies and how certain the estimate of safety is. QMRA gives a detailed breakdown of the contribution of each step in the chain from catchment-to-tap to the overall risk (reduction), along with the potential effects of hazardous events (such as those following heavy rainfall) and some indication of data variability and uncertainty. Water utilities can use this information to decide where optimisation or additional controls would be most effective (Schijven et al., 2011) and to determine whether treatment is meeting health-based targets with the required level of certainty (Smeets et al., 2010).

The Australian Drinking Water Guidelines 2011 (ADWG) (NHMRC–NRMMC 2011) contain mostly qualitative information on treatment requirements. The ADWG promote having appropriate disinfection and filtration, and a ‘catchment to consumer’ multi-barrier approach to manage and assess risks as discussed above. The ADWG also provide some general guidance on targets for these treatments, but do not set out a quantifiable approach to defining treatment adequacy (Deere et al., 2014).

In contrast, the Australian Guidelines for Water Recycling (2006; 2008; 2009; the AGWR series) (NRMMC-EPHC-AHMC-NHMRC 2006-2009) stipulate that recycled water should be treated to reduce human infectious pathogen concentrations by a quantifiable extent, often termed the pathogen "log reduction value" (LRV), to meet a quantifiable "health-based target" (HBT). A tolerable disease burden, expressed in units of disability-adjusted life year (DALY) is used to provide the HBT to be achieved through the LRV delivered by treatment. The DALY was developed by Harvard University for the World Bank to provide a consistent framework to quantify and compare the health burden of a wide range of diseases and injuries on populations (World Bank, 1993). This measure was developed as an alternative approach to simply using the number of deaths (mortality) or illnesses (morbidity) to rank the effects of diseases on populations. The DALY integrates disease impacts including premature death, degree of disability caused by an illness, and the length of time lived with disability into a single measure, which can be used to compare the importance of different diseases, injuries, and risk factors as part of health decision-making and planning processes (Sinclair et al., 2015). The so-called "tolerable" disease burden is defined as the exposed population not experiencing more than one additional DALY per million exposed persons annually - on average. This is often expressed as the 1 μDALY tolerable disease burden target.

While the ADWG currently does not have numerical targets for microbial safety of drinking water, it is anticipated that this discrepancy will soon be addressed and that a combination of QMRA (to determine the likelihood of infection and illness occurring from exposure to specific pathogens contained in water) and the metric of DALYs (to convert the likelihood of illness into impacts or burdens of disease) will be used to define microbial safety (O'Toole et al., 2015). The target being considered is 1 μDALY per person per year, the same target as defined in the World Health Organization guidelines for drinking water quality (WHO GDWQ) (WHO, 2011) and already adopted in Australian guidelines for water recycling

(AGWR) (NRMMC/EPHC/AHMC 2006-2009). This allows for up to approximately 6% of diarrhoeal disease caused by *Cryptosporidium* to be associated with consumption of drinking water (O'Toole et al., 2015) and a $\log_{10}$ reduction of 6.15 of *Cryptosporidium* in drinking water to meet annual 1 μDALY health targets (Sinclair, 2012). As a result of this, accurate modelling and risk assessments will become even more important to the water industry. Thus, data about the prevalence of human-infectious *Cryptosporidium* species in source waters and catchments will become increasingly important for QMRA processes. However, currently, in the case of *Cryptosporidium*, standard monitoring data for QMRA analysis are based on fluorescence microscopy, which does not include species and subtype identification (or information on viability). This results in inaccurate risk assessments that either under or overestimate the public health risk. To perform accurate QMRA analysis, quantitative data on oocyst excretion rates for the different hosts in various catchments, quantitative data on the prevalence of zoonotic and non-zoonotic species in the various hosts over time and space, host prevalence data, infectivity levels (i.e. percentage that are still viable) under different climatic conditions, are essential to accurately model oocyst inputs into source waters.

6.4 Practical benefits of the present research

The data generated by the present thesis will be applied to QMRA models for each catchment to provide site-specific analysis and highlight key risks that need to be addressed. As sampling for this project occured over two years, it enables authorities to investigate the fate of oocysts during different seasons and different weather events from drought to heavy rainfall. This is essential, as the risk of waterborne infections varies widely over time depending not only on the *Cryptosporidium* load coming from different hosts and the prevalence of zoonotic species, but also on the environmental conditions, as *Cryptosporidium* oocysts are

greatly affected by temperature and rainfall (King and Monis, 2007). An increased *Cryptosporidium* load, which enters the water source from surface runoff, may put higher pressure on water treatment and, as a result increased risk of waterborne infections. The risk outcome generated by QMRA analysis of the present data can then be compared to the soon to be implemented Australian health based targets and improved management strategies devised. In addition to providing essential data to improve management strategies, QMRA analysis can also be used retrospectively to gain insights about conditions leading to past outbreak and to model outbreaks while they are occurring to estimate the outbreak magnitude and the potential effectiveness of additional interventions (Seto et al., 2007).

The development of more targeted control measures against *Cryptosporidium*, resulting from QMRA modeling based on the much more accurate input data generated from the present study, will reduce the transmission of *Cryptosporidium* and limit outbreaks of disease. For example, QMRA will allow water authorities to: (1) identify relative risks from various contamination sources; (2) identify accurate detection limits that would be needed to monitor *Cryptosporidium* at levels of public health relevance; (3) characterise differential risks to sensitive subpopulations (e.g. children); (4) the relative risks of temporally varying inputs etc.; (5) provide quantitative insights into managing control points; (6) determine the value of interventions (i.e. is it better to spend money in the catchment to limit oocyst inputs, or better to invest in treatment barriers?); and (7) to develop "what if" scenarios and model alternative management scenarios to provide the ability to generate scientifically defensible, site specific criteria for improved management. In addition, recommendations for more targeted, cost-effective control strategies will be disseminated via liaison with (1) the major utilities, (2) Water research Australia (WaterRA) (3) the Water Services Association of Australia (WSAA) and via (4) co-ordination with community-based water catchment management groups.

6.5 Limitations of the present study and future needs for the water industry

Despite the recent developments in the molecular epidemiology of *Cryptosporidium* spp., including the newly gained knowledge reported in the present thesis on quantitative prevalence and genotypes of zoonotic *Cryptosporidium* spp. in Australian watersheds, there are still research gaps in the understanding of the public health significance of *Cryptosporidium* in animals in drinking water catchments.

The viability/infectivity of *Cryptosporidium* oocysts shed by animals is vital for accurate QMRA analysis in order to better understand transmission dynamics of *Cryptosporidium* spp. and the associated human health risks. However, current monitoring methods (methods 1622 and 1623) do not determine viability of oocysts, yet anecdotal evidence suggest that some species may be more robust than others and the viability of oocysts under the very different climatic conditions may vary. Solar inactivation, desiccation, temperature and residence time in the catchment and water, and transport, including hydro-dynamically-driven accumulation, settlement, dispersion, dilution can all affect final concentration and the viability of *Cryptosporidium* oocyst in the drinking water (King et al., 2008), and therefore should be factored into risk assessments in future studies. In addition, obtaining sufficient quantities of *C. hominis* oocysts for viability/infectivity analysis requires either growing the parasite in gnotobiotic pigs or purifying the parasite from large quantities of faeces from an infected person or animal, both of which are impractical and costly. This has been a major barrier both to the development of detection and infectivity assays for *C. hominis*.

Cryptosporidium oocyst viability has conventionally been determined by using *in vitro* excystation, while mouse infectivity assays have been utilized to determine whether oocysts are infectious (Ryan and Hijjawi, 2015). The majority of *in vitro* cultivation studies to date have used human adenocarcinoma (HCT-8) cells, however these studies have been mainly

hampered by lack of reproducibility and difficulties in continuous long-term proliferation of *Cryptosporidium* spp. in *in vitro* culture, which requires urgent attention. Recently, a hollow-fibre culture system and a 3D human intestinal tissue model have been established for long-term culture of *C. parvum,* but neither model currently support growth of other species, in particular *C. hominis* (the main species infecting humans) (Moranda et al., 2016; DeCicco RePass et al., 2017). In addition, limitations of the hollow-fibre culture system and 3D model culture systems include that they are: (1) not suitable for viability assays; (2) expensive (up to US$5,000 per experiment); (3) complicated to use (i.e. the hollow-fibre system requires daily monitoring and replacement of expensive growth medium every 2 days); (4) growth inside the hollow-fibre cartridge cannot be observed directly using a microscope; (5) they do not generate precise constant culture conditions; and (6) the conditions for *C. hominis* growth in these models has not been yet determined.

Lack of suitable subtyping tools is another major hurdle to study *Cryptosporidium* transmission patterns and population genetic structure (Feng et al., 2014). Application of multilocus sequence typing (MLST) based on both length polymorphism and SNP potentially can increase the resolution of typing. However, in the case of *Cryptosporidium*, current MLST tools have been developed based on microsatellites from the genome of *C. parvum* and *C. hominis*, and can only be applied to genetically related species. Therefore, there is an urgent need for more extensive whole genome sequence analysis of a more diverse range of *Cryptosporidium* species, in particular zoonotic strains, to develop MLST tools for high-resolution subtyping of all other species. In addition, whole genome sequencing of *Cryptosporidium* spp. will greatly support research into the within-host genetic diversity of *Cryptosporidium* subtypes, as well as evolution of the genus, virulence and mechanisms controlling host specificity of *Cryptosporidium*. This in turn will promote the development of vaccines and new anti-*Cryptosporidium* therapeutics (Striepen, 2013).

Continued improvements in monitoring and removal methods for *Cryptosporidium* in water (e.g. recreational water, drinking source water, and treated and/or untreated wastewater), more rigorous enumeration practices, and enhanced analytical and risk assessment techniques are essential for the water industry to ensure safe water distribution. In particular, there is a need for further monitoring to fill spatial and temporal data gaps regarding the occurrence of *Cryptosporidium* in source water. This will greatly help with generating more accurate data for better microbial risk assessments.

Lastly, given the public health significance of water-associated outbreaks of cryptosporidiosis, substantial enhancement in surveillance of community-wide cryptosporidial infection is required. Currently, in Australia, due to considerable variation in testing practices and reporting procedures between different States and Territories, it is difficult to establish a comprehensive picture of the true burden of community-wide cryptosporidiosis. This highlights a need to develop a national practice protocol for surveillance of *Cryptosporidium* species in the community. The surveillance should include a systematic approach to elevated rates of diarrhoeal illness across the community in particular among public institutions like childcare facilities and nursing homes, a follow-up of individual cases of cryptosporidiosis, active contact with laboratories, and possible activation of other sentinel systems, including school absenteeism, general practice systems and oncology units. Geographic Information Systems (GIS) mapping of such data may be also useful as an adjunct to identify and track outbreaks. In addition, a well-validated system is required to accurately extrapolate the number of laboratory-defined cases to the number of cases in the community.

6.6 Concluding remarks

This thesis used innovative approaches to understand the epidemiology and public health risks of *Cryptosporidium* species in animals in Australian drinking water catchments across three states including NSW, QLD and WA. For the first time in Australia, cutting-edge molecular tools were used on a large scale to address key knowledge gaps on the lack of quantitative prevalence and genotyping data on zoonotic *Cryptosporidium* species infecting animal hosts in Australian drinking water catchments over time and space. The data generated in this thesis will be used to conduct modeling and quantitative microbial risk assessments (QMRA) for the various catchments.

As a result of this thesis, five papers have been published, with another manuscript under review. Lastly, work carried out during my PhD tenure has also contributed to four additional publications in the field of *Cryptosporidium* research (*see* Appendix 7-10).

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Appendices

Appendix 1 - Zahedi, A., Paparini, A., Jian, F., Robertson, I., Ryan, U., 2016. Public health significance of zoonotic *Cryptosporidium* species in wildlife: critical insights into better drinking water management. Int. J. Parasitol. Parasites Wildl. 5, 88-109. (Invited review).

Appendix 2 - Ryan, U., Zahedi, A., Paparini, A., 2016. *Cryptosporidium* in humans and animals-a one health approach to prophylaxis. Parasite Immunol. 38, 535-547. (Invited review).

Appendix 3 - Zahedi, A., Monis, P., Aucote, S., King, B., Paparini, A., Jian, F., Yang, R., Oskam, C., Ball, A., Robertson, I., Ryan, U., 2016. Zoonotic *Cryptosporidium* Species in Animals Inhabiting Sydney Water Catchments. PLoS ONE. 11(12), e0168169.

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Appendix 1

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Invited review

Public health significance of zoonotic *Cryptosporidium* species in wildlife: Critical insights into better drinking water management

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ABSTRACT

Cryptosporidium is an enteric parasite that is transmitted via the faecal–oral route, water and food. Humans, wildlife and domestic livestock all potentially contribute *Cryptosporidium* to surface waters. Human encroachment into natural ecosystems has led to an increase in interactions between humans, domestic animals and wildlife populations. Increasing numbers of zoonotic diseases and spill over/back of zoonotic pathogens is a consequence of this anthropogenic disturbance. Drinking water catchments and water reservoir areas have been at the front line of this conflict as they can be easily contaminated by zoonotic waterborne pathogens. Therefore, the epidemiology of zoonotic species of *Cryptosporidium* in free-ranging and captive wildlife is of increasing importance. This review focuses on zoonotic *Cryptosporidium* species reported in global wildlife populations to date, and highlights their significance for public health and the water industry.

Contents

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Appendix 1

1. Introduction

More than 15% of the world's population has no access to safe drinking water (Cauchie et al., 2014). Waterborne parasitic protozoan diseases with worldwide distribution, result in four billion cases of diarrhoea, 1.6 million deaths annually (www.who.int) and 62.5 million Disability Adjusted Life Years (DALYs) worldwide (Wright and Gundry, 2009; WHO, 2009). Yet, despite the latest advances made in water treatment measures, protecting drinking water supplies against waterborne pathogens remains by far, as one of the most challenging concerns for the entire drinking water supply chain worldwide (Cotruva et al., 2004; Betancourt and Rose, 2004; Thompson and Smith, 2011; Plutzer, 2013; Burnet et al., 2014). In response to this, in 2009, the World Health Organization has developed guidelines for water suppliers on how to implement "Water Safety Plans" (WSPs), in the hope of halving the number of people without safe access to drinking water by the end of 2015 (WHO, 2009).

In less developed countries, lack of basic infrastructure for providing safe drinking water is considered a major cause of poor water quality which contributes to the spread of endemic/epidemic waterborne diseases. However, even in industrialized nations, highly advanced infrastructures are not yet a protective factor against outbreaks (Cummins et al., 2010; Smith and Nichols, 2010; Castro-Hermida et al., 2010; Burnet et al., 2014; Smolders et al., 2015). This appears to be largely due to a lack of knowledge about the epidemiology and transmission dynamics of waterborne pathogens (e.g. from animals ranging within the catchments) which leads to poor management practices for drinking water catchments (Gormley et al., 2011; Castro-Hermida et al., 2010).

Waterborne parasitic protozoans are responsible for the majority of waterborne outbreaks worldwide, with socio-economic impacts even in developed countries (Cotruva et al., 2004; Pond, 2005; Baldursson and Karanis, 2011; Cauchie et al., 2014). Of these, *Cryptosporidium* was the etiological agent in 60.3% (120) of the waterborne protozoan parasitic outbreaks that have been reported worldwide between 2004 and 2010 (Baldursson and Karanis, 2011). For the global water industry, therefore, *Cryptosporidium* represents the major public health concern, as its oocyst (the environmentally stable stage) is able to survive and penetrate routine wastewater treatment and is resistant to inactivation by commonly used drinking water disinfectants (Fayer et al., 2001; Baldursson and Karanis, 2011; Burnet et al., 2014). As a result of these waterborne outbreaks of cryptosporidiosis, *Cryptosporidium* testing in source or finished water is now mandatory in most industrialised nations. For example, the U.S. EPA, working with the U.S. public water supply industry, developed and implemented the Long-term Stage 2 Enhanced Surface Water Treatment Rule (LT2ESWTR), known as LT2 to control *Cryptosporidium* in public water supplies (US EPA, 2006). LT2 requires all public water suppliers using surface water sources and serving populations >10,000 to monitor their sources for *Cryptosporidium* by analysing at least 24 consecutive monthly samples. In the UK, the Drinking Water Inspectorate (DWI) requires that water companies carry out risk assessments on all their water supply sites to ascertain the level of risk *Cryptosporidium* poses to the final treated water quality. Those at high risk need additional treatment (in the form of properly controlled coagulation/flocculation filtration systems or membrane or UV treatment systems). The UK regulations also require companies to design and continuously operate adequate treatment and disinfection. A proven failure to comply with this is now an offence (DWI, 2010).

Cryptosporidium species are able to infect a broad range of hosts including humans, domestic and wild animals (mammals, birds, fish, marsupials, reptiles and amphibians) worldwide (Table 1), causing asymptomatic or mild to severe gastrointestinal disease in their host species (Monis and Thompson, 2003; Hunter et al., 2007; Ryan and Power, 2012; Xiao, 2010; Kváč et al., 2014a; Ryan et al., 2014). Current treatment options for cryptosporidiosis are limited and only one drug, nitazoxanide (NTZ), has been approved by the United States (US) Food and Drug Administration (FDA). This drug, however, exhibits only moderate clinical efficacy in children and immunocompetent people, and none in people with HIV (Abubakar et al., 2007; Amadi et al., 2009; Pankiewicz et al., 2015; Gargala, 2008; Rossignol, 2010).

Oocyst transport to surface water can occur by deposition of manure directly in the water or surface runoff. Hence, humans, wildlife and domestic livestock all potentially contribute *Cryptosporidium* contamination to water systems (Ryan et al., 2014). Identification of the sources/carriers of human pathogenic strains is therefore essential for accurate risk assessment and optimal catchment management. However, most studies to date have focused on humans and the potential role of livestock in the epidemiology of zoonotic cryptosporidiosis, while wildlife as a potential risk factor to source water, has only been studied opportunistically. Thus, the aim of this review is to summarize available information about molecular characterisation of *Cryptosporidium* species in wildlife with emphasis on the public health significance of zoonotic species.

2. Taxonomy and zoonotic potential of *Cryptosporidium* species

The application of advanced molecular techniques has led to an improved taxonomy and systematics, and better understanding of *Cryptosporidium* phylogeny (Ryan et al., 2014). Given the morphological similarity of oocysts by microscopy, these advances are crucial for confident identification, description of host/parasite intractions and ultimately accurate risk assessment. Currently, 29 *Cryptosporidium* species have been recognised as valid (Table 1), including the recently described *C. rubeyi* in ground-dwelling squirrels (*Spermophilus* sp.) (Li et al., 2015a). More than 17 species have been identified in humans (Table 1). Of these, *C. parvum* and *C. hominis* are by far the most common species reported in humans worldwide (Xiao, 2010; Ryan and Xiao, 2014) and have been responsible for the majority of waterborne outbreaks typed to date with the exception of a waterborne outbreak in the UK caused by *C. cuniculus* from rabbits (*Oryctolagus cuniculus*) (Chalmers et al., 2009; Xiao, 2010; Ryan et al., 2014).

The most widely molecular markers used for typing of *Cryptosporidium* isolates are the 18S ribosomal RNA (18S rRNA) gene and the 60-kDa glycoprotein (gp60) gene. The latter locus encodes a

Appendix 1

Table 1
Valid *Cryptosporidium* species confirmed by molecular analysis.

Species name	Author(s)	Type host(s)	Major host(s)	Reports in humans
C. rubeyi	Li et al., 2015a	*Spermophilus beecheyi* (California ground squirrel)	Squirrels	None reported
C. scophthalmi	Alvarez-Pellitero et al., 2004; Costa et al., 2015	*Scophthalmus maximus* (Turbot)	Turbot	None reported
C. huwi	Ryan et al., 2015	*Poecilia reticulata* (Guppy), *Paracheirodon innesi* (Neon tetra) and *Puntius tetrazona* (Tiger barb)	Fish	None reported
C. erinacei	Kváč et al., 2014b	*Erinaceus europaeus* (European hedgehog)	Hedgehogs, horses	Kváč et al., 2014a
C. scrofarum	Kváč et al., 2013	*Sus scrofa* (Pig)	Pigs	Kváč et al., 2009a, 2009b
C. viatorum	Elwin et al., 2012	*Homo sapiens* (Human)	Humans	Elwin et al., 2012; Insulander et al., 2013
C. tyzzeri	Tyzzer, 1912; Ren et al., 2012	*Mus musculus* (Mouse)	Rodents	Rasková et al., 2013
C. cuniculus	Robinson et al., 2010	*Oryctolagus cuniculus* (European rabbit)	Rabbits	Chalmers et al., 2009; Anon, 2010; Molloy et al., 2010; Chalmers et al., 2011; Anson et al., 2014; Koehler et al., 2014; Chalmers, 2012
C. ubiquitum	Fayer et al., 2010	*Bos taurus* (Cattle)	Ruminants, rodents, primates	Commonly reported (cf. Fayer et al., 2010; Elwin et al., 2012)
C. xiaoi	Fayer et al., 2010	*Ovis aries* (Sheep)	Sheep and goats	Adamu et al., 2014
C. ryanae	Fayer et al., 2008	*Bos taurus* (Cattle)	Cattle	None reported
C. macropodum	Power and Ryan, 2008	*Macropus giganteus* (Kangaroo)	Marsupials	None reported
C. fragile	Jirků et al., 2008	*Duttaphrynus melanostictus* (Toad)	Toads	None reported
C. fayeri	Ryan et al., 2008	*Macropus rufus* (Kangaroo)	Marsupials	Waldron et al., 2010
C. bovis	Fayer et al., 2005	*Bos taurus* (Cattle)	Cattle	Khan et al., 2010; Ng et al., 2012; Helmy et al., 2013
C. suis	Ryan et al., 2004	*Sus scrofa* (Pig)	Pigs	Xiao et al., 2002a; Leoni et al., 2006; Cama et al., 2007; Wang et al., 2013a
C. galli	Pavalasek, 1999; Ryan et al., 2003	*Spermestidae, Frangillidae, Gallus gallus, Tetrao urogallus, Pinicola enucleator* (Birds)	Birds	None reported
C. hominis	Morgan Ryan et al., 2002	*Homo sapiens* (Human)	Humans	Most common species in humans
C. molnari	Alvarez-Pellitero and Sitjà-Bobadilla, 2002	*Sparus aurata* (Gilt-head sea bream) and *Dicentrarchus labrax* (European seabass)	Fish	None reported
C. canis	Fayer et al., 2001	*Canis familiaris* (Dog)	Dogs	Many reports (cf. Lucio-Forster et al., 2010)
C. andersoni	Lindsay et al., 2000	*Bos taurus* (Cattle)	Cattle	Leoni et al., 2006; Morse et al., 2007; Waldron et al., 2011; Agholi et al., 2013; Jiang et al., 2014; Liu et al., 2014
C. varanii	Pavlásek et al., 1995	*Varanus prasinus* (Emerald Monitor)	Lizards	None reported
C. baileyi	Current et al., 1986	*Gallus gallus* (Chicken)	Birds	None reported
C. parvum	Tyzzer, 1912	*Bos taurus* (Cattle)	Ruminants	Commonly reported in humans
C. meleagridis	Slavin, 1955	*Meleagris gallopavo* (Turkey)	Birds and humans	Commonly reported in humans
C. serpentis	Levine, 1980	*Elaphe guttata, E. subocularis, Sanzinia madagascarensus* (Snakes)	Snakes and lizards	None reported
C. felis	Iseki, 1979	*Felis catis* (Cat)	Cats	Many reports (cf. Lucio-Forster et al., 2010)
C. wrairi	Vetterling et al., 1971	*Cavia porcellus* (Guinea pig)	Guinea pigs	None reported
C. muris	Tyzzer, 1907; and 1910	*Mus musculus* (House mouse)	Rodents	Many reports – Guyot et al., 2001; Gatei et al., 2002; Tiangtip and Jongwutiwes, 2002; Gatei et al., 2003; Palmer et al., 2003; Gatei et al., 2006; Leoni et al., 2006; Muthusamy et al., 2006; Azami et al., 2007; Al-Brikan et al., 2008; Neira et al., 2012; Hasajová et al., 2014; Petrincová et al., 2015; Spanakos et al., 2015

precursor protein, that is cleaved to produce mature cell surface glycoproteins (gp45/gp40 and gp15) implicated in zoite attachment to, and invasion of enterocytes (Xiao, 2010; Ryan et al., 2014). Most of the genetic heterogeneity in the gp60 gene is the variation in the number of a tri-nucleotide repeat (TCA, TCG or TCT) in the 5′ end (gp40) of the coding region, although extensive sequence polymorphism is also present in the rest of the gene. The repeats are used to define the subtype families within a species, whereas the remaining polymorphic sites are used to identify subtypes within a subtype family (Ryan et al., 2014).

3. Wildlife associated outbreaks and water contamination

Relatively little is known about the distribution of zoonotic and non-zoonotic *Cryptosporidium* species and subtypes in wildlife populations (Appelbee et al., 2005; Ziegler et al., 2007; Ryan et al., 2014). Conclusive molecular evidence, linking contamination of water supplies by wild animals in catchments with outbreaks of cryptosporidiosis in human populations is scant. However, a recent waterborne outbreak in the UK caused by *C. cuniculus* from rabbits has highlighted the importance of wildlife in the dissemination of *Cryptosporidium* to drinking water sources and the associated human health risk (Chalmers et al., 2009; Elvin et al., 2012).

A wide range of *Cryptosporidium* species and genotypes have been identified in drinking source water, storm water runoff, stream sediment, wastewater and seawater in various geographic locations including *C. hominis*, *C. parvum*, *C. andersoni*, *C. muris*, *C. cuniculus*, *C. meleagridis* and *C. canis* as well as various wildlife

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Table 2
Cryptosporidium species and genotypes identified by molecular tools in wild terrestrial mammals and their zoonotic importance.

Cryptosporidium species/ genotypes	Wildlife hosts	Zoonotic importance	gp60 subtypes reported in wildlife	References
C. hominis	Fallow deer (*Dama dama*), Dugong (*Dugong dugon*), Chinchillas (*Chinchilla lanigera*), Baboons (*Pabio anubis*), Chimpanzees (*Pan troglodytes schweinfurthii*), Red colobus (*Procolobus rufomitratus*), Black-and-white colobus (*Colobus guereza*), Rhesus macaque (*Macaca mulatta*), Cynomolgus monkey (*Macaca fascicularis*), Francois' leaf monkey (*Trachypithecus francoisi*), Lemurs (*Lemur sp.*), Bandicoots (*Isoodon obesulus*), Bushtail possums (*Trichosurus vulpecula*), Estern grey kangaroos (*Macropus giganteus*), Brush-tailed rock-wallabies (*Petrogale penicillata*), Wild dingo (*Canis lupus dingo*), Squirrel monkey (*Saimiri sciureus*)	Main *Cryptosporidium* species infecting humans	IbA12G3, IbA10G2R2, IiA17, IfA12G2, IaA13R7, IaA13R8, IaA14R7, IdA20, IeA11G3T3, IfA16G2, IkA7G4 (novel subtype)	Morgan et al., 2002; Salyer et al., 2012; Ye et al., 2012; Ng et al., 2011; Dowle et al., 2013; Nolan et al., 2013; Karim et al., 2014; Ryan et al., 2014; Liu et al., 2015b; Parsons et al., 2015; Zahedi et al., 2015
C. parvum	Alpaca (*Lama pacos*), Swamp deer (*Cervus duvauceli*), Red deer (*Cervus elaphus*), Roe deer (*Capreolus capreolus*), Fallow deer (*Dama dama*), Addaxes (*Addax nasomaculatus*), Arabian oryx (*Oryx leucoryx*), Gemsboks (*Oryx gazella*), Sable antelopes (*sable antelopes*), White-tailed deer (*Odocoileus Virginianus*), Game grey wolves (*Canis lupus*), Racoon dog (*Nyctereutes procyonoides viverrinus*), Rabbit (*Oryctolagus cuniculus*), Nutria (*Myocastor coypus*), Prezewalski's wild horse (*Equus przewalskii*), Alpaca (*Lama quanico pacos*), Eastern grey squirrel (*Sciurus carolinensis*), Ground Squirrels (*Spermophilus beecheyi*), Siberian chipmunk (*Tamias sibiricus*), Hamsters (*Cricetinae*), Wood mice (*Apodemus sylvaticus*), White-footed mouse (*Peromyscus leucopus*), Yellow-bellied marmot (*Marmota flaviventris*), Bamboo rats (*Rhizomys sinensis*), Small brown bat (*Myotis lucifugus*), Campbell hamster (*Phodopus campbelli*), Golden hamster (*Mesocricetus auratus*), Capybara (*Hydrochoerus hydrochaeris*), Racoon dog (*Nictereutes procyonoides viverrinus*), Red fox (*Vulpes vulpes*), Rhesus macaques (*Macaca mulatta*), Toque macaques (*Macaca sinica sinica*), Grey langurs (*Semnopithecus priam thersites*), Purple-faced langurs (*Trachypithecus vetulus philbricki*), Common dolphins (*Delphinus delphis*), Golden takins (*Budorcas taxicolor bedfordi*), Eastern grey kangaroos (Macropus giganteus), Asian house rat (*Rattus tanezumi*), Brown rat (*Rattus norvegicus*), Bamboo rats (*Rhizomys sinensis*)	Major	IIdA15G1, IIdA18G1, IIdA19G1, IIaA15G2R1, IIaA19G2R1, IIaA19G3R1, IIaA19G4R1, IIaA20G3R1, IIaA20G3R2, IIaA20G4R1, IIaA21G3R1, IIaA16G2R1, IIaA14G1R1, IIaA14G2R1, IIaA16G3R1, IIcA5G3, IIcA5G3a, IIoA13G1, IIpA9 (novel subtype)	Morgan et al., 1999a; Atwill et al., 2001; Perez and Le Blancq, 2001; Matsui et al., 2000; Matsubayashi et al., 2004; Ryan et al., 2003, 2004; Ekanayake et al., 2007; Feng et al., 2007; Meireles et al., 2007; Paziewska et al., 2007; Starkey et al., 2007; Ziegler et al., 2007; Cinque et al., 2008; Lv et al., 2009; Feng, 2010; Gómez-Couso et al., 2012; Ravaszova et al., 2012; Ye et al., 2012; Dowle et al., 2013; Nolan et al., 2013; Liu et al., 2014; Lv et al., 2009; Reboredo-Fernández et al., 2014; Bodager et al., 2015; Liu et al., 2015a; Montecino-Latorre et al., 2015; Qi et al., 2015; Wang et al., 2015; Wells et al., 2015; Zahedi et al., 2015; Zhao et al., 2015a, 2015b
C. cuniculus	European rabbit (*Oryctolagus cuniculus*), Eastern grey kangaroo (*Macropus giganteus*) (single report)	Responsible for several waterborne outbreaks and sporadic cases of cryptosporidiosis in the UK and has been identified in a human in Australia	VaA18,VbA18, VbA19, VbA21, VaA22, VbA24, VbA26, VbA29, VbA32, VbA22R4, VbA23R3, VbA24R3, VbA25R4,VbA26R4	Xiao et al., 2002a; Ryan et al., 2003;Chalmers, 2012; Nolan et al., 2010; Robinson et al., 2010; Elwin et al., 2012; Zhang et al., 2012; Nolan et al., 2013; Kapuke et al., 2014; Koehler et al., 2014; Liu et al., 2014; Puleston et al., 2014
C. ubiquitum	Swamp deer (*Cervus duvauceli*), Deer mouse (*Peromyscus*), Eastern grey squirrels (*Sciurus carolinensis*), Red	Emerging human pathogen	XIIa, XIIb, XIIc, XIId, XIIe, XIIf	Perez and Le Blancq, 2001; da Silva et al., 2003; Ryan et al., 2003; Feng et al., 2007; Karanis et al., 2007; Ziegler

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Table 2 (*continued*)

Cryptosporidium species/ genotypes	Wildlife hosts	Zoonotic importance	gp60 subtypes reported in wildlife	References
	squirrel (*Sciurus vulgaris*), Eastern chipmunk (Tamias striatus), Lemur (Lemuroidea), North American beaver (*Castor canadensis*), Woodchuck (*Marmota monax*), Raccoon (*Procyon lotor*), White-tailed deer (*Odocoileus virginianus*), Sika deer (*Cervus Nippon*), Roe deer (*Capreolus capreolus*), Blesbok (*Damaliscus pygargus phillipsi*), Ibex (*Capara sibirica*), Nyala (*Niyala anagasii*), Coquerel's sifaca (*Propithecus coquereli*), Large Japanese field mouse (*Apodemus speciosus*), Foxes			et al., 2007; Wang et al., 2008; Fayer et al., 2010; Cinque et al., 2008; Feng, 2010; Robinson et al., 2010, 2011; Feng et al., 2012; Abu Samraa et al., 2013; Nolan et al., 2013; Murakoshi et al., 2013; Li et al., 2014; Ma et al., 2014; Perec-Matysiak et al., 2015; Qi et al., 2015a, 2015b; Stenger et al., 2015b
C. muris	Wild rats (*Rattus* sp.), Mice (*Mus* sp.), Greater bilblies (*Macroties lagotis*), Girrafes house mice (*Mus musculus*), Eastern grey squirrel (*Sciurus carolinensis*), Golden hamster (*Mesocricetus auratus*), Rock hyrax (*Procavia capensis*), Large footed mouse-eared bat (*Myotis adversus*), Japanese field mouse (*Apodemus argenteus*), Bilbies (*Macrotis lagotis*), Bank voles (*Clethrionomys glareolus*), Campbell hamster (*Phodopus campbelli*), Siberian hamster (*Phodopus sungorus*), Golden hamster (*Mesocricetus auratus*), Mountain goats (*Oreamnos americanus*), Cynomolgus monkeys (*Macaca fascicularis*), East African mole rat (*Tachyoryctes splendens*), Ringed seal (*Pusa hispida*), Donkey (*Giraffa camelopardalis*), Ringed seal (*Phoca hispida*), Large Japanese field mouse (*Apodemus speciosus*), Cynomolgus monkey (*Macaca fascicularis*), Slow loris (*Nycticebus coucang*), Ostriches (*Struthio camelus*), Mountain gorillas (*Gorilla beringei beringei*), Asian house rat (*Rattus tanezumi*), Brown rat (*Rattus norvegicus*), House mouse (*Mus musculus*)	Numerous reports in humans	Chalmers et al., 1997; Hurkova et al., 2003	Dubey et al., 2002; Morgan et al., 1999a; Xiao et al., 2002a, 2004b; Warren et al., 2003; Nakai et al., 2004; Hikosaka and Nakai, 2005; Santín et al., 2005; Azami et al., 2007; Brikan et al., 2008; Kvac et al., 2008; Lupo et al., 2008; Lv et al., 2009; Kodadkova et al., 2010; Feng, 2010; Murakoshi et al., 2013; Yang et al., 2011, 2013; Ng-Hublin et al., 2013; Karim et al., 2014, Qi et al., 2014; Sak et al., 2014; Du et al., 2015; Laatamna et al., 2015; Petrincová et al., 2015, Zhao et al., 2015b
C. andersoni	Bacterian camel (*Camelus bactrianus*), European wisent (*Bison bonasus*), Marmots Campbell hamster (*Phodopus campbelli*), Golden hamster (*Mesocricetus auratus*), Golden takins (*Budorcas taxicolor bedfordi*), Giant panda (*Ailuropoda melanoleuca*), *Macaca mulatta* (*Rhesus macaque*), American mink (*Mustela vison*)	Minor	–	Matsubayashi et al., 2005; Wang et al., 2008; Lv et al., 2009; Stuart et al., 2013; Du et al., 2015; Wang et al., 2015; Zhao et al., 2015a
C. felis	Rhesus macaques (*Macaca mulatta*); Pallas's cat (*Felis Manul*)	Numerous reports in humans	–	Lucio-Forster et al., 2010; Ye et al., 2012; Beser et al., 2015; Ebner et al., 2015; Li et al., 2015c
C. canis dog genotype	Unidentified fox, Coyote (*Canis latrans*)	Numerous reports in humans	–	Xiao et al., 2002a; Ryan et al., 2004; Zhou et al., 2004; Trout et al., 2006; Ziegler et al., 2007; Elwin et al., 2012; Koompapong et al., 2014
C. canis fox genotype	Fox	No reports in humans to date	–	Zhou et al., 2004; Swaffer et al., 2014
C. canis coyote genotype	Coyotes	No reports in humans to date	–	Xiao et al., 2002a; Zhou et al., 2004
C. erinacei	European hedgehog (*Erinaceus europaeus*), Horses	One report in humans	XIIIaA21R11, XIIIaA22R9, XIIIaA21R10, XIIIa20R10, XIIIaA19R12, XIIIaA22R11	Dyachenko et al., 2010; Laatamna et al., 2013; Nolan et al., 2013; Kváč et al., 2014a, 2014b; Meredith and Milne, 2009
C. fayeri	Southern brown bandicoot (*Isodon obesulus*), Western-barred bandicoot (*Permeles bougainville*), Koala (*Phascolarctos cincerus*), Red kangaroo (*Macropus rufus*), Eastern grey	Minor	IVaA9G4T1R1, IVaA10, IVaA7, IVbA9G1T1, IVcA8G1T1, IVdA7G1T1, IVfA12G1T1	Power et al., 2005; Ryan et al., 2008; Yang et al., 2008, 2011; Power, 2010; Waldron et al., 2010; Feng et al., 2011b; Nolan et al., 2013; Swaffer et al., 2014; Vermeulen et al., 2015

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Table 2 (*continued*)

Cryptosporidium species/ genotypes	Wildlife hosts	Zoonotic importance	gp60 subtypes reported in wildlife	References
	kangaroo (*Macropus giganteus*), Yellow footed rock wallaby (*Petrogale xanthopus*), Western grey kangaroo (*Macropus fuliginosus*), Koalas (*Phascolarctos cinereus*)			
Opossum genotype I (*C. fayeri*)	Opossum (*Didelphimorphia*)	No reports in humans to date	XIaA4G1T1	Feng et al., 2011b
Opossum genotype II	Virginia opossum (*Didelphis virginiae*)	No reports in humans to date	–	Xiao et al., 2002b; Oates et al., 2012
C. meleagridis	Mountain gorillas (*Gorilla beringei beringei*), Brush-tailed rock wallabies (*Petrogale penicillata*), deermouse (*Peromyscus* sp.)	Major	IIIbA, IIIgA (closest match to IIIeA19G2R1)	Morgan et al., 2000; Cama et al., 2003; Gatei et al., 2006; Leoni et al., 2006; Muthusamy et al., 2006; Feng et al., 2007; Elwin et al., 2012; Silverlås et al., 2012; Kurniawan et al., 2013; Adamu et al., 2014; Ryan and Xiao, 2014; Ghaffari and Kalantari, 2014; Sak et al., 2014; Rahmouni et al., 2014; Wang et al., 2014; Stensvold et al., 2015; Vermeulen et al., 2015
C. tyzerri	Mice (*Mus musculus*), Brown rats (*Rattus norvegicus*), Large-footed bat (*Myotus adversus*), Yellow-necked mouse (*Apodemus flavicollis*), Bank vole (*Myodes glareolus*), Common vole (*Microtus arvalis*), Red panda (*Ailurus fulgens*), Leopard (*Panthera pardus*), Takin (*Budorcas taxicolor*), Prairie bison (*Bison bison*), Lesser panda (*Ailurus fulgens*), Black leopards (*Pantera pardus*), Bobcats (*Lynx rufus*)	Occasionally reported in humans	IXaA5R2, IXaA6R1, IXaA6R2, IXaA6R3, IXbA6, IXbA6R2	Morgan et al., 1999a, Xiao et al., 2002a; Bajer et al., 2003; Alves et al., 2005; Foo et al., 2007; Karanis et al., 2007; Ziegler et al., 2007; Lv et al., 2009; Feng et al., 2011b; Carver et al., 2012; Kváč et al., 2012; Ren et al., 2012; Rasková et al., 2013; Silva et al., 2013; Swaffer et al., 2014
C. macropodum	Red kangaroo (*Macropus rufus*), Eastern grey kangaroo (*Macropus giganteus*), Swamp wallaby (*Wallabia bicolor*), Western grey kangaroos (*Macropus fuliginosus*)	No reports in humans to date	–	Power et al., 2004, 2005; Power and Ryan, 2008; Power, 2010; Yang et al., 2011; Nolan et al., 2013
C. bovis	Yaks, foxes, Gorillas (single report), Roe deer (*Capreolus capreolus*)	Occasionally reported in humans	–	Robinson et al., 2011; Helmy et al., 2013; García-Presedo et al., 2013b; Sak et al., 2013; Qi et al., 2015b; Qin et al., 2014
C. ryanae	Roe deer (*Capreolus capreolus*), Water buffaloes (*Bubalus bubalis*)	No reports in humans to date	–	Feng et al., 2012; García-Presedo et al., 2013b
C. wrairi	Guinea pig (*Cavia porcellus*), California ground squirrels (*Spermophilus beecheyi*)	No reports in humans to date	VIIaA13T1, VIIaA17T1, VIIaA16T1	Atwill et al., 2004; Feng et al., 2007, 2011b, Lv et al., 2009
C. scrofarum	Asian house rat (*Rattus tanezumi*), Brown rat (*Rattus norvegicus*), Eurasian wild boars (*Sus scrofa*)	Occasionally reported in humans	–	García-Presedo et al., 2013a; Ng-Hublin et al., 2013, Nemeic et al., 2013; Bodager et al., 2015; Parsons et al., 2015
C. suis	Chimpanzees (*Pan troglodytes schweinfurthii*), Eurasian wild boars (*Sus scrofa*), Rodents	Occasionally reported in humans	–	Nemeic et al., 2012, 2013; Bodager et al., 2015; Parsons et al., 2015
C. suis-like	Asian house rat (*Rattus tanezumi*)	No reports in humans to date	–	Ng-Hublin et al., 2013
C. rubeyi	California ground squirrel (*S. beecheyi*), Belding's ground squirrel (*S. beldingi*), Golden Mantled ground squirrel (*S. lateralis*)	No reports in humans to date	–	Pereira et al., 2010; Li et al., 2015a
Bear genotype	Black bear (*Ursus americanus*)	No reports in humans to date	–	Xiao et al., 2000
Bat genotype I	Chinese rufous horseshoe bat (*Rhinolophus sinicus*), Stoliczka's trident bat (*Aselliscus stoliczkanus*)	No reports in humans to date	–	Wang et al., 2013b
Bat genotype II	Chinese rufous horseshoe bat (*Rhinolophus sinicus*), Fulvus roundleaf bat (*Hipposideros fulvus*), Leschenault's rousette (*Rousettus leschenaultii*)	No reports in humans to date	–	Wang et al., 2013b
Bat genotype III	Big brow bat (*Eptesicus fuscus*)	No reports in humans to date	–	Kváč et al., 2015
Bat genotype IV	Western barbastelle (*Barbastella barbastellus*)	No reports in humans to date		Kváč et al., 2015
Beaver genotype	North American beaver (*Castor canadensis*)	No reports in humans to date	–	Feng et al., 2007
Brushtail possum I	Brushtail possum (*Trichasuris vulpecula*)	No reports in humans to date	–	Hill et al., 2008

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Table 2 (*continued*)

Cryptosporidium species/ genotypes	Wildlife hosts	Zoonotic importance	gp60 subtypes reported in wildlife	References
Chipmunk genotype I	Chipmunk sp. (*Tamias* sp.), Eastern grey squirrel (*Sciurus carolinensis*), Deer mice (*Peromyscus maniculatus*)	Emerging human pathogen	XIVaA18G2T1, XIVaA18G2T2	Jiang et al., 2005; Feltus et al., 2006; Feng et al., 2007; ANOFEL, 2010; Insulander et al., 2013; Lebbad et al., 2013; Guo et al., 2015
Chipmunk genotype II	Eastern chipmunk (*Ramias striatus*)	No reports in humans to date	–	Feng et al., 2007; Stenger et al., 2015a
Chipmunk genotype III	Siberian chipmunk (*Tamias sibiricus*)	No reports in humans to date	–	Lv et al., 2009
Deer mouse genotype I	Deer mouse (*Peromyscus*)	No reports in humans to date	–	Xiao et al., 2002b; Feng et al., 2007, 2011b
Deer mouse genotype II	Deer mouse (*Peromyscus*)	No reports in humans to date	–	Xiao et al., 2002b; Feng et al., 2007
Deer mouse genotype III	Deer mouse (*Peromyscus*)	No reports in humans to date	–	Feng et al., 2007; Stenger et al., 2015b
Deer mouse genotype IV	Deer mouse (*Peromyscus*)	No reports in humans to date	–	Feng et al., 2007
Ferret genotype	Ferret (*Mustelidae*), Siberian chipmunk (*Tamias sibiricus*), River otters (Lontra canadensis), Black-footed ferret (*Mustela nigripes*), Red squirrel (*Sciurus vulgaris*)	No reports in humans to date	VIIIaA5G2	Xiao et al., 2002a; Abe and Iseki, 2003; Gaydos et al., 2007; Kváč et al., 2008; Lv et al., 2009; Feng et al., 2011b
Giant panda genotype	Giant panda (*Ailuropoda melanoleuca*)	No reports in humans to date	–	Liu et al., 2013
Squirrel genotypes I–III	Golden-mantled ground squirrels (*Callospermophilus lateralis*), Belding's ground squirrels (*Urocitellus beldingi*), California ground squirrels (*Otospermophilus beecheyi*), Black-tailed prairie dog (*Cynomys ludovicianus*)	No reports in humans to date	–	Atwill et al., 2004; Pereira et al., 2010; Stenger et al., 2015b
Hamster genotype	Siberian hamster (*Phodopus sungorus*)	No reports in humans to date	–	Lv et al., 2009
Horse genotype	Przewalski's wild horse (*Equus przewalski*), Four-toed hedgehog (*Atelerix albiventris*)	Identified in humans in the UK	VIaA11G3, VIbA13	Ryan et al., 2003; Robinson et al., 2008; Abe and Matsubara, 2015
Mink genotype	River otter (*Lontra canadensis*), American minks (*Mustela vison*), Ermine (*Mustela ermine*)	Several reports in humans	XaA5G1	Feng et al., 2007, Wang et al., 2008; Feng et al., 2011b; Ng-Hublin et al., 2013; Stuart et al., 2013; Ebner et al., 2015
Mouse genotype II	House mouse (*Mus musculus*)	No reports in humans to date	–	Foo et al., 2007; Silva et al., 2013
Mouse genotype III	House mouse (*Mus musculus*)	No reports in humans to date	–	Silva et al., 2013
Muskrat genotype I	Muskrat (*Ondatra zibethicus*), Boreal red-backed vole (*Myodes rutilus*)	No reports in humans to date	–	Xiao et al., 2002a; Zhou et al., 2004; Feng et al., 2007
Muskrat genotype II	Muskrat (*Ondatra zibethicus*), Red fox (*Vulpus vulpus*), Deer mouse (*Peromyscus maniculatus*), Meadow vole (*Microtus pennsylvanicus*)	No reports in humans to date	–	Ziegler et al., 2007; Robinson et al., 2011
Naruko genotype	Large Japanese field mouse (*Apodemus speciosus*)	No reports in humans to date	–	Murakoshi et al., 2013
Rat genotype I	Brown rat (*Rattus norvegicus*)	No reports in humans to date	–	Ng-Hublin et al., 2013
Rat genotype II	Brown rat (*Rattus tanezum*), Wild black rat (*Rattus rattus*), Brown rat (*Rattus norvegicus*).	No reports in humans to date	–	Lv et al., 2009; Paparini et al., 2012; Ng-Hublin et al., 2013; Silva et al., 2013
Rat genotype III	Asian house rat (*Rattus tanezumi*), Wild black rat (*Rattus rattus*).	No reports in humans to date	–	Lv et al., 2009; Paparini et al., 2012; Ng-Hublin et al., 2013; Silva et al., 2013
Rat genotype IV	Tanezumi rat (*Rattus tanezumi*), Asian house rat (*Rattus tanezumi*), Brown rat (*Rattus norvegicus*)	No reports in humans to date	–	Ng-Hublin et al., 2013
Seal genotypes I and II	Ringed seals (*Phoca hispida*), Harbour seals (*Phoca vituline*), Hooded seal (*Cystophora cristata*)	No reports in humans to date	–	Santín et al., 2005; Bass et al., 2012
Seal genotype III	Harp seal (*Pagophilus groenlandicus*)	No reports in humans to date	–	Bass et al., 2012
Seal genotype IV (similar to skunk genotype)	Southern elephant seal (*Mirounga leonina*)	No reports in humans to date	–	Rengifo-Herrera et al., 2011, 2013
Seal genotype V (Weddell seal genotype)	Weddel seal (*Leptonychotes weddellii*)	No reports in humans to date	–	Rengifo-Herrera et al., 2013
Shrew genotype		No reports in humans to date	–	

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Table 2 (*continued*)

Cryptosporidium species/ genotypes	Wildlife hosts	Zoonotic importance	gp60 subtypes reported in wildlife	References
	Northern short-tailed shrew (*Blarina brevicauda*), Wildebeest (*Connochaetes*), White-toothed shrew (*Crocidura russula*), Common shrew (*Sorex araneus*), Masked shrew (*Sorex scinereus*), Pygmy shrew (*Sorex minutus*), Brewer's mole (*Parascalops brewer*), Ermine (*Mustela ermine*)			Torres et al., 2000; Alves et al., 2005; Feng et al., 2007; Ziegler et al., 2007
Skunk/skunk-like genotype	Striped skunk (*Mephitis mephitis*), Raccoon (*Procyon lotor*), Eastern grey squirrel (*Sciurus carolinensis*), River otter (*Lontra canadensis*), Raccoon (*Procyon lotor*), Southern elephant seal (*Mirounga leonina*), Raccoon (*Procyon lotor*), Shunk (*Mephitis mephitis*), American red (*Tamiasciurus hudsonicus*), Fox squirrel (*Sciurus niger*)	Has been reported in humans	–	Xiao et al., 2002b; Zhou et al., 2004; Feng et al., 2007; Ziegler et al., 2007; Robinson et al., 2008; Chalmers et al., 2009; Feng et al., 2011b; Rengifo-Herrera et al., 2011; Elwin et al., 2012; Stenger et al., 2015b
Vole genotype	Meadow vole (*Microtus pennynsylvanicus*)	No reports in humans to date	–	Feng et al., 2007
Wildbeast genotype	Black wildbeast (*Connochaetos*)	No reports in humans to date	–	Alves et al., 2005

adapted genotypes and unidentified "environmental sequences" which probably represent as yet unidentified wildlife genotypes and which also highlight the potential for contamination of water supplies by wildlife (Zhou et al., 2004; Jiang et al., 2005; Yang et al., 2008; Jellison et al., 2004; Nichols et al., 2010; Koompapong and Sukthana, 2012; Van Dyke et al., 2012; Xiao et al., 2012; Galván et al., 2014; Li et al., 2014; Mahmoudi et al., 2015). For example, studies on *Cryptosporidium* contamination from wildlife from New York watersheds have shown that wildlife are the major source of *Cryptosporidium* in protected drinking source water, including some emerging human pathogens such as *C. ubiquitum* and chipmunk genotype I (Jiang et al., 2005; Feng et al., 2007).

3.1. *Cryptosporidium in mammals*

Due to the morphological similarity of *Cryptosporidium* oocysts from different host species, initial findings of *Cryptosporidium* infections in wild animals were assumed to be due to *C. parvum* leading to an overestimation of the potential role of wildlife as reservoirs of human disease (Appelbeea et al., 2005). However, with the assistance of advanced molecular techniques, many of these species were identified as host-adapted genotypes (Table 2). Both wild terrestrial and marine mammals have been studied as potential reservoirs for human-infectious *Cryptosporidium* species and genotypes using molecular tools (Table 2). The prevalence of *Cryptosporidium* in wild placental mammal hosts has been reported in detail in a recent review (Feng, 2010) and varies widely between mammalian hosts.

3.1.1. *Cryptosporidium hominis*

Although humans are the major host species for *C. hominis*, it has been reported in a number of wildlife hosts including a dugong and non-human primates (Table 2) (Xiao et al., 1999; Ye et al., 2012; Karim et al., 2014; Bodager et al., 2015; Parsons et al., 2015). *C. hominis/Cryptosporidium parvum*-like sequences were identified in red and black-and-white colobus monkeys in Uganda (Salyer et al., 2012). However, typing was obtained using a short fragment of the *Cryptosporidium* oocyst wall protein (COWP) gene, which is not reliable for differentiating *Cryptosporidium* species. In Australia, a number of recent studies have also identified *C. hominis/C. parvum*-like isolates at the 18S locus in marsupials including bandicoots, brushtail possums, eastern grey kangaroos and brush-tailed rock-wallabies (Hill et al., 2008; Ng et al., 2011; Dowle et al., 2013; Vermeulen et al., 2015). However, despite efforts, the identification of *C. hominis/C. parvum* could not be confirmed at other loci. This might be due to low numbers of oocysts and the multi copy nature of the 18S rRNA gene. Another study reported a *C. hominis*-like sequence at the 18S locus in a wild dingo, but was also unable to confirm this at other loci (Ng et al., 2011).

Subtyping of *C. hominis* at the gp60 locus has identified nine subtype families (Ia to Ij) (Ryan et al., 2014). To date, few *C. hominis* subtypes have been reported in wild mammals but include subtype IbA12G3 in Rhesus macaques, subtype IiA17 in Cynomolgus monkeys and Rhesus monkeys and subtype IfA12G2 in baboons and Mitumba chimpanzees (Feng et al., 2011b; Karim et al., 2014; Bodager et al., 2015; Parsons et al., 2015).

Recently, *C. hominis* has been identified and enumerated from eastern grey kangaroos and cattle faecal samples from Sydney catchments and characterised at multiple loci (Zahedi et al., 2015). In that study, *C. hominis* isolates were typed at three loci (18S, a novel mucin-like glycoprotein that contains a C-type lectin domain and the gp60 gene) (Zahedi et al., 2015). The *C. hominis* IbA10G2 subtype was identified in the marsupials and cattle (Zahedi et al., 2015), which is the main subtype associated with outbreaks of cryptosporidiosis by *C. hominis* (Xiao, 2010).

3.1.2. *C. parvum*

C. parvum was first described in mice (Tyzzer, 1912) and is primarily a parasite of artiodactyls and humans (Xiao, 2010). *C. parvum* has however been frequently reported in wildlife, infecting a broad range of wild species including various rodents, bovids, camelids, equids, canids, non-human primates and marine mammals (Table 2) (Morgan et al., 1999a; Atwill et al., 2001; Perez and Le Blancq, 2001; Matsubayashi et al., 2004; Ryan et al., 2004; Appelbee et al., 2005; Feng et al., 2007; Meireles et al., 2007; Paziewska et al., 2007; Starkey et al., 2007; Ziegler et al., 2007; Gómez-Couso et al., 2012; Ye et al., 2012; Abu Samraa et al., 2013; Liu et al., 2013; García-Presedo et al., 2013b; Reboredo-Fernández et al., 2014; Montecino-Latorre et al., 2015; Wells et al., 2015; Matsui et al., 2000).

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Few studies have identified *C. parvum* in captive wild mammals but red deer, fallow deer, addaxes, Arabian oryx, gemsboks, and sable antelopes are among mammals to be infected with *C. parvum* in captivity (Perez and Le Blancq, 2001; Ryan et al., 2003; Hajdusek et al., 2004; Abe et al., 2006; Feng et al., 2007; Meireles et al., 2007; Matsubayashi et al., 2004; Bodager et al., 2015; Wang et al., 2015; Zhao et al., 2015a).

Subtyping of *C. parvum* at the gp60 locus has identified fourteen subtype families (IIa to IIo (Ryan et al., 2014)). Few studies which identified *C. parvum* in wild mammals have conducted typing at the gp60 locus but a variety of *C. parvum* subtypes including IIdA15G1, IIdA18G1, IIdA19G1 have been reported from golden takins, lemurs, chipmunks and hamsters, and IIaA15G2R1, IIaA19G2R1, IIaA19G3R1, IIaA19G4R1, IIaA20G3R1, IIaA20G4R1, IIaA20G3R2 and IIaA21G3R1 have been reported from deer and Eastern grey kangaroos (Lv et al., 2009; Bodager et al., 2015; Montecino-Latorre et al., 2015; Zhao et al., 2015a; Zahedi et al., 2015). These are all *C. parvum* subtypes that have been reported in humans (Xiao, 2010).

3.1.3. *Cryptosporidium cuniculus*

C. cuniculus (previously known as rabbit genotype) was first described in rabbits by Inman and Takeuchi (1979), who described the microscopic detection and ultra-structure of endogenous *Cryptosporidium* parasites in the ileum of an asymptomatic female rabbit. Molecular characterisation of *C. cuniculus* was first conducted on rabbit faecal samples from the Czech Republic (Ryan et al., 2003) and *C. cuniculus* was formally re-described as a species in 2010 (Robinson et al., 2010). Since then, it has been described from rabbits across a wide geographic area including Australia, China, the UK, the Czech Republic, Poland, France and Nigeria (Ryan et al., 2003; Nolan et al., 2010; Shi et al., 2010; Chalmers et al., 2011; Zhang et al., 2012; Nolan et al., 2013; Liu et al., 2014; Koehler et al., 2014; Puleston et al., 2014; Zahedi et al., 2015). *C. cuniculus* has a close genetic relationship with *C. hominis* and its zoonotic potential became clear in 2008, when it was responsible for a drinking-water associated outbreak of cryptosporidiosis in the UK (Chalmers et al., 2009; Robinson et al., 2011; Puleston et al., 2014) and has also been identified in many sporadic human cases of cryptosporidiosis (Robinson and Chalmers, 2011; Chalmers et al., 2011; Elwin et al., 2012; Koehler et al., 2014). It is also the third most commonly identified *Cryptosporidium* species in patients with diarrhoea in the UK (Chalmers et al., 2011). Subtyping at the gp60 locus has identified two distinct subtype families, designated Va and Vb (Chalmers et al., 2009). Most cases described in humans relate to clade Va and the first waterborne outbreak was typed as VaA22 (Robinson et al., 2008; Chalmers et al., 2009). *C. cuniculus* has been reported in rabbits and humans (subtypes VaA9–VaA22 and VbA20–VbA37 – see Wang et al., 2012) but has recently been identified in marsupials (subtype VbA26) (and a human – subtype VbA25) in Australia (Nolan et al., 2013; Koehler et al., 2014). The widespread occurrence of *C. cuniculus* genotypes in rabbits and the fact that it has been now been identified in marsupials in Australia suggests that *C. cuniculus* might be a species more ubiquitous than previously thought, and might be able to spread to other mammals as well as humans. Therefore, there is a need to diligently monitor for *C. cuniculus* in the vicinity of drinking water catchments and in drinking water.

3.1.4. *Cryptosporidium ubiquitum*

C. ubiquitum (previously cervine genotype, cervid, W4 or genotype 3) was first identified by Xiao et al. (2000) in storm water samples in lower New York State (storm water isolate W4, GenBank accession no. AF262328). Subsequently, Perez and Le Blancq (2001) identified this genotype in white-tailed deer-derived isolates from lower New York State and referred to it as genotype 3. Since then it has been described in a wide variety of hosts worldwide including humans and was formally described as a species in 2010 (Fayer et al., 2010). *C. ubiquitum* is of public health concern because of its wide geographic distribution and broad host range (Li et al., 2014). In addition to domestic animals (in particular sheep) and wildlife, *C. ubiquitum* has been frequently reported from drinking source water, storm water runoff, stream sediment and wastewater in various geographic locations, suggesting potential contamination of water sources with oocysts of *C. ubiquitum* shed by animals inhabiting water catchments (Nolan et al., 2013; Li et al., 2014). *C. ubiquitum* is considered an emerging zoonotic pathogen (Li et al., 2014), as it has been identified in many human cases of cryptosporidiosis in the United Kingdom, Slovenia, the United States, Canada, Spain, New Zealand, Venezuela and Nigeria (Charlmers et al., 2011; Wong and Ong, 2006; Fayer et al., 2010; Cieloszyk et al., 2012; Elwin et al., 2012; Blanco et al., 2015; Qi et al., 2015a).

In wildlife, *C. ubiquitum* has been reported sporadically in rodents, wild ruminants, carnivores, marsupials and primates (Table 2) (Perez and Le Blancq, 2001; da Silva et al., 2003; Ryan et al., 2003; Feng et al., 2007; Feng, 2010; Karanis et al., 2007; Ziegler et al., 2007; Wang et al., 2008; Fayer et al., 2010; Cinque et al., 2008; Robinson et al., 2011; Feng et al., 2011b; Abu Samraa et al., 2013; Mi et al., 2013; Murakoshi et al., 2013; Li et al., 2014; Ma et al., 2014; Perec-Matysiak et al., 2015; Qi et al., 2015a, 2015b; Stenger et al., 2015b; Vermeulen et al., 2015).

Because *C. ubiquitum* is genetically distant from *C. hominis* and *C. parvum*, until recently, gp60 homologs had not been sequenced. However, the gp60 gene of *C. ubiquitum* was identified by whole-genome sequencing and six subtype families (XIIa–XIIf) within *C. ubiquitum* were identified (Li et al., 2014). Application of this new tool to human, animal, and environmental (water) isolates has suggested that sheep and rodents are a key source of *C. ubiquitum* transmission to humans, through either direct human contact with infected animals or by contamination of drinking source water (Li et al., 2014). For example, in the US, all *C. ubiquitum* specimens from humans characterized belonged to the same subtype families found in wild rodents in the US (XIIb, XIIc and XIId) (Li et al., 2014). However, as persons in the United States usually have little direct contact with wild rodents, the authots concluded that transmission of *C. ubiquitum* to humans from rodents was likely to come from drinking untreated water contaminated by wildlife (Li et al., 2014).

3.1.5. *Cryptosporidium muris*

C. muris is a gastric parasite and was first identified in the gastric glands of mice in 1907 by Tyzzer (1907). Since then, molecular tools have shown that it has a wide host range, including various mammals (rodents, canids, felids, suids, giraffida, equids, non-human primates and marsupials) and birds (Tables 1 and 2). *C. muris* is considered a zoonotic species as there have been numerous reports of *C. muris* in humans and one report in human sewage (Guyot et al., 2001; Gatei et al., 2002; Tiangtip and Jongwutiwes, 2002; Gatei et al., 2003; Palmer et al., 2003; Gatei et al., 2006; Leoni et al., 2006; Muthusamy et al., 2006; Azami et al., 2007; Al-Brikan et al., 2008; Neira et al., 2012; Hasajová et al., 2014; Petrincová et al., 2015; Spanakos et al., 2015; Hurkova et al., 2003).

In a recent human infectivity study, *C. muris* was examined in six healthy adults (Chappell et al., 2015). Volunteers were challenged with 10^5 *C. muris* oocysts and monitored for 6 weeks for infection and/or illness. All six patients became infected. Two patients experienced a self-limited diarrhoeal illness. *C. muris* oocysts shed during the study ranged from 6.7×10^6 to 4.1×10^8, and *C. muris*-infected subjects shed oocysts longer than occurred with other species studied in healthy volunteers. Three volunteers shed oocysts for 7 months (Chappell et al., 2015). The authors concluded

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that healthy adults are susceptible to *C. muris*, which can cause mild diarrhoea and result in persistent, asymptomatic infection (Chappell et al., 2015), which confirms the zoonotic status of *C. muris* and highlights the public health risks of finding *C. muris* in wildlife in drinking water catchments.

3.1.6. Cryptosporidium andersoni

Like *C. muris*, *C. andersoni* is also a gastric parasite and primarily infects the abomasum of cattle and to a lesser extent, sheep and goats (Ryan et al., 2014; Wang et al., 2012). *C. andersoni* produces oocysts that are morphologically similar to, but slightly smaller than those of *C. muris* (7.4–8.8 × 5.8–6.6 μm vs 8.2–9.4 × 6.0–6.8 μm, respectively) and was originally mistakenly identified in cattle as *C. muris* based on its oocyst size. In 2000, it was described as a new species based on the location of endogenous stages in the abomasum, its host range, and genetic distinctness at multiple loci (Lindsay et al., 2000). It has only occasionally being detected in wild animals (Table 2) (Ryan et al., 2004; Wang et al., 2008, 2015; Lv et al., 2009; Feng et al., 2010; Zhao et al., 2015a). Several studies have reported that *C. andersoni* is the dominant species in source and tap water (Feng et al., 2011; Nichols et al., 2010), suggesting that cattle may be the primary source of contamination. Interestingly, in a recent study, it was found at a prevalence of 15.6% (19/122) and 0.5% (1/200) in captive and wild giant pandas, respectively in China (Wang et al., 2015). It is occasionally detected in humans (Leoni et al., 2006; Morse et al., 2007; Waldron et al., 2011; Agholi et al., 2013; Jiang et al., 2014; Liu et al., 2014). Two studies in China by the same research group have reported that *C. andersoni* was the most prevalent *Cryptosporidium* species detected in humans (Jiang et al., 2014; Liu et al., 2014). However, further research is required to better understand the zoonotic importance of *C. andersoni*.

3.1.7. Cryptosporidium canis

C. canis (previously dog genotype 1) was first identified as the dog genotype by Xiao et al. (1999) and described as a species in 2001 (Fayer et al., 2001), on the basis that *C. canis* oocysts were infectious for calves but not mice and were genetically distinct from all other species. *C. canis* and its sub-genotypes (*C. canis* fox genotype and *C. canis* coyote genotype) have been reported in dogs, foxes and coyotes (Table 2) (Xiao et al., 2002a; Zhou et al., 2004; Fayer, 2010; Feng, 2010). *C. canis* has also been reported worldwide in humans (Lucio-Forster et al., 2010; Fayer, 2010; Elwin et al., 2012; Mahmoudi et al., 2015; Parsons et al., 2015).

3.1.8. Cryptosporidium erinacei

Little is known about epidemiology and pathogenicity of zoonotic *C. erinacei* in wildlife. *C. erinacei* (previously known as hedgehog genotype) was first identified morphologically in a captive four-toed hedgehog (*Ateletrix albiventris*) in 1998 (Graczyk et al., 1998). An isolate from a European hedgehog originating from Denmark was typed in 2002 (Enemark et al., 2002) and shown to be distinct. Subsequent studies have identified *C. erinacei* in hedgehogs, horses and humans (Dyachenko et al., 2010; Laatamna et al., 2013; Kváč et al., 2014a, 2014b; Meredith and Milne, 2009). At the gp60 locus, *C. erinacei* isolates are identified as subtype family XIII (Dyachenko et al., 2010; Laatamna et al., 2013; Lv et al., 2009; Kváč et al., 2014b). Previously reported *C. erinacei* subtypes include XIIIaA20R10 (KF055453), XIIIaA21R10 (GQ214085), XIIIaA22R9 (KC305644), XIIIaA19R12 (GQ214081), and XIIIaA22R11 (GQ259140) Kváč et al., 2014b).

3.1.9. Cryptosporidium fayeri and Cryptosporidium macropodum

The two main species identified in a wide range of marsupials are *C. fayeri* and *C. macropodum* (previously marsupial genotype I and II) (Table 2) (Morgan et al., 1997; Power et al., 2004, 2005; Power and Ryan, 2008; Ryan et al., 2008; Nolan et al., 2010; Power, 2010; Ng et al., 2011a; Yang et al., 2011; Ryan and Power, 2012; Nolan et al., 2013; Vermeulen et al., 2015; Zahedi et al., 2015). Neither of these species is associated with diarrhoea in their marsupial hosts (Ryan and Power, 2012). *C. macropodum* has not been reported in humans but cryptosporidiosis caused by *C. fayeri* has been reported in a 29-year-old female patient in Australia (Waldron et al., 2010). The woman was immunocompetent but suffered prolonged gastrointestinal illness. The patient resided in a national forest on the east coast of New South Wales, Australia, an area where marsupials are abundant. She had frequent contact with partially domesticated marsupials (Waldron et al., 2010). Identification of *C. fayeri* in a human patient is a concern for water catchment authorities in the Sydney region. The main water supply for Sydney, Warragamba Dam, covers 9050 km^2 and is surrounded by national forest inhabited by diverse and abundant marsupials. At the gp60 locus, the subtype family IV has been identified with 6 subtypes (IVa–IVf) (Power et al., 2009). Subtyping of the human-derived isolate of *C. fayeri* identified IVaA9G4T1R1, which has also been identified in eastern grey kangaroos in Warragamba Dam, suggesting possible zoonotic transmission (Power, 2010; Waldron et al., 2010).

In addition to *C. fayeri* and *C. macropodum*, there have been several other host-adapted genotypes identified in Australian marsupials. Possum genotype I has been described in brushtail possums, a host species found in a range of habitats throughout Australia (Hill et al., 2008) and the novel kangaroo genotype I in western grey kangaroos (Yang et al., 2011). Possum genotype I and kangaroo genotype I have not been reported in humans or other animals and their zoonotic potential is unknown.

3.1.10. Cryptosporidium meleagridis

Although primarily a bird parasite (see section 3.2.1 and Table 3), *C. meleagridis* has been identified in deermice, mountain gorillas and marsupials (Feng et al., 2007; Sak et al., 2014; Vermeulen et al., 2015). It is also the third most prevalent species infecting humans (Morgan et al., 2000; Cama et al., 2003; Gatei et al., 2006; Muthusamy et al., 2006; Leoni et al., 2006; Berrilli et al., 2012; Elwin et al., 2012; Neira et al., 2012; Silverlås et al., 2012; Kurniawan et al., 2013; Sharma et al., 2013; Wang et al., 2014; Adamu et al., 2014; Ghaffari and Kalantari, 2014; Ryan and Xiao, 2014; Ghaffari and Kalantari, 2014; Rahmouni et al., 2014; Wang et al., 2014; Stensvold et al., 2014, 2015). In some studies, *C. meleagridis* prevalence is similar to that of *C. parvum* (Gatei et al., 2002; Cama et al., 2007). The ability of *C. meleagridis* to infect humans and other mammals, and its close relationship to *C. parvum* and *C. hominis* at multiple loci, has led to the suggestion that mammals actually were the original hosts, and that the species has later adapted to birds (Xiao et al., 2002a,). Subtyping at the gp60 locus has identified seven subtype families (IIIa to IIIg) (Stensvold et al., 2015). More details on transmission dynamics will be discussed in section 3.2.1.

3.1.11. Other Cryptosporidium species and genotypes reported in wild mammals

A number of other *Cryptosporidium* species and genotypes have been identified in wildlife (Table 2). Most are host-adapted genotypes that are not of public health significance, however several have been identified in humans (Table 2). Of these, the chipmunk genotype I is considered an emerging human pathogen (Jiang et al., 2005; Feltus et al., 2006; Feng et al., 2007; ANOFEL, 2010; Insulander et al., 2013; Lebbad et al., 2013; Guo et al., 2015). At the gp60 locus, 15 different subtypes have been identified but subtypes differ only in the number of tandem repeats (TCA/TCG/

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Table 3
Cryptosporidium species and genotypes in avian hosts confirmed by molecular analysis (Modified from Ryan and Xiao, 2014).

Species name	Major host(s)	Site of infection	References
C. meleagridis	Turkey (*Meleagris gallopavo*), Indian ring-necked parrot (*Psittacula kameri*), Red-legged partridge (*Alectoris rufa*), Cocktails (*Nymphicus hollandicus*), Bohemian waxwing (*Bombycilla garrulous*), Rufousturle dove (*Streptopelia orientalis*), Fan-tailed pigeon (*Columba livia*), Chicken (*Gallus gallus*), Quails (*Coturnixcoturnix japonica*), Pekin ducks (*Anas platyrhynchos*), Domestic Pigeons (*Columba livia domestica*), European turtle dove (*Streptopelia turtur*), Red-legged partridge (*Alectoris rufa*)	Intestine	Morgan et al., 2000; Glaberman et al., 2001; Abe and Iseki, 2004; Abe and Makino 2010; Wang et al., 2010; Qi et al., 2011; Berrilli et al., 2012; Wang et al., 2012; Baroudi et al., 2013; Wang et al., 2014; Koompapong et al., 2014; Maca and Pavlasek, 2015; Reboredo-Fernandez et al., 2015
C. baileyi	Turkey (*Meleagris gallopavo*), Chicken (*Gallus gallus*), Brown squail (*Synoicus australis*), Cocktails (*Nymphicus hollandicus*), Whooping crane (*Grus vipio*), Grey-bellied bulbul (*Pycnonotus* spp.), Black vulture (*Coragyps atratus*), Saffron finch (*Sicalis flaveola*), Mixed-bred falcons (*Falcorusticolus x Falco cherrug*), Reddy Shelduck (*Tadornaferruginea*), Red-billed leiothrixes (*Leiothrix lutea*), Pekin ducks (*Anas platyrhynchos*), Buffy-fronted seedeater (*Sporophila frontalis*), Javva sparrows (*Padda oryzivora*), Mynas (*Acridotheres tristis*), Zebra finches (*Taeniopygia guttata*), Crested Lark (*Galerida cristana*), Gouldian finch (*Chloebia gouldiae*), Black-billed magpie (*Pica pica*), Ostriches (*Struthio camelus*), Quails (*Coturnixcoturnix japonica*), Red grouse (*Lagopus lagopus scotica*), Red-crowned crane (*Grus japonenis*)	Cloaca, bursa, trachea	Morgan et al., 2001; Abe and Iseki, 2004; Ng et al., 2006; Huber et al., 2007; Kimura et al., 2004; Nakamura et al., 2009; Abe and Makino, 2010; Wang et al., 2010; Qi et al., 2011; Wang et al., 2012; Baroudi et al., 2013; Baines et al., 2014; Hamidinejat et al., 2014; Wang et al., 2014; Li et al., 2015c; Maca and Pavalasek, 2015
C. galli	Chicken (*Gallus gallus*), Finches (*Spermestidae* and *Fringillidae*), Capercaille (*Tetrao urogallu*), Pine grosbeak (*Pinicola enuncleator*), Turqoise parrots (*Neophema pulchella*), Cuban flamingo (*Phoenicopterus ruber ruber*), Rhinoceros hornbill (*Buceros rhinoceros*), Red-cowled cardinal (*Paroaria dominicana*), Zebra finches (*Taeniopygia guttata*), Chocolate parson finches (*Peophila cincta*), Chesnut finches (*Lonchura castaneothorax*), Painted firetali finches (*Ebmlema picta*), Canaries (*Serinus* sp.), Glosters (*Serinus canaria*), Green-winged saltatros (*Saltator similis*), Slate-collard seedeater (*Sporophila schistaceca*), Great-billed seed-fench (*Oryzoborus maximiliani*), Ultermarine grosbeak (*Cyanocompsa brissonii*), Bohemian waxwing (*Bombycilla garrulous*), Silver-eared Mesia (*Leiothrix argentauris*), Cockatiel (*Nymphicus hollandicus*), Chopi blackbird (*Gnorimopsar chopi*), Green-winged saltator (*Saltator similis*), Rufous-collared sparrow (*Zonotrichia capensis*)	Preventriculus	Ryan et al., 2003; Ng et al., 2006; Antunes et al., 2008; Nakamura et al., 2009; da Silva et al., 2010; Qi et al., 2011; Nakamura et al., 2014
Avian genotype I	Red factor canary (*Serinus canaria*), Canary (*S. canaria*), Indian peafowl (*Pavo cristatus*)	–	Ng et al., 2006; Nakamura et al., 2009
Avian genotype II	Eclectus (*Eclectus roratus*), Galah (*Eolophus roseicapilla*), Cockatiel (*Nymphicus hollandicus*), Major Mitchel Cockatoo (*Cavcatua lead beater*), Ostriches (*Struthio camelus*), White-eyed parakeet (*Aratinga leucophthalma*)	–	Meireles et al., 2006; Ng et al., 2006; Nakamura et al., 2009; Seva et al., 2011; Nguyen et al., 2013
Avian genotype III	Galah (*Eolophus roseicapilla*), Cockatiel (*Nymphicus hollandicus*), Java sparrow (*Padda oryzivora*), Son conure (*Aratinga solstitialis*), Peach faced lovebirds (*Agapornis roseicollis*), Seagull (*Laridae* sp), Blue-fronted amazon (*Amazona aestival*), Cockatiel (*Nymphicus hollandicus*), Rufous-collared sparrow (*Zonotrichia capensis*), Lovebird (*Agapornis species*), Cockatiel (*Nymphicus hollandicus*)	–	Ng et al., 2006; Nakamura et al., 2009; Makino et al., 2010; Koompapong et al., 2014; Nakamura et al., 2014; Ravich et al., 2014; Li et al., 2015c; Gomes et al., 2012
Avian genotype IV	Japanese white-eye (*Zosterops japonica*)	–	Abe and Makino, 2010; Qi et al., 2011
Avian genotype V	Cockatiel (*Nymphicus hollandicus*), Budgerigar (*Melopsittacus undulates*)	–	Abe and Makino, 2010; Qi et al., 2011; Zhang et al., 2015
Duck genotype	Black dock (*Anus rubripes*), Canada geese (*Branta canadensis*)	–	Jellsison et al., 2004; Zhou et al., 2004
Eurasian woodcock genotype	Eurasian woodcock (*Scolopax rusticola*)	–	Ryan et al., 2003; Ng et al., 2006
Goose genotype I	Canada geese (*Branta canadensis*)	–	Xiao et al., 2002b; Jellison et al., 2004; Zhou et al., 2004
Goose genotype II	Canada geese (*Branta canadensis*)	–	Jellison et al., 2004; Zhou et al., 2004
Goose genotype III	Canada geese (*Branta canadensis*)	–	Jellison et al., 2004
Goose genotype IV	Canada geese (*Branta canadensis*)	–	Jellison et al., 2004
Goose genotype V	Canada geese (*Branta canadensis*)	–	Jellison et al., 2004

Appendix 1

TCT) and comprise a single subtype family (XIVa). Analysis indicates that subtypes from humans and wildlife are genetically similar and zoonotic transmission might play a potential role in human infections (Guo et al., 2015). The skunk and mink genotypes have also been reported in a few human cases of cryptosporidiosis (Robinson et al., 2008; Chalmers et al., 2009; Rengifo-Herrera et al., 2011; Elwin et al., 2012; Ng-Hublin et al., 2013; Ebner et al., 2015).

3.2. *Cryptosporidium* in birds

The mobility of migratory birds, together with their distribution and ability to form large colonies, makes them potentially suitable to spread pathogens. Due to their easy access to drinking water catchments and other water sources, wild birds are believed to be a potential risk to drinking water safety. The epidemiology of avian cryptosporidiosis, in particular zoonotic *Cryptosporidium* species infecting birds is therefore of public health importance. Currently only three avian *Cryptosporidium* spp. are recognised; *C. meleagridis, C. baileyi* and *C. galli* (Table 3) (Ryan and Xiao, 2014).

3.2.1. *C. meleagridis*

C. meleagridis infects the intestinal (small and large intestine and bursa) epithelial cells of a wide range of birds (Table 3) (Ryan and Xiao, 2014). It was first detected in a wild turkey (*Meleagris gallopavo*) by Tyzzer in 1929, but named as a valid *Cryptosporidium* species in 1955 (Slavin, 1955). *C. meleagridis* oocysts have been experimentally infected into broiler chickens, ducks, turkeys, calves, pigs, rabbits, rats and mice (Darabus and Olariu, 2003; Ryan and Xiao, 2014). It has also been reported as one of the most commonly detected human-infectious *Cryptosporidium* species in wastewater (Feng et al., 2007, 2011a; Li et al., 2012).

Molecular analysis has revealed that *C. meleagridis* has relatively low host specificity, and many *C. meleagridis* subtypes at other loci have been found in both birds and humans and both anthroponotic and zoonotic transmission routes have been suggested (Cama et al., 2003; Elwin et al., 2012; Silverlås et al., 2012). Subtyping at the gp60 locus has identified seven subtype families (IIIa–IIIg) and the likely occurrence of cross-species transmission of *C. meleagridis* between birds and humans (Wang et al., 2014). Human volunteer studies have shown that healthy adults can be infected and become ill from ingestion of *C. meleagridis* oocysts (Chappell et al., 2011). In the study by Chappell et al., five volunteers were challenged with 10^5 *C. meleagridis* oocysts and monitored for six weeks for faecal oocysts and clinical manifestations. Four volunteers had diarrhoea; three had detectable faecal oocysts; and one infected volunteer remained asymptomatic. All infections were self-limited and oocysts were cleared within $\leq$12 days of challenge (Chappell et al., 2011).

3.2.2. *Cryptosporidium baileyi*

C. baileyi is generally associated with the respiratory form of cryptosporidiosis in birds and has been predominantly reported in broiler chickens. Compared to *C. meleagridis*, *C. baileyi* is capable of infecting a larger spectrum of avian hosts (Table 3), targeting various sites of infection mostly associated with digestive and respiratory tracts (Ryan and Xiao, 2014). Experimental cross-transmission of *C. baileyi* to other birds has been successfull, however there has been no reports of cross-transmission between birds and other vertebrates (Lindsay and Blagburn, 1990; Cardozo et al., 2005), except for a single unsubstantiated report of human infection with *C. baileyi* which did not include any molecular analysis (Ditrich et al., 1991). Therefore, *C. baileyi* is not considered to be of public health significance.

3.2.3. *Cryptosporidium galli*

Unlike other avian species, *C. galli* is a gastric species with endogenous developmental stages occurring in the glandular epithelial cells of the proventriculus (Pavlásek, 1999, 2001; Ryan et al., 2003; Ng et al., 2006; Ryan and Xiao, 2014). It predominantly infects birds of the family Spermestidae, Fringilidiae and domestic chickens (*Gallus gallus*), and seems to be more prevalent among songbirds (Table 3). Successful experimental cross-transmission of *C. galli* to other chickens have been reported, however the full extent of its host range is still unknown (Ryan, 2010). It has not been reported in humans.

3.2.4. *Other Cryptosporidium species and genotypes reported in birds*

In addition to *C. meleagridis*, other zoonotic species of *Cryptosporidium* reported in birds include *C. hominis*, *C. parvum*, *C muris* and *C. andersoni* (Zylan et al., 2008; Jellison et al., 2009; Ryan, 2010; Reboredo-Fernandez et al., 2015; Gomes et al., 2012). In addition, twelve genotypes; avian genotypes I–V, the black duck genotype, the Eurasian woodcock genotype and goose genotypes I–V have been reported (Table 3). To date, there is no evidence of human cryptosporidiosis caused by these genotypes.

3.3. *Cryptosporidium* in fish and marine mammals

Cryptosporidium has been described in both fresh and marine water piscine species with parasitic stages located either on the stomach or intestinal surface, or deep within the epithelium (Table 4). The first account of *Cryptosporidium* in a piscine host was *Cryptosporidium nasorum*, identified in a Naso tang, a tropical fish species (Hoover et al., 1981). However, currently only three species are recognized; *C. molnari*, *C. scophthalmi* and *C. huwi* (previously known as piscine genotype I) (Alvarez-Pellitero and Sitja-Bobadilla, 2002; Alvarez-Pellitero et al., 2004; Palenzuela et al., 2010; Costa et al., 2015; Ryan et al., 2015), none of which have been reported in humans. In fish hosts, *Cryptosporidium* fish species and genotypes are typically located either in the stomach or intestine and the parasite can cause clinical manifestations, such as emaciation, decrease in growth rate, anorexia, whitish faeces, abdominal swelling, and ascites (Alvarez-Pellitero et al., 2004; Ryan et al., 2015). Most studies on *Cryptosporidium* in fish have been reported in farmed or aquarium fish (Table 4) and little data are currently available regarding the molecular identification of *Cryptosporidium* species and genotypes in wild fish populations and, in particular, in edible fish (Palenzuela et al., 2010; Reid et al., 2010; Barugahare et al., 2011; Gibson-Keuh et al., 2011; Koinari et al., 2013; Certard et al., 2015).

In addition to the three recognized species of *Cryptosporidium* in piscine hosts, numerous *Cryptosporidium* species and genotypes have been reported in fish including; piscine genotypes 2 to 8, unnamed novel genotypes (n = 5), rat genotype III, *C. parvum*, *C. hominis*, *C. xiaoi* and *C. scrofarum* (Table 4). Of these, only *C. parvum*, *C. hominis* and *C. scrofarum* are of public health interest. *Cryptosporidium scrofarum* was identified in a whiting (Reid et al., 2010); *C. parvum* was found in School whiting, Nile tilapias, a Silver barb, Arctic char and European whitefish and *C. hominis* was reported in Mackerel scad (Reid et al., 2010; Gibson-Kueh et al., 2011; Koinari et al., 2013; Certad et al., 2015). In one of the most recent studies, *C. parvum* was identified in freshwater fish from Lake Geneva (Lac Léman) by both histology and molecular analysis (Certad et al., 2015). In that study, the overall prevalence of *Cryptosporidium* was 36.6% (15/41); the prevalence of *C. parvum* and *C. molnari* was 86.7% (13/15) and 6.7% (1/15), respectively, while 6.7% (1/15) were mixed *C. parvum* and *C. molnari* infections (Certad et al., 2015). Histological analysis identified *C. parvum*

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Table 4
Cryptosporidium sp. reported in fish using molecular tools (modified from Ryan et al., 2014).

Species	Host	Site of infection	Reference
C. molnari	Gilthead sea bream (*Sparus aurata*), European sea bass (*Dicentrarchus labrax*), Murray cod (*Maccullochella peelii peelii*)	Stomach (and intestine)	Palenzuela et al., 2010; Barugahare et al., 2011; Certad et al., 2015
C. scophthalmi	Turbot (*Scophthalmus maximus*)	Intestine	Costa et al., 2015
C. huwi (previously piscine genotype 1)	Guppy (*Poecilia reticulata*)	Stomach	Ryan et al., 2004; Ryan et al., 2015
Piscine genotype 2	Angelfish (*Pterophyllum scalare*)	Stomach	Murphy et al., 2009
Piscine genotype 3	Mullet (*Mugil cephalus*)	Intestine	Reid et al., 2010
Piscine genotype 4	Golden algae eater (*Crossocheilus aymonieri*), Kupang damsel (*Chrysiptera hemicyanes*), Oscar fish (*Astronatus ocellatis*), Neon tetra (*Paracheirodon innesi*)	Intestine	Reid et al., 2010; Morine et al., 2012
Piscine genotype 5	Angelfish (*Pterophyllum scalare*), Butter bream (*Monodactylidae*), Golden algae eater (*Crossocheilus aymonieri*)	–	Zanguee et al., 2010
Piscine genotype 6/genotype 6-like	Guppy (*Poecilia reticulata*), Gourami (*Trichogaster trichopterus*)	–	Zanguee et al., 2010; Morine et al., 2012
Piscine genotype 7	Red eye tetra (*Moenkhausia sanctaefilomenae*)	–	Morine et al., 2012
Piscine genotype 8	Oblong silver biddy (*Gerres oblongus*)	–	Koinari et al., 2013
Rat genotype III, *C. hominis, C. parvum, C. xiaoi* and *C. scrofarum*	Whiting (*Sillago vittata*), Barramundi (*Lates calcarifer*), Arctic char (*Salvelinus alpinus*), Nile tilapias (*Oreochromis niloticus*), Silver barb (*Puntius gonionotus*), Mackerel scad (*Decapterus macarellus*), European whitefish (*Coregonus lavaretus*), School whiting (*Sillago vittata*)	–	Reid et al., 2010; Gibson-Kueh et al., 2011; Koinari et al., 2013; Certad et al., 2015
Novel un-named genotypes (n = 5)	Orange clownfish (*Amphiprion percula*), Azure damsel (*Chrysiptera hemicyanea*), Blue tang (*Paracanthurus hepatus*), Platyfish (*Xiphophorus maculatus*), Oscar (*Astronotus ocellatus*), Goldfish (*Carassius auratus*)	–	Yang et al., 2015

developmental stages in the stomach and intestine suggesting that *C. parvum* was infecting the fish, rather than being passively carried which has important public health implications.

Subtyping of *Cryptosporidium* isolates in fish has identified *C. parvum* subtype IIaA18G3R1 in School whiting from Australia (Reid et al., 2010), three *C. parvum* subtypes (IIaA14G2R1, IIaA15G2R1 and IIaA19G4R1) in Nile tilapia, silver barb and mackerel scad and a *C. hominis* subtype (IdA15G1) in mackerel scad in Papua New Guinea (Koinari et al., 2013), and *C. parvum* subtypes IIaA15G2R1, IIaA16G2R1 and IIaA17G2R1 in Arctic char and European whitefish from France (Certad et al., 2015). All of these *C. parvum* subtypes are zoonotic and commonly found in cattle and humans (Xiao, 2010). The identification of the *C. hominis* subtype probably reflects human sewage contamination of the water. Clearly further studies in this area are required to better understand the transmission dynamics of *Cryptosporidium* in fish.

3.4. *Cryptosporidium* in amphibians and reptiles

Little is known about *Cryptosporidium* species infecting amphibians. Of the three orders of amphibians; *Anura*, *Caudata* and *Gymnophonia*, *Cryptosporidium* has been only reported in Anura which includes frogs and toads and only one species, *C. fragile* is recognised (Table 5) (Jirků et al., 2008). In transmission experiments, *C. fragile* was not infective in one fish species (*Poecilia reticulate*), four amphibian species (*Bufo bufo*, *Rana temporaria*, *Litoria caerulea* and *Xenopus laevis*), one species of reptile (*Pantherophis guttatus*) and SCID mice (Jirků et al., 2008). This species has not been reported in humans.

Cryptosporidium infections are ubiquitous in reptiles and have been reported in more than 57 reptilian species (O'Donoghue, 1995; Ryan and Xiao, 2014). Unlike in other animals in which *Cryptosporidium* infection is usually self-limiting in immunocompetent individuals, cryptosporidiosis in reptiles is frequently chronic and sometimes lethal in some snakes. Both intestinal and gastric cryptosporidiosis has been described in snakes and lizards. To date, two species are recognised; *C. serpentis* (gastric) and *C. varanii* (*C. saurophilum*) (intestinal) (Levine, 1980; Pavlasek et al., 1995; Koudela and Modry, 1998; Pavlásek and Ryan, 2008); neither of which have been reported in humans, but *C. serpentis* has been identified in cattle (Azami et al., 2007; Chen and Qiu, 2012). A new intestinal species, *Cryptosporidium ducismarci* (tortoise genotype II) has been reported in several species of tortoises, snakes and lizards (Traversa, 2010). Because only molecular data are presented, this species is regarded as a nomen nudum, pending the support of morphological and biological data.

C. parvum, *C. muris* and *Cryptosporidium tyzzeri* are also commonly reported in reptiles, particularly snakes but this is thought to be due to mechanical transmission due to predation of infected rodents and is not thought to present a substantial zoonotic risk (Morgan et al., 1999; Xiao et al., 2004b; Pedraza-Diaz et al., 2009; Díaz et al., 2013; da Silva et al., 2014). In addition, various host-adapted genotypes have been identified including tortoise genotype I and snake genotypes I and II (cf. Ryan and Xiao, 2014), which have not been reported in humans (Table 5) (Xiao et al., 2004b; Pedraza-Diaz et al., 2009; Traversa, 2010; Seva Ada et al., 2011; Richter et al., 2011; Rinaldi et al., 2012; da Silva et al., 2014; Abe and Matsubara, 2015). There is also a single report of avian genotype V from green iguanas (*Iguana inguana*) (Kik et al., 2011).

4. The role of urbanisation in the transmission of zoonotic *Cryptosporidium* species from wildlife

The risk of waterborne outbreaks of cryptosporidiosis depends on a complex interplay of factors, associated with both the environment and the biology and ecology of host and parasite. *Cryptosporidium* detection in an animal faecal sample does not necessarily mean active infection in the host, nor does this guarantee that the parasite prevalence and the host-population dynamics are conducive to an outbreak. For these reasons the epidemiological potential of detection of *Cryptosporidium* in wildlife cannot be easily and fully extrapolated. An increased epidemiological risk, however, can be identified when there is an overlap between humans and the distribution and dispersal of animal hosts. This is largely due to human encroachment into wildlife-populated areas, which, by extension, also includes conversion of natural environments to drinking water catchments. Similarly, urban environments may also represent attractive new habitats for animals harbouring zoonotic *Cryptosporidium* spp. Thus, it is clear

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Table 5
Amphibian and reptile *Cryptosporidium* species and genotypes and their hosts confirmed by molecular analyses (modified from Ryan et al., 2014).

Species/genotype	Amphibian/Reptile host species	Site of infection	Reference
C. fragile	Black-spined toads (*Duttaphrynus melanostictus*)	Stomach	Jirků et al., 2008
C. serpentis	Amazon tree boa (*Corallus hortulanus*), Black rat snake (*Elaphe obsoleta obsolete*), Bornmueller's viper (*Vipera bornmuelleri*), Bull snake (*Pituophis melanoleucus melanoleucus*), California kingsnake (*Lampropeltis getulus californiae*), Cornsnake (*Elaphe guttata guttata*), Common death adder (*Acanthophis antarticus*), Desert monitor (*Varanus griseus*), Eastern/Mainland Tiger snake (*Notechis scutatus*), Frilled lizard (*Chlamydosaurus kingui*), Giant madagascar or Oustalet's chameleon (*Chamaeleo oustaleti*), Leopard gecko (*Eublepharis macularius*), Mexican black kingsnake (*Lampropeltis getulus nigritus*), Milk snake (*Lampropeltis triangulum*), Mountain viper (*Vipera wagneri*), Python (*Python molurus*), Savannah monitor (*Varanus exanthematicus*), Skink (*Mabuya perrotetii*), Taipan (*Oxyuranus scutellatus*), Red-tailed boa (*Boa constrictor constrictor*), Rainbow boa (*Epicrates cenchria cenchria*)	Stomach	Kimbell et al., 1999; Morgan et al., 1999b; Hajdusek et al., 2004; Xiao et al., 2004b; Pedraza-Díaz et al., 2009; Richter et al., 2011; Sevá-Ada et al., 2011; Rinaldi et al., 2012; Díaz et al., 2013; da Silva et al., 2014; Abe and Matsubara, 2015
C. varanii	African fat-tailed gecko (*Hemitheconyx caudicinctus*), Leopard gecko (*Eublepharis macularius*), Boa constrictor (*Boa constrictor*), Cornsnake (*Elaphe guttata guttata*), Leopard gecko (*Eublepharis macularius*), Desert monitor (*Varanus griseus*), Gecko (*Gekkoninae* sp.), Green iguana (*Iguana iguana*), *Lampropeltis* sp; Louisiana pine snake (*Pituophis ruthveni*), Plated lizard (*Gerrhosaurus* sp.), Schneider's Skink (*Eumeces schneideri*), Taipan (*Oxyuranus scutellatus*), Baron's green racer (*Philodryas baroni*), Yellow anaconda (*Eunectes notaeus*), Cornsnake (*Elaphe guttata guttata*), Mato Grosso lancehead (*Bothrops matogrossensis*)	Intestine and Cloaca	Koudela and Modry, 1998; Morgan et al., 1999b; Hajdusek et al., 2004; Xiao et al., 2004b; Plutzer and Karanis, 2007; Pedraza-Díaz et al., 2009; Richter et al., 2011; da Silva et al., 2014; Abe and Matsubara, 2015
Lizard genotype/*C. serpentis*-like	Leopard gecko (*Eublepharis macularius*), Cornsnake (*Pantherophis guttatus*), Chinese wonder gecko (*Teratoscincus scincus*)	–	Xiao et al., 2004b; Richter et al., 2011, Abe and Matsubara, 2015
Tortoise genotype I	Indian star tortoises (*Geochelone elegans*), Herman's tortoise (*Testudo hermanii*), Ball python (*Python regius*), Russian tortoise (*Agrionemys [Testudo] horsfieldii*)	Stomach	Xiao et al., 2002b, 2004b, Alves et al., 2005; Pedraza-Díaz et al., 2009; Griffin et al., 2010; Richter et al., 2012
Tortoise genotype II (*C. duismarci*)	Marginated tortoise (*Testudo marginata*), Ball python (*Python regius*), Veiled chameleon (*Chamaeleo calyptratus*), Pancake tortoise (*Malacochersus tornieri*), Russian tortoise (*Agrionemys [Testudo] horsfieldii*)	Intestine	Traversa et al., 2008; Pedraza-Díaz et al., 2009; Griffin et al., 2010; Traversa, 2010; Richter et al., 2012
Snake genotype I	New Guinea Viper boa (*Candoia asper*), Japanese grass snakes (*Rhabdophis tigris*)	–	Xiao et al., 2002b; Kuroki et al., 2008
Snake genotype II	Boa constrictor (*Boa constrictor ortoni*)	–	Xiao et al., 2004b

that wildlife-associated *Cryptosporidium* is an increasing concern for cryptosporidiosis in humans.

During the last 100 years in many countries of the world, there have been dramatic changes in natural/rural landscapes due to urbanization (Mackenstedt et al., 2015). Although urbanization is one of the leading causes of species extinction (McKinney, 2006), for adaptable species, urban and periurban areas can be very attractive due to increased food and water resources (waste food, pet food, garden produce, water tanks etc) (Mackenstedt et al., 2015). In these environments, wildlife species may reach far higher population densities than in more natural or rural landscapes (Bradley and Altizer, 2007), potentially increasing the faecal–oral transmission of oocysts between wildlife and humans and contamination of drinking water catchments.

Shifting boundaries between wildlife and humans have been responsible for the emergence of species like *C. ubiquitum* and chipmunk genotype I in human populations. For example, squirrels host *C. ubiquitum*, chipmunk genotype I, the skunk genotype and other *Cryptosporidium* genotypes associated with human disease (Feng et al., 2007; Kváč et al., 2008; Ziegler et al., 2007; Stenger et al., 2015b), and because they frequently share habitats with humans they may be a significant reservoir of human infection. Squirrels can reach relatively high densities in suitable habitats, resulting in high rates of environmental loading of *Cryptosporidium* oocysts (Atwill et al., 2001). For example, California ground squirrels can reach densities as high as 92 adults hectare^{-1} (Owings et al., 1977; Boellstorff and Owings, 1995), which when combined with shedding of up to 2×10^5 oocysts animal^{-1} day^{-1} results in rates of environmental loading equivalent to 1×10^7 oocysts hectare^{-1} day^{-1} (Atwill et al., 2004). Further analysis of squirrel populations however has suggested that most tree squirrels host zoonotic species and genotypes while ground squirrels host species and genotypes that are tribe-specific and unlikely to cause human disease, despite overlapping ranges (Stenger et al., 2015b). This highlights the importance of extensive molecular epidemiological studies of wildlife to better understand the public health risks.

While urban-environment-induced increases in wildlife

population densities are conducive to elevated rates of *Cryptosporidium* transmission, the host specificity of some wildlife species and genotypes may limit the potential for spillover of wildlife genotypes to sympatric populations of humans. For example, in Australia, the common brushtail possum is one of the most abundant native marsupials in urban environments, having successfully adapted to utilise anthropogenic resources (Hill et al., 2008). A higher *Cryptosporidium* prevalence in urban compared to woodland possum populations (11.3 versus 5.6%) has been reported, but the majority of possums sampled shed low numbers of host adapted (possum genotype) oocysts (1 to 10^2) (Hill et al., 2008). However, the finding a *C. fayeri* clinical infection in a human, which had previously been thought to be a host-adapted species (Waldron et al., 2010), highlights our lack of knowledge about the human infectious potential of many species and genotypes of *Cryptosporidium* infecting wildlife.

5. Perspectives for the water industry

Management of *Cryptosporidium* public health risks for the drinking water industry requires the implementation of a holistic approach including research, monitoring *Cryptosporidium* oocysts in animals and source water and catchment management (e.g., access protection, vegetation cover, etc). As watersheds are vulnerable to contamination with both zoonotic and non-zoonotic species from wildlife, sensitive detection of *Cryptosporidium* oocysts in water and correct identification of oocysts to the species/genotype level are essential for source water management and risk assessment (Li et al., 2015b). The routine practice of assessing *Cryptosporidium* contamination of catchments and drinking water supplies using total oocyst counts based on the U.S. Environmental Protection Agency (EPA) Method 1622/1623, cannot differentiate *Cryptosporidium* species and cannot reliably access viability (infectivity). This microscopy-based method, therefore overestimates the human health risk, as wildlife in catchments frequently carry non-zoonotic genotypes and species and not all oocysts are viable.

The introduction of molecular identification techniques has therefore been an important advance for water management and quantification of the risk to drinking water supplies from *Cryptosporidium*-infected wildlife (Nolan et al., 2013; Zahedi et al., 2015). Identification of *Cryptosporidium* to the species/genotype level is especially challenging for environmental (faecal and water) samples because of the usual presence of very low numbers of oocysts and high concentrations of PCR inhibitors and non-target organisms (Li et al., 2015b). It is essential however, for the assessment of the public health importance of *Cryptosporidium* oocysts from wildlife. Recently, the use of fluorescence resonance energy transfer (FRET) probes combined with melt curve analysis has been used for rapid and sensitive differentiation of zoonotic from non-zoonotic species in water samples (Li et al., 2015b). Another study of a drinking water supply in Australia, found no *C. hominis* in any water sample tested, but *Cryptosporidium* genotypes associated with native and non-native wildlife made up 70% of all isolates typed (Swaffer et al., 2014). Similarly, Ruecker et al. (2012) reported that non-zoonotic wildlife species and genotypes of *Cryptosporidium* accounted for 64.3% of *Cryptosporidium* identified in environmental water samples in Canada and that only 7.2% of human-infectious species were detected. A low prevalence of *C. hominis* and *C. parvum* was also reported by Nolan et al. (2013) in Melbourne catchments, who detected *C. hominis* and *C. parvum* in only 0.6% of samples, despite screening >2000 animal faecal samples. However, the human-infectious potential of many wildlife-adapted *Cryptosporidium* is currently unknown and the UK outbreak caused by *C. cuniculus* should act as a caution against assuming these unusual species and genotypes are not significant (Chalmers et al., 2009; Robinson et al., 2011).

Accurate, quantitative identification of *Cryptosporidium* in wildlife excreta is an essential starting point for estimating catchment loads (Davies et al., 2003). Quantitative PCR (qPCR) (real-time PCR) therefore represents an invaluable tool that enables rapid, high-throughput and cost-effective detection and quantitation of *Cryptosporidium* oocysts and is increasingly being used to monitor oocyst shedding by animals in catchments (Yang et al., 2014a). Due to the intrinsic constraints of qPCR however, standards of known concentration are required to generate calibration curves used to estimate the concentration of pathogens in a sample (Hindson et al., 2013; Rački et al., 2014). Therefore the quantification of the target molecules in the unknown sample is only as good as that of the standards used. Droplet digital PCR (ddPCR) (Hindson et al., 2013) is the third-generation implementation of conventional PCR that facilitates the quantitation of nucleic acid targets without the need for calibration curves (Vogelstein and Kinzler, 1999). A recent study compared ddPCR with qPCR for the quantitative detection of *Cryptosporidium* DNA in animal and human faecal samples (Yang et al., 2014b) and revealed that ddPCR appeared to be less sensitive to inhibitors than qPCR and that inaccurate calibration of qPCR standards resulted in qPCR overestimating the numbers of oocysts present (Yang et al., 2014b). This has important implications for catchment risk management. However, qPCR is cheaper and provides better throughput and therefore using ddPCR to precisely quantify qPCR standards would be one way to combine the advantages of the two technologies and provide more accurate assessment of *Cryptosporidium* catchments loads from wildlife faecal samples.

Besides quantitative considerations, measuring the infectivity is also important for adjusting the risk profile of oocysts from wildlife in source waters (Swaffer et al., 2014). For example, a recent study has shown that the infectivity fraction of oocysts within source water samples in South Australian catchments was low (~3.1%), which provided a much more accurate water quality risk assessment (Swaffer et al., 2014). This low infectivity fraction is consistent with source water infectivity reported by Di Giovanni et al. (1999) of 4.9% and Lalancette et al. (2012) of 0%. The ability to routinely measure oocyst infectivity has been hampered by a number of issues including the distribution and low numbers of oocysts, costs and reproducibility (Di Giovanni and LeChevallier, 2005; Swaffer et al., 2014). However, recent improvements in cell culture immunofluorescence assays have led to the development of a single format assay that provides information on method performance (recovery rate), oocyst number, oocyst infectivity and genotype of infectious oocysts, overcoming these obstacles (King et al., 2015). This assay should therefore enable a more comprehensive understanding of *Cryptosporidium* risk for different water sources, assisting in the selection of appropriate risk mitigation measures (King et al., 2015). It is however important to remember that the detection of non-viable oocysts in the 10–20 L of the water column that is usually sampled, does not mean that other oocysts in the water body are also non-viable.

Factors that affect the viability of *Cryptosporidium* oocyst load in faecal samples from wildlife in the catchment and water (runoffs, water column and sediments), include solar inactivation, desiccation, temperature and residence time in catchments and these dynamics should be factored into risk assessments (Hijen et al., 2006; King and Monis, 2007; Monis et al., 2014). Transport, including hydrodynamically-driven accumulation, settlemement, dispersion, dilution etc. can also affect oocyst concentrations in the water, either positively or negatively. Peak flow periods (when the maximum area of catchment is contributing to stream flow), are a major driver behind the transport of oocysts to surface water.

Appendix 1

Therefore monitoring the distribution of *Cryptosporidium* during elevated flow conditions caused by rainfall run-off is important given the demonstrated positive and significant correlation between *Cryptosporidium* concentration with flow and turbidity (Swaffer et al., 2014). Measuring the infectivity of different wildlife-derived *Cryptosporidium* species under different climactic conditions is therefore crucial for accurate risk assessment of public health implications, particularly as more extreme precipitation is predicted globally (IPCC, 2013 – www.ipcc.ch) (Ryan et al., 2014).

There are still many research gaps in our understanding of the public health significance of wildlife in drinking water catchments and taxonomic and molecular epidemiological studies on *Cryptosporidium* spp. in wildlife, especially those in watersheds are still scarce. Whole genome studies in *Cryptosporidium* species will assist with the development of gp60 and other typing tools to better access the zoonotic potential and transmission dynamics of *Cryptosporidium* in wildlife. Morphological and biological data including pathogenicity and oocyst shedding rates are not yet available for some common zoonotic *Cryptosporidium* species and genotypes in wildlife. There is also a need to confirm if molecular detection of zoonotic *Cryptosporidium* species in wildlife is commonly associated with actual infections or mechanical transmission (Ryan et al., 2014). *C. cuniculus* is the only species besides *C. hominis* and *C. parvum*, known to be associated with a waterborne outbreak of human cryptosporidiosis, yet little is known about the prevalence and oocyst shedding rates of *C. cuniculus* in rabbits.

The evolution of methods to enumerate and genotype oocysts and determine oocyst infectivity provides much-needed tools to refine the human health risk from wildlife in catchments and future studies will provide water quality managers with much more accurate and informed data for modelling and quantitative microbial risk assessments (QMRA) of wildlife in various catchments.

Conflict of interest

None.

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COMMISSIONED REVIEW ARTICLE

WILEY Parasite Immunology

Cryptosporidium in humans and animals—a one health approach to prophylaxis

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Summary

Cryptosporidium is a major cause of moderate-to-severe diarrhoea in humans worldwide, second only to rotavirus. Due to the wide host range and environmental persistence of this parasite, cryptosporidiosis can be zoonotic and associated with foodborne and waterborne outbreaks. Currently, 31 species are recognized as valid, and of these, *Cryptosporidium hominis* and *Cryptosporidium parvum* are responsible for the majority of infections in humans. The immune status of the host, both innate and adaptive immunity, has a major impact on the severity of the disease and its prognosis. Immunocompetent individuals typically experience self-limiting diarrhoea and transient gastroenteritis lasting up to 2 weeks and recover without treatment, suggesting an efficient host antiparasite immune response. Immunocompromised individuals can suffer from intractable diarrhoea, which can be fatal. Effective drug treatments and vaccines are not yet available. As a result of this, the close cooperation and interaction between veterinarians, health physicians, environmental managers and public health operators is essential to properly control this disease. This review focuses on a One Health approach to prophylaxis, including the importance of understanding transmission routes for zoonotic *Cryptosporidium* species, improved sanitation and better risk management, improved detection, diagnosis and treatment and the prospect of an effective anticryptosporidial vaccine.

KEYWORDS

Cryptosporidium, diagnosis, prophylaxis, risk management, treatment, vaccines

1 | INTRODUCTION

Cryptosporidium species are protozoan parasites that infect a broad range of hosts including humans, domestic and wild animals worldwide, causing asymptomatic or mild-to-severe gastrointestinal disease in their host species.[1–6] Currently, 31 *Cryptosporidium* species have been recognized as valid, and of these, by far the most common species reported in humans worldwide are *C. parvum* and *C. hominis*.[7–12]

Human cryptosporidiosis is frequently accompanied by abdominal pain, fever, vomiting, malabsorption and diarrhoea that may sometimes be profuse and prolonged.[13,14] The immune status of the host, both innate and adaptive immunity, has a major impact on the severity of the disease and its prognosis. Immunocompetent individuals typically experience self-limiting diarrhoea and transient gastroenteritis lasting up to 2 weeks and recover without treatment, suggesting an efficient host antiparasite immune response. Immunocompromised individuals, including HIV/AIDS patients (not treated with antiretroviral therapy), often suffer from intractable diarrhoea, which can be fatal.[15] An effective vaccine for cryptosporidiosis is not yet available.

The Global Enteric Multicenter Study (GEMS) study, which was a three-year matched case–control study of moderate-to-severe diarrhoea in over 22 000 infants and children at seven sites across Africa and Asia aged 0–59 months, found that *Cryptosporidium* was second only to rotavirus as a major cause of severe diarrhoea.[16,17] More recent

matched case–control studies of diarrhoea have confirmed this.[18] Similarly, a birth cohort study conducted by a Global Network for the Study of Malnutrition and Enteric Diseases (MAL-ED) has assessed pathogen-specific burdens in diarrhoeal and nondiarrhoeal stool specimens from 2145 children aged 0–24 months, over five years at eight community sites in Africa, Asia and South America, and identified *Cryptosporidium* spp. as one of the five highest attributable burdens of diarrhoea in the first year of life.[19] Globally, cryptosporidiosis is estimated to be responsible for the majority of deaths among children under 5 years of age [20–22] and *Cryptosporidium* infection in children is also associated with malnutrition, persistent growth retardation, impaired immune response and cognitive deficits.[23,24] The mechanism by which *Cryptosporidium* affects child growth seems to be associated with inflammatory damage to the small intestine.[25] Undernutrition (particularly in children) is both a sequela of and a risk factor for cryptosporidiosis, particularly in low-income countries.[26–31] FAO's executive summary of the State of Food Insecurity in the World (http://www.fao.org/docrep/018/i3458e/i3458e.pdf) indicates there are 842 million chronically malnourished persons worldwide, which significantly contributes to impaired immunity and thus increased susceptibility to infection with *Cryptosporidium*, perpetuating the cycle of chronic diarrhoea and malnutrition. In developed countries, *Cryptosporidium* is less common [15] and accounts for ~9% of diarrhoeal episodes in children.[32]

Cryptosporidiosis is a highly prevalent and extremely widespread disease,[6] and several factors contribute to this. Infected individuals shed large numbers of oocysts, which are environmentally very robust, resistant to inactivation by commonly used drinking water disinfectants including chlorine treatment and are able to survive routine wastewater treatments.[33,34] *Cryptosporidium* oocysts are highly infectious; in human volunteer studies, as few as 10 or less *Cryptosporidium* oocysts can produce disease in healthy adults.[35,36] A quantitative risk assessment has estimated that ingestion of a single oocyst of the *C. parvum* IOWA isolate will result in clinical disease in 2.79% of immunologically normal persons.[37] Another contributing factor to the high prevalence and widespread distribution of *Cryptosporidium* is the lack of treatment options. Only one drug, nitazoxanide (NTZ, Alinia; Romark Laboratories, Tampa, FL, United States), has been approved by the US Food and Drug Administration (FDA). This drug, however, exhibits only moderate clinical efficacy in malnourished children and immunocompetent people, and none in immunocompromised individuals like people with HIV.[38,39]

Because oocysts of *Cryptosporidium* species from humans and animals are ubiquitous in the environment, cryptosporidiosis can be acquired through multiple routes (reviewed by Robertson et al.[40]). Transmission of oocysts is by the faecal–oral route, either directly or indirectly. For humans, direct transmission can be from person to person primarily due to poor hygiene among household members and attendees in day care centres, aged care facilities and other institutions, or from animals to persons such as farmworkers and pet owners. Most indirect transmission is from contaminated drinking or recreational water. Contaminated food can also be a source of transmission, and contamination can occur at every step throughout the food preparation process, from farm to table.[41,42] Findings from animal models, human case reports and a few epidemiological studies suggest that *Cryptosporidium* may also be transmitted via inhalation of aerosolized droplets or by contact with fomites contaminated by coughing (see [43]).

The "One Health" approach to tackle zoonotic diseases, defined as "One Medicine" by Schwabe[44], is a worldwide strategy to improve health and well-being through the mitigation and prevention of disease risks that originate at the interface between humans, animals and their various environments. This review focuses on a One Health approach to prophylactic prevention of cryptosporidiosis, including improved detection, diagnosis and treatment, the importance of understanding zoonotic transmission, better environmental and risk management and the prospect of an effective anticryptosporidial vaccine.

2 | DETECTION, DIAGNOSIS AND TREATMENT

Cryptosporidium presents many challenges for detection and diagnosis. The use of different diagnostic methods and the inconsistent application of typing techniques can make direct comparisons difficult or even impossible between clinical, veterinary and environmental testing or between different regions and countries.[45] Detection of *Cryptosporidium* in clinical pathology laboratories is still based mainly on microscopic detection via stains and/or fluorescent antibodies (IFA) and other antigenic detection methods. Although microscopy needs relatively simple instruments and cheap consumables, it is labour intensive, requires a skilled operator and lacks sensitivity and specificity.[45] Morphological characters for identifying *Cryptosporidium* are few,[46,47] and differential staining techniques are usually required due to the fact that oocysts are similar in size and shape to yeasts, faecal components and other debris.[47,48] Acid fast (AF)-modified Ziehl–Neelsen staining is one of the most common differential staining techniques.[45,48] However, the detection limits of conventional microscopy for *Cryptosporidium* have been reported to be as low as 50 000 to 500 000 oocysts per gram of human faeces,[49] resulting in low levels of infection or sporadic shedding possibly going unnoticed when conventional methods of detection are used. Sporadic shedding is such that studies have shown that three separate faecal samples should be examined for immunocompetent patients and two samples for patients with AIDS for confident diagnosis of cryptosporidial infections using acid-fast staining.[50] IFA stains offer superior sensitivity; in some studies, about 97% sensitivity compared with only about 75% sensitivity for acid-fast staining.[51] However, IFA is more expensive than acid-fast staining and requires a fluorescence microscope and trained staff.[51] This is particularly problematic in resource-poor areas where cryptosporidiosis is a major health problem. A recent study proposed the use of phase-contrast microscopy (PCM) as a specific and inexpensive method for detection of *Cryptosporidium*; however, this method still lacks sensitivity.[52]

Other antigen detection formats such as enzyme-linked immunosorbent assays (ELISAs), enzyme immunoassays (EIAs) and immunochromatographic (dipstick) assay for *Cryptosporidium* are also

commercially available and have the advantage of reducing assay times and being amenable to automation. However, diagnostic sensitivities are variable (70%–100%)[51,53–55]; some rapid tests have reduced specificity and sensitivity for species other than *C. parvum* or *C. hominis*,[56,57] and confirmation of positive reactions is needed.[55] Biosensor chips, that detect and quantitate *C. parvum* in real-time via anti-*C. parvum* IgM binding, have also been developed[58,59]; however, detection limits are relatively high (100 or more oocysts) and they have yet to be fully evaluated on water or faecal samples. Another major limitation of both conventional microscopy and antigen detection methods is that they cannot identify to species or subtype level, which is essential for understanding transmission dynamics and outbreaks, in particular for zoonotic species.

Polymerase chain reaction (PCR)-based techniques have permitted specific and sensitive detection and differentiation of *Cryptosporidium* spp. for clinical diagnosis and environmental monitoring.[45] Real-time or quantitative PCR (qPCR) assays have been developed to quantitate the numbers of *Cryptosporidium* oocysts present in human and animal faecal and water samples [60–63] with 100% specificity and sensitivities as low as 200 oocysts per gram of faeces, which equates to 2 oocysts per PCR.[60] Multiplex qPCR assays have also been developed for the detection of *Cryptosporidium* and other common causes of diarrhoea such as *Giardia duodenalis* and *Entamoeba histolytica*, which have the advantage of identifying mixed infections.[64–66] The most widely used molecular markers for identification and typing of *Cryptosporidium* species are the 18S ribosomal RNA (18S rRNA) gene and the 60-kDa glycoprotein (gp60) gene, respectively.[4,10] Miniaturized fluidic devices, which can detect to species level, have also been developed, mainly for the water industry (reviewed by Bridle et al.[67]), but as with antibody-based biosensor chips, have yet to be fully validated and are costly.

New drug targets for *Cryptosporidium* are urgently needed, as the only FDA-approved drug, nitazoxanide, does not provide benefit for malnourished children and immunocompromised patients with cryptosporidiosis. However, *Cryptosporidium* has completely lost the plastid-derived apicoplast present in many other apicomplexans, and the remnant mitochondrion lacks the citrate cycle and cytochrome-based respiratory chain.[68] Therefore, many classic drug targets are unavailable in *Cryptosporidium*. Progress in developing anticryptosporidial drugs has also been affected by the inability to generate large numbers of *Cryptosporidium* oocysts in vitro and an inability to genetically manipulate the organism.[69,70] The recent development of a hollow-fibre in vitro culture system to generate large numbers of oocysts (up to 10^8 oocysts per day) [71] and advances in genetically engineering *Cryptosporidium* [72] will transform the development of novel therapeutics.

To date, the best studied drug target is the bacterial-derived inosine 5′-monophosphate dehydrogenase (IMPDH) gene, as *Cryptosporidium* does not contain guanine salvage enzymes and is totally dependent on this enzyme to convert adenosine salvaged from the host into guanine nucleotides.[73–76] This coupled with the parasite's high metabolic demand for nucleotides due to the complicated life cycle of this parasite make IMPDH an important drug target.[77–85]

Other drug targets include long-chain fatty acyl-coenzyme A synthetases (LC-ACS), which are essential in fatty acid metabolism,[68] and a recent study reported good efficacy of the ACS inhibitor triacsin C against cryptosporidial infection in mice.[86] A parasite cysteine protease inhibitor was also effective in vitro and in an animal model.[87] Other studies have focused on repurposing existing drugs to overcome the prohibitive costs of de novo drug development (estimated to be between $500 million and $2 billion per compound successfully brought to market).[88] For example, several compounds from the Medicines for Malaria Venture (MMV) Open Access Malaria Box have exhibited activity against *C. parvum* [89] and drugs such as the human 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase inhibitor, itavastatin and Auranofin (Ridaura®) initially approved for the treatment of rheumatoid arthritis and have been shown to be effective against *Cryptosporidium* in vitro,[90,91] which holds promise for further in vivo testing in animals and humans.

3 | ZOONOTIC *CRYPTOSPORIDIUM* SPECIES

Due to the morphological similarity of *Cryptosporidium* oocysts from different host species, initial findings of *Cryptosporidium* infections in both domestic and wild animals were assumed to be due to *C. parvum* leading to an overestimation of the potential role of animals as reservoirs of human disease.[92] However, with the assistance of advanced molecular techniques, many of these species in wildlife particularly were identified as host-adapted genotypes.[6,10] Of the 31 *Cryptosporidium* species that have been recognized as valid, more than 20 species and genotypes have been identified in humans including *C. hominis*, *C. parvum*, *C. meleagridis*, *C. felis*, *C. canis*, *C. cuniculus*, *C. ubiquitum*, *C. viatorum*, *C. muris*, *C. suis*, *C. fayeri*, *C. andersoni*, *C. bovis*, *C. scrofarum*, *C. tyzzeri*, *C. erinacei* and *Cryptosporidium* horse, skunk and chipmunk I genotypes, with *C. hominis* and *C. parvum* most commonly reported.[4,6,10] These *Cryptosporidium* spp. infect both immunocompetent and immunocompromised persons.[6,10] Of these, *C. parvum* and *C. hominis* are by far the most common species reported in humans worldwide [4,93] and are responsible for most cryptosporidiosis outbreaks, with *C. hominis* responsible for more outbreaks than *C. parvum* in most regions.[4] Although humans are the major host species for *C. hominis*, there have been isolated reports in domestic animals and wildlife hosts including sheep, goats, cattle, a dugong, non-human primates and kangaroos[94–102] (Zahedi, A., Monis, P., Aucote, S., King, B., Paparini, A., Jian, F., Yang, R., Oskam, C Ball, A., Robertson, I., Ryan, unpublished) and in fish.[103] *Cryptosporidium parvum* is primarily a parasite of artiodactyls and humans [4] but has also been frequently reported in wildlife, including various rodents, bovids, camelids, equids, canids, non-human primates and marine mammals (see 10) and in fish.[103–106] *Cryptosporidium meleagridis*, although primarily a bird parasite, is the third most prevalent species infecting humans,[4,6,93] and in some studies, *C. meleagridis* prevalence is similar to that of *C. parvum*.[107,108] *Cryptosporidium cuniculus* (previously known as rabbit genotype) was responsible for a drinking water-associated outbreak of cryptosporidiosis in the UK [109–111] and has also been

identified in many sporadic human cases of cryptosporidiosis.[112–115] It is also the third most commonly identified *Cryptosporidium* species in patients with diarrhoea in the UK.[112] Human infections with *C. canis* and *C. felis* have been reported mainly in studies conducted in children in developing countries [6] where they are responsible for as much as 3.3% and 4.4%, respectively, of overall cryptosporidiosis cases.[116] *Cryptosporidium muris* is also considered a zoonotic species as there have been numerous reports of *C. muris* in humans and one report in human sewage.[117–129] In a recent human infectivity study, *C. muris* was examined in six healthy adults.[130] Volunteers were challenged with 10^5 *C. muris* oocysts and monitored for 6 weeks for infection and/or illness. All six patients became infected. Only two of the infected volunteers had a diarrhoeal illness (a 33% illness attack rate). Three other volunteers passed an occasional unformed stool or typically had a single soft stool per day without any accompanying gastrointestinal symptoms.[130] Like *C. muris*, *C. andersoni* is also a gastric parasite and primarily infects the abomasum of cattle and to a lesser extent, sheep and goats.[6,93] It is occasionally detected in humans (cf. 10). Two studies in China by the same research group have reported that *C. andersoni* was the most prevalent *Cryptosporidium* species detected in humans.[131,132] However, further research is required to better understand the zoonotic importance of *C. andersoni*.

Cryptosporidium ubiquitum is of public health concern because of its wide geographic distribution and broad host range.[133] It has been frequently reported from drinking source water and wastewater in various geographic locations, and is considered an emerging zoonotic pathogen as it has been identified in many human cases of cryptosporidiosis.[133] Subtyping at the gp60 locus has suggested that sheep and wild rodents are a key source of *C. ubiquitum* transmission to humans, through either direct human contact with infected animals or by contamination of drinking source water.[133] It is thought that human encroachment into wildlife territories has been responsible for the emergence of *C. ubiquitum* and other genotypes such as chipmonk genotype I and to a lesser extent, skunk and mink genotypes in human populations.[113,133–142] This highlights the importance of extensive molecular epidemiological studies of wildlife to better understand the public health risks.

4 | RISK MANAGEMENT

A key part of a One Health approach to *Cryptosporidium* prophylaxis is a better understanding of environmental, epidemiological and aetiological factors associated with cryptosporidial infections to enable more targeted risk management. The far-reaching One Health strategy aims at integrating multidisciplinary knowledge and evidence, and at coordinating the interventions, to create a global synergism catering for all aspects of health care for humans, animals and the environment (the One Health Triad).

Under an environmental perspective, the prophylaxis of waterborne cryptosporidiosis must consider optimal management (or design) of source, recycled and recreational waters. Protection of source water and swimming pools is a key element of *Cryptosporidium* prevention as contamination of drinking water and swimming pools is a major mode of transmission (see ref 33,143,144) and is often achieved by restricting the access to catchments and water bodies, while swimming pools are designed and monitored according to construction standards and guidelines. Infection prevention and management, however, can only be achieved through a deep understanding of the routes of transmission, sources of contamination (human and animal), disease prevalence in the population and the risk factors in the final host.

The link between *Cryptosporidium* in drinking water and sporadic infections is well documented[33,143,144]; however, the association between drinking water contamination and endemic cryptosporidiosis is not well established. For example, some studies reporting drinking unsafe water as a risk factor for endemic cryptosporidial infection [145,146] and others report no association.[147–149] Seasonal patterns are also thought to be associated with an increased transmission risk,[150,151] such as when recreational waters are more heavily utilized.

High-precipitation events favour the transfer and survival of oocysts in surface waters and/or groundwater.[152,153] This may result in contamination of source water and increased risk of cryptosporidiosis depending on the source of contamination.[153] Indeed, the average odds of identifying *Cryptosporidium* oocysts in fresh surface waters is 2.61 (95% CI=1.63–4.21; I^2=16%) times higher during and after extreme weather events.[154] Shifts in precipitation patterns (intensity and location) is one of the climate change predictions for the future,[155] and this will clearly impact both waterborne and foodborne transmissions of *Cryptosporidium*, and therefore, future human exposures may differ significantly from current patterns as the climate changes.[156] Hydrodynamic modelling has been shown to represent a valid and cost-effective support, for decision-making and understanding of events.[157] Quantitative microbial risk assessment (QMRA) is another widely used tool to estimate health impacts from exposure to *Cryptosporidium* and other pathogens [154] and has been applied to climate change.[158] The tool, called CC-QMRA (Climate Change Quantitative Microbial Risk Assessment), quantifies the anticipated impacts in terms of relative infection risks under climate change scenarios for *Cryptosporidium* and other pathogens and can be used to evaluate impacts of climate change on infection risks from waterborne and foodborne transmissions of *Cryptosporidium*.[159] For example, CC-QMRA can be used to evaluate the effectiveness of interventions such as upgrading wastewater and drinking water treatment and strengthening drinking water and bathing water regulations.

Quantification and identification of *Cryptosporidium* in wildlife excreta is an essential starting point for estimating catchment loads.[160] Variables such as soil physicochemical properties, hydrology, orography and meteorology can all affect oocyst viability, transport and fate. Source water contamination can be avoided or reduced by the implementation of management strategies such as wildlife population control, revegetation, landscaping and soil conditioning. In addition, effective risk management cannot overlook the prevalence, infectivity and zoonotic potential of *Cryptosporidium* isolates in the animal populations within the catchment. Similarly, recreational waters such as swimming pools, sauna, spas, aquatic parks are also potential sources of outbreaks, depending on the age and health status of the users

(and maintenance). Personal hygiene practices (e.g. showering before swimming in public swimming pools, washing hands before cooking, eating and after defecation and washing fruits and vegetables before consumption) are an essential part of any prevention strategy and can prevent/reduce disease transmission.[161–164] Enforcing and encouraging similar practices, however, become absolutely crucial during outbreaks and in the presence of hypersusceptible final hosts.

It has been shown that an important host risk factor includes HIV status. *Cryptosporidium* is an important pathogen regardless of HIV prevalence[16]; however, HIV-positive children are between 3 and 18 times more likely to have *Cryptosporidium* than those who were HIV negative.[165–167] With the widespread availability of antiretroviral therapy, particularly in industrialized countries, the incidence of cryptosporidiosis has decreased among people living with AIDS.[168] However, the increasing number of transplant recipients and those receiving immunosuppressive drugs may contribute significantly to the burden in the future.[169,170] Malnutrition is also a risk factor for both diarrhoea and prolonged diarrhoea caused by *Cryptosporidium*, with significantly higher rates of infection in malnourished children controlling for HIV status.[171–174] An unknown number of individuals experience asymptomatic *Cryptosporidium* infection.[175] This clinically silent infection may remain undetected and untreated and therefore may contribute not only to parasite transmission but also to malnutrition and the associated clinical sequelae. Breastfeeding may provide some protection, as a recent study of Bangladeshi infants reported that protection from *Cryptosporidium* infection was associated with high anti-*Cryptosporidium* IgA in breastmilk.[176]

5 | VACCINES

The development of vaccines for cryptosporidiosis, particularly in vulnerable populations such as children and malnourished populations, is urgent, but has been hampered by an incomplete understanding of the host immune response to *Cryptosporidium*.[177,178] Therefore, a better understanding of host–parasite interactions is crucial for the development of an effective vaccine.[177] Given that adults in highly endemic areas are partly immune to reinfection, and human challenge studies have shown that previous infection or exposure leads to a higher infectious dose [ID_{50}],[179,180] development of a successful vaccine should be possible. It is known that both innate and adaptive host response are important in the control of *Cryptosporidium* infection.[181–183] Yet the nature of these responses, particularly in humans, is not completely understood.[178,184]

Early mediators of innate immune protection include the thick mucus layer of the small intestine, intestinal epithelial cells and chemokines, cytokines and antimicrobial peptides secreted into the intestinal lumen and/or underlying submucosa and bloodstream.[178] Important cytokines include γ-interferon (IFN-γ), which is secreted early in infection by natural killer (NK) cells, macrophages and dendritic cells, which are thought to play a major role in orchestrating both the innate and adaptive immune responses.[178,182] Th1 inflammatory response and cytokines, such as interleukin 12, 15 and 18, are also important in the resistance and recovery to *Cryptosporidium* infection.[185–190] Treatment of both immunocompetent and immunodeficient mice with IL-12 before infection prevented or greatly reduced the severity of infection and was attributed to a decrease in IFN-γ reduction.[190] Data suggest that IL-15 has an important role in activating an NK cell-mediated pathway that leads to the elimination of *Cryptosporidium* from the intestine.[186] IL-18 is produced by epithelial cells in the gut and a number of different immune cells and is upregulated in response to *C. parvum* infection, and it has been proposed that one of the functions of IL-18 is to promote IFN-γ expression by macrophages.[187] Toll-like receptors expressed by epithelial cells have been shown to be important in modulation of the host immune response and subsequent parasite clearance.[191–197]

MicroRNA (miRNA) regulation also appears to play an important role in host cell protection against *Cryptosporidium*.[198–203] miRNA are small RNA molecules of 23 nucleotides that result in gene silencing via translational suppression or mRNA degradation and are a mechanism to fine-tune cellular responses to the environment, and may be regulators of host antimicrobial immune responses.[201] More than 700 miRNAs have been identified in humans and are postulated to control 20%–30% of human genes. miRNA-mediated post-transcriptional gene regulation may regulate expression of genes critical to epithelial antimicrobial defence, and one cellular miRNA (*let-7i*) has been shown to target Toll-like receptor 4 (TLR4) and regulate TLR4-mediated anti-*C. parvum* defence.[198] Functional manipulation of select miRNA expression levels in epithelial cells has been shown to alter *C. parvum* infection burden in vitro.[201,202] The intercellular adhesion molecule-1 (ICAM-1; CD54) is a 90-kDa member of the Ig superfamily expressed by several cell types including endothelial cells and epithelial cells and is thought to facilitate adhesion and recognition of lymphocytes at infection sites as ICAM-1 is constitutively present on endothelial cells and epithelial cells, but its expression is increased by pro-inflammatory cytokines or following microbe infection. Evidence has shown that miR-221-mediated translational suppression controls ICAM-1 expression through targeting the ICAM-1 3′-untranslated region (UTR), in epithelial cells in response to *C. parvum* infection, as transfection of an miR-221 precursor in an vitro model of human biliary cryptosporidiosis abolished *C. parvum*-stimulated ICAM-1 protein expression.[201]

Mannose-binding lectin (MBL) is an evolutionarily conserved protein, secreted by hepatocytes, that functions in human innate immunity by binding to microbial surfaces and promoting opsonophagocytosis. MBL has been shown to be important in the protection against cryptosporidiosis, as children and HIV-infected adults with mannose-binding lectin deficiency have increased susceptibility to cryptosporidiosis and more severe disease.[204–206] The genetic contribution to deficient or low serum levels of MBL results from polymorphisms in the MBL2 gene (MBL1 is a pseudogene), which create low MBL-producing MBL2 genotypes in ~5% of the world's population.[206] In one study on a cohort of preschool children from Dhaka, Bangladesh, polymorphisms in the MBL2 gene (and corresponding haplotypes) and deficient serum levels of MBL were associated with increased susceptibility to infection with *Cryptosporidium*. MBL deficiency of <500 ng/mL was associated with single and multiple symptomatic episodes of *Cryptosporidium* infection, with an OR of 7.6 for children with multiple symptomatic

infections with *Cryptosporidium*.[206] The mechanism by which MBL controls *Cryptosporidium* infection and protects children from it is still not clearly understood.

Adaptive immunity creates immunological memory after an initial response to *Cryptosporidium* and leads to an enhanced response to subsequent encounters with *Cryptosporidium*. For example, antibodies to the parasite antigen gp15 were associated with protection against reinfection.[207] The adaptive immune response to *Cryptosporidium* is characterized as a T-helper 1 (Th1) response,[188] and the importance of the adaptive immune response during *Cryptosporidium* infection is highlighted by the susceptibility of patients with AIDS to cryptosporidiosis, as well as the resolution of infection observed following CD4+ T lymphocyte cell reconstitution in patients given antiretroviral therapy.[184,208] Low absolute CD4+ T cell counts in patients with HIV/AIDS were thought to be responsible for persistent and severe cryptosporidiosis; however, research with Simian immunodeficiency virus (SIV)-infected macaques reported that persistent cryptosporidiosis was more dependent on SIV load and profound viral damage to gut lymphoid tissue and rapid depletion of mucosal CD4+ T cells during the acute phase of viral infection, than on declining circulating CD4+ T cell levels during chronic SIV infection.[209] This suggests that depletion of local CD4+ T cells may be more predictive of disease severity than absolute CD4+ T cell numbers. The importance of other T cells such as CD8+ has not been extensively studied but do appear to play a role.[178] The role of humoral immunity in protection from cryptosporidiosis is not well understood, and no clear surrogate marker of protective immunity exists (reviewed in 46, 179).

The ideal *Cryptosporidium* vaccine should provide rapid lifelong immunity in all vaccinated individuals, be broadly protective against the most common species and subtypes of *Cryptosporidium*, prevent disease transmission, and be readily accessible, stable and cheap.[177,178] Ensuring cross-reaction against the most common species infecting humans, however, will be difficult, as more than 20 *Cryptosporidium* species and genotypes can infect humans as discussed above. For example, a recent study showed that infection of gnotobiotic pigs with *C. hominis* resulted in complete protection against subsequent infection with *C. hominis*, but incomplete protection against infection with *C. parvum*[210]; therefore, multiple species will need to be targeted to provide sufficient cross-protection. In addition, as children, malnourished and immunocompromised individuals are the most important vaccine targets, and they may not be able to develop a strong and sustained immune-mediated protection in response to vaccination. Indeed, malnutrition has been cited as an important factor underlying limited efficacy of vaccines.[211] It is therefore likely that adjuvants such as TLR ligands [212] will be required to enhance the immune response in target populations.[192,195]

Several antigens, aimed at raising immunoglobulin G antibodies, are being developed as vaccine candidates.[177] Some of the best studied are *gp*15,[213–218] *cp*15 [219–224] and *cp*23.[222,225,226] The *gp*15 antigen is derived from the glycoprotein *gp*60, which is cleaved by a parasite serine proteinase into two surface proteins—*gp*15 and *gp*40, both of which play an essential role in parasite motility and attachment to and invasion of host epithelial cells,[227] and can stimulate γ-interferon production by peripheral blood mononuclear cells of those previously infected.[213] The *gp*15 antigen is relatively conserved between *C. parvum* and *C. hominis*, and studies in Bangladesh indicated that there is a significant cross-reactivity between them and that antibodies to *gp*15 were associated with shorter duration of illness.[216] Similarly, in a study in Kenya, AIDS patients without diarrhoea had significantly higher serum IgG levels to *gp*15 than those with diarrhoea.[228]

*cp*15 is an immunodominant protein present on the oocyst surface and is associated with internal structures and bears no apparent similarity to *gp*15.[227] Immunization of pregnant goats with *cp*15 vaccines protected offspring.[229] The impact of malnutrition, however, on vaccination was demonstrated in recent research on intranasal vaccination of nourished and malnourished mice, with the *cp*15 antigen primed with a live enteric bacterial vector.[224] The authors reported that malnutrition blunted antigen-specific cell-mediated responses to *cp*15 and that vaccination resulted in only transient reduction in stool shedding of *Cryptosporidium* and was not protective against disease.[224]

*cp*23 is an immunodominant protein, geographically conserved among *C. parvum* isolates, is present in both the sporozoite and merozoite stages,[177] and antibodies to it are frequently detected following *Cryptosporidium* infection.[228,230] Serum antibodies to both *gp*15 and *cp*23 are associated with protection from diarrhoea in immunocompetent adult human volunteers infected with *Cryptosporidium*.[180,207,231,232] Thus, a multivalent vaccine, incorporating multiple antigens or antigenic epitopes, may enhance protection against infection. For example, a divalent *cp*23 and *cp*15 vaccine prolonged the prepatent period and decreased oocyst shedding in mice vaccinated with the divalent vaccine compared with vaccination with *cp*23 alone.[222] Similarly, a reverse vaccinology approach based on genome mining that included three antigens; the well-characterized *cp*15, a calcium-activated apyrase involved in the invasion process of *Cryptosporidium* and profilin, an agonist of the innate immune system through its recognition by Toll-like receptors, induced specific and potent humoral and cellular immune responses in mice; however, further studies are necessary to verify the protection induced by these antigens.[223] The development of an effective vaccine against *Cryptosporidium* is still a challenge and a better understanding of which immune responses are necessary for protection is essential to the development of immune-based interventions.

6 | CONCLUSIONS

Recent developments have improved our understanding of both the genetics of and immune response in cryptosporidial infections. However, many knowledge gaps remain. Current diagnostic tests each have their limitations in cost, performance, differentiation of clinical significance and assessment of co-infections with other pathogens. Inaccessibility of diagnostic testing in nonindustrialized has meant that the knowledge of the epidemiology of *Cryptosporidium* infection in early infancy is scarce, and as a result, the burden of cryptosporidiosis is under-reported and underestimated, which reinforces ineffective clinical and public health management of *Cryptosporidium*. Rapid, cost-effective and reliable diagnostic tests therefore need to be developed for nonindustrialized countries to improve detection, reporting and interpretation of results

in the setting of multiple infections. With the recent improvements in cell culture and genetic manipulation, identification of novel or repurposed therapeutics should be radically transformed. Vaccines have the potential to reduce the significant burden of disease, but the extent and types of immunity necessary, and the methods by which to administer and induce protective immunity need further study.

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DISCLOSURES

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RESEARCH ARTICLE

Zoonotic *Cryptosporidium* Species in Animals Inhabiting Sydney Water Catchments

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Abstract

Cryptosporidium is one of the most common zoonotic waterborne parasitic diseases worldwide and represents a major public health concern of water utilities in developed nations. As animals in catchments can shed human-infectious *Cryptosporidium* oocysts, determining the potential role of animals in dissemination of zoonotic *Cryptosporidium* to drinking water sources is crucial. In the present study, a total of 952 animal faecal samples from four dominant species (kangaroos, rabbits, cattle and sheep) inhabiting Sydney's drinking water catchments were screened for the presence of *Cryptosporidium* using a quantitative PCR (qPCR) and positives sequenced at multiple loci. *Cryptosporidium* species were detected in 3.6% (21/576) of kangaroos, 7.0% (10/142) of cattle, 2.3% (3/128) of sheep and 13.2% (14/106) of rabbit samples screened. Sequence analysis of a region of the 18S rRNA locus identified *C. macropodum* and *C. hominis* in 4 and 17 isolates from kangaroos respectively, *C. hominis* and *C. parvum* in 6 and 4 isolates respectively each from cattle, *C. ubiquitum* in 3 isolates from sheep and *C. cuniculus* in 14 isolates from rabbits. All the *Cryptosporidium* species identified were zoonotic species with the exception of *C. macropodum*. Subtyping using the 5' half of *gp60* identified *C. hominis* IbA10G2 (n = 12) and IdA15G1 (n = 2) in kangaroo faecal samples; *C. hominis* IbA10G2 (n = 4) and *C. parvum* IIaA18G3R1 (n = 4) in cattle faecal samples, *C. ubiquitum* subtype XIIa (n = 1) in sheep and *C. cuniculus* VbA23 (n = 9) in rabbits. Additional analysis of a subset of samples using primers targeting conserved regions of the MIC1 gene and the 3' end of *gp60* suggests that the *C. hominis* detected in these animals represent substantial variants that failed to amplify as expected. The significance of this finding requires further investigation but might be reflective of the ability of this *C. hominis* variant to infect animals. The finding of zoonotic *Cryptosporidium* species in these animals may have important implications for the management of drinking water catchments to minimize risk to public health.

preparation of the manuscript. The specific roles of WaterNSW authors [AB] are articulated in the 'author contributions' section.

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Introduction

Cryptosporidium is one of the most prevalent waterborne parasitic infections [1] and represents a public health concern of water utilities in developed countries, including Australia. Currently, 31 *Cryptosporidium* species have been recognised based on biological and molecular characteristics including two recently described species; *C. proliferans* and *C. avium* [2, 3, 4, 5, 6]. Of these, *C. parvum* and *C. hominis* have been responsible for all waterborne outbreaks typed to date, with the exception of a single outbreak in the UK caused by *C. cuniculus* [7, 8, 9].

In Australia, marsupials, rabbits, sheep and cattle are the dominant animals inhabiting drinking water catchments and can contribute large volumes of manure to water sources [10]. Therefore, it is important to understand the potential contribution from these animals in terms of *Cryptosporidium* oocyst loads into surface water. A number of genotyping studies have been conducted on animals in Australian water catchments to date and have reported a range of species including *C. parvum*, *C. hominis*, *C. cuniculus*, *C. ubiquitum*, *C. bovis*, *C. ryanae*, *C. canis*, *C. macropodum*, *C. fayeri*, *C. xiaoi*, *C. scrofarum*, and *C. andersoni* [11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23]. To date, in humans in Australia, *C. hominis*, *C. parvum*, *C. meleagridis*, *C. fayeri*, *C. andersoni*, *C. bovis*, *C. cuniculus*, a novel *Cryptosporidium* species most closely related to *C. wrairi* and the *Cryptosporidium* mink genotype have been reported [24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42]. The aim of the present study was to use molecular tools to identify the *Cryptosporidium* sp. infecting the kangaroos, rabbits, cattle and sheep population inhabiting Sydney's drinking water catchments and so better understand the potential health risks they pose.

Materials and Methods

Sample collection and processing

Animal faecal samples were collected by WaterNSW staff from watersheds within the WaterNSW area of operations. Sampling was carried out either on land owned by WaterNSW or on private land owned by farmers who gave permission to WaterNSW staff to conduct this study on their property. To minimize cross-contamination and avoid re-sampling the same animals, animals were observed defecating and then samples were collected randomly from freshly deposited faces from the ground, using a scrapper to expose and scoop from the center of the scat pile. Samples were collected on a monthly interval over an 18 months period (July, 2013 to February, 2015) into individual 75 ml faecal collection pots, and stored at 4˚C until required (no animal was sacrificed). As faecal samples were collected from the ground and not per rectum, animal ethics approval was not required. Instead, an animal cadaver/tissue notification covering all the samples collected was supplied to the Murdoch University Animal Ethics Committee. The animal sources of the faecal samples were confirmed by watching the host defecate prior to collection and also with the aid of a scat and tracking manual published for Australian animals [43]. Faecal samples were collected from two previously identified hotspot zones from eastern grey kangaroos (*Macropus giganteus*) (n = 576), cattle (n = 142), sheep (n = 128) and rabbits (n = 106). This study did not involve collecting samples from endangered or protected animal species. Samples were shipped to Murdoch University and stored at 4˚C until required.

Enumeration of *Cryptosporidium* oocysts in faecal samples

Enumeration of *Cryptosporidium* oocysts by microscopy was conducted in duplicate for a subset of samples (n = 8) by Australian Laboratory Services (Scoresby, Vic). To quantify recovery efficiency, each individual faecal composite or homogenate was seeded with ColorSeed

(Biotechnology Frontiers Ltd. [BTF], Sydney, Australia). *Cryptosporidium* oocysts were purified from faecal samples using immunomagnetic separation (IMS) employing the Dynal GC Combo kit (Dynal, Oslo, Norway) as described by Cox et al., (2005) [44]. Oocysts were stained with Easystain and 4',6',-diamidino-2-phenylindole (DAPI; 0.8 μg.ml^{-1}) (Biotechnology Frontiers Ltd. [BTF], Sydney, Australia) and examined with an Axioskop epifluorescence microscope (Zeiss, Germany) using filter set 09 (blue light excitation) for Easystain (BTF), filter set 02 (UV light excitation) for DAPI staining, and filter set 15 (green light excitation) for ColorSeed (BTF). The identification criteria described in U.S. EPA method 1623 [45] were used for Easystain-labeled and DAPI-stained objects.

DNA isolation

Genomic DNA was extracted from 250mg of each faecal sample using a Power Soil DNA Kit (MO BIO, Carlsbad, California). A negative control (no faecal sample) was used in each extraction group.

PCR amplification of the 18S rRNA gene

All samples were screened for the presence of *Cryptosporidium* at the 18S rRNA locus using a quantitative PCR (qPCR) previously described [46, 47]. qPCR standards were *Cryptosporidium* oocysts (purified and haemocytometer counted), diluted to a concentration of 10,000 oocysts/μl. DNA was extracted from this stock using a Powersoil DNA extraction kit (MO BIO, Carlsbad, California, USA). The 10,000 oocyst/μl DNA stock was then serially diluted to create oocyst DNA concentrations equivalent to 1000, 100, 10, 1 oocysts/μl DNA respectively to be used for standard curve generation using Rotor-Gene 6.0.14 software. Absolute numbers of *Cryptosporidium* oocysts in these standards were determined using droplet digital PCR (ddPCR) at the 18S locus using the same primer set and these ddPCR calibrated standards were used for qPCR as previously described [47]. Each 10 μl PCR mixture contained 1x Go Taq PCR buffer (KAPA Biosystems), 3.75 mM $MgCl_2$, 400 μM of each dNTP, 0.5 μM 18SiF primer, 0.5 μM 18SiR primer, 0.2 μM probe and 1U/reaction Kapa DNA polymerase (KAPA Biosystems). The PCR cycling conditions consisted of one pre-melt cycle at 95˚C for 6 min and then 50 cycles of 94˚C for 20 sec and 60˚C for 90 sec.

Samples that were positive by qPCR were amplified at the 18S locus using primers which produced a 611 bp product (Table 1) as previously described [48] with minor modifications; the annealing temperature used in the present study was 57˚C for 30 sec and the number of cycles was increased from 39 to 47 cycles for both primary and secondary reactions. PCR contamination controls were used including negative controls and separation of preparation and amplification areas. A spike analysis (addition of 0.5 μL of positive control DNA into each sample) at the 18S locus by qPCR, was conducted on randomly selected negative samples from each group of DNA extractions to determine if negative results were due to PCR inhibition, by comparing the Ct of the spike and the positive control (both with same amount of DNA).

PCR amplification of the lectin (*Clec*) gene

Samples that were typed as *C. parvum*, *C. hominis* and *C. cuniculus* at the 18S locus were also typed using sequence analysis at a unique *Cryptosporidium* specific gene (*Clec*) that codes for a novel mucin-like glycoprotein that contains a C-type lectin domain [55, 56]. Hemi-nested primers were designed for this study using MacVector 12.6 (http://www.macvector.com). The external primers Lectin F1 5′ TCAACTAACGAAGGAGGGGA 3′ and Lectin R1 5′ GTGGTGT AGAATCGTGGCCT 3′ produced a fragment size of 668 bp for *C. hominis* and 656 bp for *C.*

Table 1. List of primers used in this study to amplify *Cryptosporidium* species at 18S, lectin (*Clec*), *gp60*, *lib13* and *MIC1* gene loci.

Gene	Forward Primer	Reverse Primer	Reference
18S	5′ ACCTATCAGCTTTAGACGGTAGGGTAT 3′	5′ TTCTCATAAGGTGCTGAAGGAGTAAGG 3′	[48]
	5′ ACAGGGAGGTAGTGA CAAGAAATAACA 3′	5′ AAGGAGTAAGGAACAACCTCCA 3′	
lectin (*Clec*)	5′ TCAACTAACGAAGGAGGGGA 3′	5′ GTGGTGTAGAATCGTGGCCT 3′	Present Study
	5′ CCAACATACCATCCTTTGG 3′	5′ GTGGTGTAGAATCGTGGCCT 3′	
gp60	5′ ATAGTCTCGCTGTATTC 3′	5′ GCAGAGGAACCAGCATC 3′	[49, 50]
	5′ TCCGCTGTATTCTCAGCC 3′	5′ GAGATATATCTTGGTGCG 3′	
18S	5′ TTCTAGAGCTAATACATGCG 3′	5′ CCCATTTCCTTCGAAACAGGA 3′	[51, 52]
	5′ CCCATTTCCTTCGAAACAGGA 3′	5′ CTCATAAGGTGCTGAAGGAGTA 3′	
***gp*60**	5′ ATAGTCTCCGCTGTATTC 3′	5′ GGAAGGAACGATGTATCT 3′	[52, 53]
	5′ GGAAGGGTTGTATTTATTAGATAAAG 3′	5′ GCAGAG GAA CCAGCAT 3′	
lib13	5′ TCCTTGAAATGAATATTTGTGACTCG 3′	5′ AAATGTGGTAGTTGCGGTTGAAA 3′	[54]
	Probe: VIC-CTTACTTCGTGGCGGCGT MGB-NFQ		
MIC1	5′ TGCAGCACAAACAGTAGATGTG 3′	5′ ATAAGGATCTGCCAAAGGAACA 3′	[52]
	5′ ACCGGAATTGATGAGAAATCTG 3′	5′ CATTGAAAGGTTGACCTGGAT 3′	

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parvum. The secondary reaction consisted of primers, Lectin F2 5′ CCAACATACCATCCT TTGG 3′ and Lectin R1 5′ GTGGTGTAGAATCGTGGCCT 3′ (Table 1), which produced a fragment of 518 bp for *C. hominis*, 506 bp for *C. parvum* and 498 bp for *C. cuniculus*. The cycling conditions for the primary amplification was 94˚C for 3 min, followed by 94˚C for 30 sec, 58˚C for 30 sec, 72˚C for 1 min for 40 cycles, plus 5 min at 72˚C for the final extension. The same cycling conditions were used for the secondary PCR, with the exception that the number of cycles was increased to 47 cycles. The 25 μl PCR mixture consisted of 1 μl of DNA, 1x Go Taq PCR buffer (KAPA Biosystems), 200 μM of each dNTP (Promega, Australia), 2 mM $MgCl_2$, 0.4 μM of each primer, 0.5 units of Kapa DNA polymerase (KAPA Biosystems). The specificity of this locus for *Cryptosporidium* has been previously confirmed [41]. Enumeration of *Cryptosporidium* oocysts by qPCR was conducted using a specific *C. hominis* and *C. parvum* assay targeting the *Clec* gene as previously described [41].

PCR amplification of the *gp60* gene

Samples that were typed as *C. hominis*, *C. parvum*, *C. cuniculus* and *C. ubiquitum* at the 18S locus were subtyped at the 60 kDa glycoprotein (*gp60*) locus using nested PCR as previously described (Table 1) [57, 49 50, 58].

Sequence analysis and phylogenetic analysis

The amplified DNA from secondary PCR products were separated by gel electrophoresis and purified for sequencing using an in house filter tip method [41]. Purified PCR products from all three loci, were sequenced independently using an ABI Prism™ Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, California) according to the manufacturer's instructions at 57˚C, 58˚C and 54˚C annealing temperature for the 18S rRNA, lectin and *gp60* loci, respectively. Sanger sequencing chromatogram files were imported into Geneious Pro 8.1.6 [59], edited, analysed and aligned with reference sequences from GenBank using ClustalW (http://www.clustalw.genome.jp). Distance, parsimony and maximum likelihood trees were constructed using MEGA version 7 [60].

Independent confirmation by the Australian Water Quality Centre (AWQC)

A total of eight blinded faecal samples consisting of seven *C. hominis* positives and one *Cryptosporidium* negative were sent to the Australian Water Quality Centre (AWQC) for independent analysis. DNA was extracted using a QIAamp DNA Mini extraction kit (Qiagen, Australia). Samples were screened using primers targeting the 18S rRNA locus (Xiao et al., 2000 as modified by Webber at al., 2014) [51, 52], *gp60* using producing an approx. 871 bp secondary product (Alves et al., 2003 as modified by Webber at al., 2014) [53, 52] and an approx. 400 bp primary product [50] as well as the *lib13* [54] and *MIC1* gene loci [52] as previously described (Table 1). PCRs were conducted on a RotorGene 6000 HRM (Qiagen) or LightCycler 96 (Roche) and amplification of the correct product was determined by DNA melting curve analysis [52]. Amplicons with atypical DNA melting profiles were further characterized by capillary electrophoresis using a DNA 1000 chip on a Bioanalyzer 2100 (Agilent) as per the manufacturer's instructions. The amplicons from all positive PCRs were purified using a Qiagen PCR purification kit according to the manufacturer's instructions and submitted to the Australian Genome Research Facility for DNA sequencing using BigDye3 chemistry on an Applied Biosystems AB3730xl capillary DNA sequencer. Sequences were analyzed using Geneious Pro 6.1.8 (Biomatters).

PCR amplification of open reading frames flanking *gp60* and *MIC1*

Open reading frames flanking both ends of *gp60* and *MIC1* in the *C. parvum* genome were used in BLAST searches (http://blast.ncbi.nlm.nih.gov/) to obtain homologous *C. hominis* sequences. Alignments of the *C. parvum* and *C. hominis* open reading frame pairs were constructed using Geneious Pro 6.1.8 (Biomatters). Conserved primers were designed for each alignment using the default settings and a target amplicon size of approximately 400 bp. The resulting primers (Table 2) were subjected to BLAST searches to verify specificity.

Each 25 μl qPCR reaction contained 0.5 x GoTaq PCR Buffer (Promega), 1.5 mM $MgCl_2$, 0.2 mM dNTP, 3.3 μM SYTO 9, 100 ng GP32, 0.5 μM forward primer, 0.5 μM reverse primer, 1 unit Promega GoTaq HS, and 2 μl of DNA extract. The qPCR was performed on a Light Cycler96 (Roche), and cycling conditions consisted of one pre-melt cycle at 95˚C for 6 min and then 40 cycles of 94˚C for 45 sec, 60˚C for 45 sec and 72˚C for 60 sec. High-resolution DNA melting curve analysis was conducted from 65˚C to 97˚C using an acquisition rate of 25 reads /˚C. *Blastocystis hominis* DNA was used as a negative control and nuclease free water was used as a no template control. Positive controls included *C. parvum* Iowa 2a (BTF, Sydney, Australia) and *C. hominis* IbA10G2 (kindly provided by Ika Sari). Amplicons were sized by capillary electrophoresis using a DNA 1000 chip on a Bioanalyzer 2100 (Agilent) as per the manufacturer's instructions.

Table 2. List of primers designed in the present study to amplify regions flanking the 5' and 3' ends of *MIC1* and *gp60*.

Gene	Flanking openreading frame	Forward Primer	Reverse Primer	Product size (*C. parvum* and *C. hominis*)
MIC1	cgd6_770 Chro. 60100(3' end)hypothetical proteinCDS	5′TGCGGTTGTATGACACCATCA 3′	5′TCTCTGGTGTTTGGCCTGAC 3′	511
	cgd6_810 Chro. 60105(5' end)BRCT	5′AGACACCAAGATGGAAAAGGCA 3′	5′GGGAAGACCTTTTGATATTGCCC 3′	467
gp60	cgd6_1070 Chro. 60137(3' end) conservedhypothetical protein	5′AGCAAGACCGCAACTCAAGT 3′	5′CCCATAGTGCCCAGCTTGAA 3′	430
	cgd6_1090 Chro. 60141(5' end) hsp40	5′TATTTGGAGGTGGGGCCAAG 3′	5′AAAACGGGTTTAGGGGTGGT 3′	367

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Statistical Analysis

The prevalence of *Cryptosporidium* in faecal samples collected from each host species was expressed as the percentage of samples positive by qPCR, with 95% confidence intervals calculated assuming a binomial distribution, using the software Quantitative Parasitology 3.0 [61]. Linear coefficients of determination (R^2) and Spearman's rank correlation coefficient (Spearman's rho) were used for the analysis of agreement (correlation) between oocyst numbers per gram of faeces determined by qPCR calibrated with ddPCR standards and enumeration of *Cryptosporidium* oocysts by microscopy (IMS) using SPSS 21.0 for Windows (SPSS Inc. Chicago, USA).

Results

Prevalence of *Cryptosporidium* in faecal samples collected from various hosts

The overall PCR prevalence of *Cryptosporidium* species in 952 faecal samples collected from four different host species was 5% (48/952) (Table 3). *Cryptosporidium* species were detected in 3.6% (21/576) of the kangaroo faecal samples, 7.0% (10/142) of cattle faeces, 2.3% (3/128) of sheep faeces and 13.2% (14/106) of rabbit faecal samples based on qPCR and sequence analysis of the 18S rRNA locus (Table 3).

Cryptosporidium species detected in various hosts

Sequencing of secondary PCR amplicons at the 18S rRNA locus identified four of the 21 positive isolates from kangaroo faecal samples as *C. macropodum*, while the other 17 isolates were identified as *C. hominis* (100% similarity for 550bp) (Table 4). Of the ten positives detected in cattle faecal samples, six were *C. hominis* and four were *C. parvum* (Table 4). The three sheep positive samples were identified as *C. ubiquitum* and all fourteen positives detected in rabbit faecal samples were *C. cuniculus* (Table 4).

Sequence analysis at the lectin *(Clec)* locus was consistent with 18S gene results. Eleven of 17 *C. hominis* isolates from kangaroos were successfully amplified and confirmed as *C. hominis* sequences. Eight of the 14 positives from rabbits successfully amplified at this locus and were identified as *C. cuniculus*. Four of six *C. hominis* and all four *C. parvum* isolates from cattle were also confirmed at this locus.

Table 3. Prevalence of *Cryptosporidium* species in faecal samples collected from four different host species in Sydney water catchments*. 95% confidence intervals are given in parenthesis.

Host species	Number of samples	Number of positives	Prevalence%	Species and subtype
Eastern grey kangaroo	576	21	3.6 (95% CI: 2.3–5.5)	*C. hominis* (n = 17)**,IbA10G2 (n = 12),IdA15G1 (n = 2),*C. macropodum* (n = 4)
Cattle	142	10	7 (95% CI: 3.4–12.6)	*C. hominis* (n = 6)**,IbA10G2 (n = 4),*C. parvum* (n = 4), IIaA18G3R1 (n = 4)
Sheep	128	3	2.3 (95% CI: 0.5–6.7)	*C. ubiquitum* (n = 3)**,XIIa (1)
Rabbit	106	14	13.2 (95% CI: 7.4–21.2)	*C. cuniculus* (n = 14)**,VbA 23 (n = 9)
Total	**952**	**48**	**5 (95% CI: 3.7–6.6)**	

* Based on PCR amplification and sequencing at the 18S rRNA gene, with subtyping based on DNA sequence analysis of a 400 bp amplicon from the 5' end of the *gp60* locus.

** Not all positive samples were successfully typed.

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Table 4. Species and subtypes of *Cryptosporidium* identified in faecal samples from various hosts (and their GPS co-ordinates) at the 18S and *gp60* loci.

Host species	Southing	Easting	18S locus	*gp*60 locus
Eastern grey kangaroo 1	-34.18861	150.2918	*C. hominis*	*C. hominis* IbA10G2
Eastern grey kangaroo 2	-34.203794	150.284394	*C. macropodum*	-
Eastern grey kangaroo 3	-34.20207	150.2742	*C. hominis*	*C. hominis* IbA10G2
Eastern grey kangaroo 4	-34.193631	150.273387	*C. macropodum*	-
Eastern grey kangaroo 5	-34.188607	150.291818	*C. macropodum*	-
Eastern grey kangaroo 6	-34.20458	150.2881	*C. hominis*	*C. hominis* IbA10G2
Eastern grey kangaroo 7	-34.61547	150.59756	*C. hominis*	no amplification
Eastern grey kangaroo 8	-34.23796	150.2598	*C. hominis*	*C. hominis* IbA10G2
Eastern grey kangaroo 9	N/A	N/A	*C. hominis*	*C. hominis* IbA10G2
Eastern grey kangaroo 10	N/A	N/A	*C. hominis*	*C. hominis* IbA10G2
Eastern grey kangaroo 11	N/A	N/A	*C. hominis*	*C. hominis* IbA10G2
Eastern grey kangaroo 12	N/A	N/A	*C. hominis*	*C. hominis* IbA10G2
Eastern grey kangaroo 13	-34.61686	150.68794	*C. hominis*	*C. hominis* IbA10G2
Eastern grey kangaroo 14	-34.63269	150.619	*C. hominis*	*C. hominis* IbA10G2
Eastern grey kangaroo 15	-34.63269	150.61897	*C. hominis*	no amplification
Eastern grey kangaroo 16	-34.61422	150.59331	*C. hominis*	*C. hominis* IbA15G1
Eastern grey kangaroo 17	-34.61415	150.59376	*C. hominis*	*C. hominis* IbA10G2
Eastern grey kangaroo 18	-34.61686	150.68794	*C. hominis*	no amplification
Eastern grey kangaroo 19	-31.60846	150.60819	*C. macropodum*	-
Eastern grey kangaroo 20	-34.61472	150.68475	*C. hominis*	*C. hominis* IbA10G2
Eastern grey kangaroo 21	-34.61472	150.68475	*C. hominis*	*C. hominis* IbA15G1
Cattle 1	-34.61278	150.585	*C. hominis*	no amplification
Cattle 2	-34.60429	150.60170	*C. hominis*	*C. hominis* IbA10G2
Cattle 3	-34.61283	150.58514	*C. hominis*	no amplification
Cattle 4	-34.60429	150.60170	*C. parvum*	*C. parvum* IIaA18G3R1
Cattle 5	-34.60642	150.60126	*C. parvum*	*C. parvum* IIaA18G3R1
Cattle 6	-34.61373	150.5876	*C. parvum*	*C. parvum* IIaA18G3R1
Cattle 7	-34.61373	150.5876	*C. hominis*	*C. hominis* IbA10G2
Cattle 8	-34.6195	150.5242	*C. hominis*	*C. hominis* IbA10G2
Cattle 9	-34.60429	150.60170	*C. hominis*	*C. hominis* IbA10G2
Cattle 10	-34.63269	150.619	*C. parvum*	*C. parvum* IIaA18G3R1
Sheep 1	-34.61556	150.68353	*C. ubiquitum*	no amplification
Sheep 2	-34.61556	150.68353	*C. ubiquitum*	no amplification
Sheep 3	-34.61743	150.68674	*C. ubiquitum*	*C. ubiquitum* XIIa
Rabbit 1	-34.61954	150.62169	*C. cuniculus*	no amplification
Rabbit 2	-34.61959	150.62172	*C. cuniculus*	*C. cuniculus* VbA23
Rabbit 3	-34.61937	150.62178	*C. cuniculus*	*C. cuniculus* VbA23
Rabbit 4	-34.61479	150.68492	*C. cuniculus*	*C. cuniculus* VbA23
Rabbit 5	-34.61954	150.62169	*C. cuniculus*	no amplification
Rabbit 6	-34.6195	150.52415	*C. cuniculus*	no amplification
Rabbit 7	-34.61937	150.62178	*C. cuniculus*	*C. cuniculus* VbA23
Rabbit 8	-34.61283	150.58514	*C. cuniculus*	*C. cuniculus* VbA23
Rabbit 9	-34.61556	150.68353	*C. cuniculus*	*C. cuniculus* VbA23
Rabbit 10	-34.61278	150.585	*C. cuniculus*	no amplification
Rabbit 11	-34.61479	150.68492	*C. cuniculus*	*C. cuniculus* VbA23
Rabbit 12	-34.60429	150.60170	*C. cuniculus*	*C. cuniculus* VbA23
Rabbit 13	-34.18951	150.2885	*C. cuniculus*	no amplification
Rabbit 14	-34.6327	150.619	*C. cuniculus*	*C. cuniculus* VbA23

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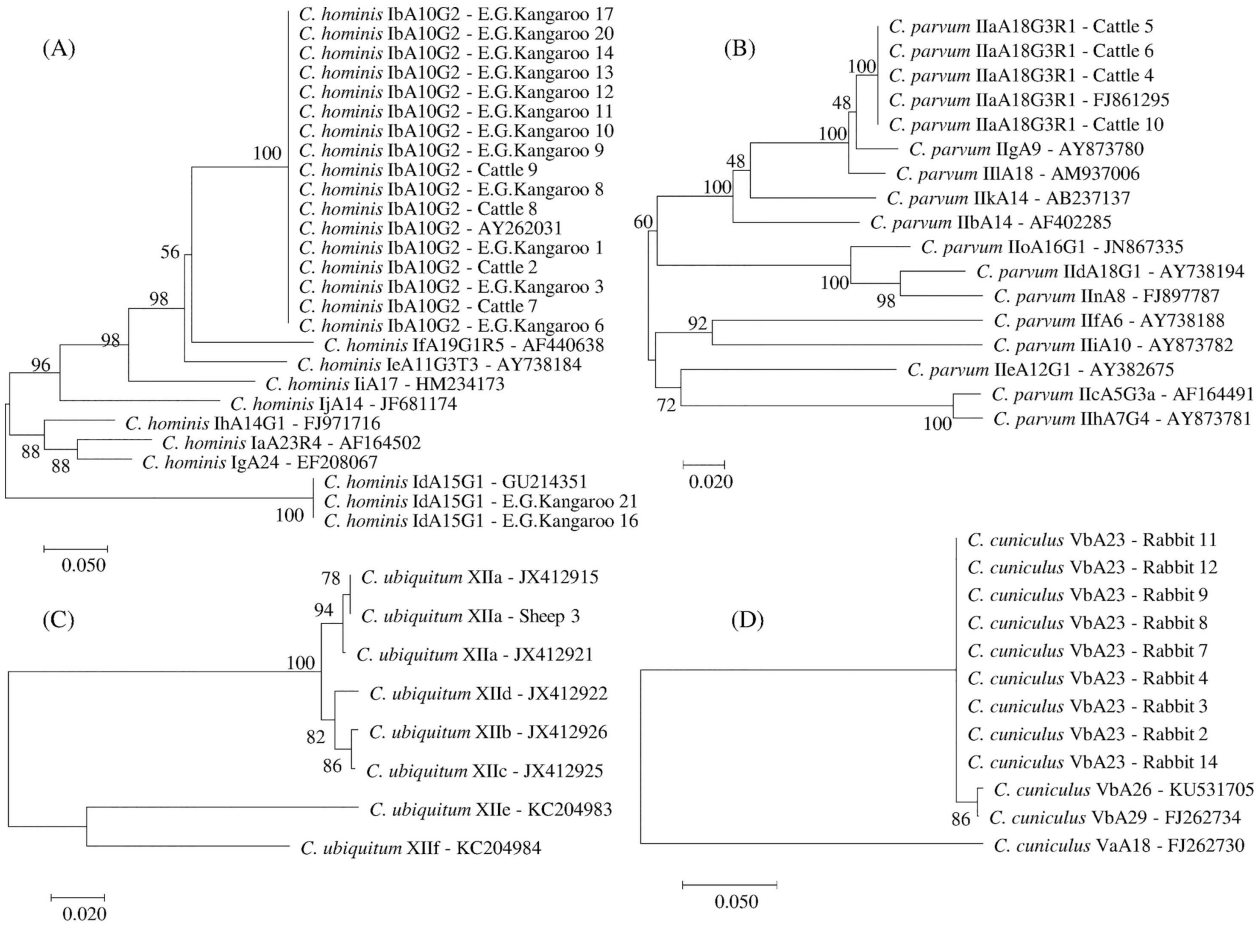

Fig 1. Phylogenetic relationships of *Cryptosporidium* subtypes inferred from Neighbor-Joining (NJ) analysis of Kimura's distances calculated from pair-wise comparisons of *gp60* sequences. (A) Relationships among *C. hominis* subtypes. (B) Relationships among *C. parvum* subtypes. (C) Relationships between *C. ubiquitum* subtypes. (D) Relationships between *C. cuniculus* subtypes. Percentage support (>50%) from 1000 pseudoreplicates from NJ analyses is indicated at the left of the supported node.

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Sequences at the *gp60* locus were obtained for 14 kangaroo and four cattle isolates that were typed as *C. hominis* at the 18S rRNA locus. These samples failed to amplify at *gp60* using the primers of Strong et al., (2000) or Alves et al., (2003) [57, 53], which amplify an approx. 832 bp fragment, but were successfully amplified using the nested primers by Zhou et al., (2003) [53], which amplify a 400 bp product. In approx. 50% of samples, the primary reaction did not produce a visible band by gel electrophoresis but a band of the correct size was visible for the secondary PCR, which was then confirmed by sequencing.

The *C. hominis* subtypes IbA10G2 and IdA15G1 were identified in 12 and 2 kangaroo samples respectively and the IbA10G2 subtype was also identified in four cattle samples (Table 4 and Fig 1A). The four *C. parvum* isolates from cattle were identified as subtype IIaA18G3R1 and the *C. cuniculus* isolates were subtyped as VbA23 (n = 9) (Table 4 and Fig 1B and 1D). Of the three *C. ubiquitum* positive isolates at 18S locus, only one isolate was successfully subtyped and identified as *C. ubiquitum* subtype XIIa (Table 4 and Fig 1C). Nucleotide sequences reported in this paper are available in the GenBank database under accession numbers; KX375346, KX375347, KX375348, KX375349, KX375350, KX375351, KX375352, KX375353, KX375354, KX375355.

Independent confirmation by the Australian Water Quality Centre (AWQC)

Blind independent analysis conducted by AWQC using the 18S rRNA nested PCR of Xiao et al., (2000) [51] identified *C. hominis* in six samples, corresponding with the six positive samples from kangaroos, and failed to detect *Cryptosporidium* in the other two samples, one of which corresponded with the negative sample. Amplification of a region of *gp60* using the protocol described by Alves et al. [53] failed to produce an amplicon for either the primary or secondary reactions. Amplification of *gp60* using the protocol described by Zhou et al., (2003) [50], failed to amplify the correct-sized product for the primary PCR but produced amplicons of the correct size for the secondary PCR for the six positive samples, which when sequenced were confirmed as *C. hominis* subtype IbA10G2. Amplification at the *lib13* locus was also successful for the six positive samples, which were confirmed as *C. hominis*. Amplification at the *MIC1* locus failed to produce any amplicons. The *gp60* and *MIC1* amplification failures were further investigated using PCR assays designed to target open reading frames (ORFs) flanking these two loci. All four primer sets produced strong amplification of the correctly sized fragments for the *C. parvum* and *C. hominis* control DNA. The cgd6-1070 ORF (located downstream of *gp60* in *C. parvum*), and cgd6-810 (upstream of *MIC1*), both amplified from four of the six samples identified as *C. hominis*. In the case of the other 2 ORFs, weak amplification was observed for one sample for cgd6-1090 (upstream of *gp60*) and for two samples for cgd6-770 (downstream of *MIC1*). While only single bands were observed for the *C. parvum* and *C. hominis* controls, most of the faecal sample extracts produced multiple bands.

Enumeration of *Cryptosporidium* oocysts in faecal samples

Oocyst numbers per gram of faeces for all PCR positive samples were determined using qPCR at the *Clec* locus for 18 *C. hominis* and 4 *C. parvum* positives and for a subset of samples (n = 8) using microscopy (Table 5). For the 8 samples for which both microscopy and qPCR data were available, there was poor correlation between the two methods ($R^2 \approx 0.0095$ and ρ (rho) = 0.2026) (Table 5). Based on qPCR, the highest numbers of oocysts was detected in Eastern grey kangaroo isolate 12 (16,890 oocysts/g^{-1}), which was identified as *C. hominis* subtype IbA10G2. No oocysts ($<2g^{-1}$) were detected by microscopy in this sample.

Discussion

The present study described the prevalence and molecular characterization of *Cryptosporidium* species in faecal samples collected from kangaroo, cattle, sheep and rabbit faecal samples from Sydney's drinking water catchments. The overall prevalence of *Cryptosporidium* species in the faecal samples collected from four animal hosts was 5% and was 3.6% in kangaroos, 7% in cattle, 2.3% in sheep and 13.2% in rabbits. Overall, the prevalence of infection with *Cryptosporidium* was generally lower than that reported previously in Sydney catchments; 25.8% [44] 6.7% [62] and 8.5% [16] and Western Australian catchments; 6.7% [13]. In the study by Ng et al., (2011b) [16], the prevalence in eastern grey kangaroos was much higher (16.9%–27/160) than the 3.6% prevalence in kangaroo faecal samples in the present study. The overall prevalence of *Cryptosporidium* species in faecal samples collected from different species in the present study was similar to the 2.8% (56/2,009) prevalence identified in faecal samples from animals in Melbourne water catchments [20]. The lower prevalence in the present study and the Melbourne study may be a consequence of testing a greater numbers of samples, seasonal and/or yearly variation in prevalence and/or proximity to agricultural land.

Table 5. *Cryptosporidium* oocyst numbers in positive samples per gram of faeces (g^{-1}) determined using microscopy and qPCR. Note: microscopy data was only available for 12 samples.

Host species	*Cryptosporidium* species (18S)	Oocyst numbers/g^{-1} microscopy	% Oocyst recovery	Oocyst numbers/g^{-1} qPCR
Eastern grey kangaroo 1	*C. hominis*	210	54	11,337
Eastern grey kangaroo 3	*C. hominis*	11,076	78	5,458
Eastern grey kangaroo 6	*C. hominis*	<2	61	9,528
Eastern grey kangaroo 8	*C. hominis*	<2	45	262
Eastern grey kangaroo 9	*C. hominis*	<2	74	648
Eastern grey kangaroo 10	*C. hominis*	<2	51	8,735
Eastern grey kangaroo 11	*C. hominis*	<2	67	131
Eastern grey kangaroo 12	*C. hominis*	<2	60	16,890
Eastern grey kangaroo 13	*C. hominis*	-	-	26
Eastern grey kangaroo 14	*C. hominis*	-	-	5,458
Eastern grey kangaroo 16	*C. hominis*	-	-	7,570
Eastern grey kangaroo 17	*C. hominis*	-	-	9,626
Eastern grey kangaroo 20	*C. hominis*	-	-	8,735
Eastern grey kangaroo 21	*C. hominis*	-	-	173
Cattle 2	*C. hominis*	-	-	144
Cattle 4	*C. parvum*	-	-	936
Cattle 5	*C. parvum*	-	-	1,819
Cattle 6	*C. parvum*	-	-	2,197
Cattle 7	*C. hominis*	-	-	4,205
Cattle 8	*C. hominis*	-	-	10,827
Cattle 9	*C. hominis*	-	-	15,804
Cattle 10	*C. parvum*	-	-	1,190

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Based on sequence analysis using the 18S rRNA locus, a total of five *Cryptosporidium* species were identified; *C. macropodum* (n = 4), *C. hominis* (n = 23), *C. parvum* (n = 4), *C. ubiquitum* (n = 3) and *C. cuniculus* (n = 14). The prospect of livestock and wildlife being reservoirs for *C. hominis* has human-health implications, so to verify this finding, a subset of faecal samples was subjected to blinded independent analysis. This additional testing initially identified *C. hominis* following sequence analysis of a large fragment of the 18S rRNA gene amplified using the Xiao et al., (2000) [51] nested PCR. It is noteworthy that the Xiao outer 18S PCR produced a clear amplification signal (threshold cycles between 24 and 29 for positive samples), suggesting the presence of reasonable numbers of oocysts with no evidence of PCR inhibition for this relatively large amplicon (approx. 1.2 kilobases). The *lib13* Taqman assay also identified *C. hominis* in these same samples. However, amplification of *gp60* using the Alves et al., (2003) [53] nested PCR failed to amplify any *Cryptosporidium*, either as a nested PCR or by direct amplification using the inner primer set. Application of the Zhou et al., (2003) [53] outer *gp60* primers (which are equivalent to the pairing of the Alves outer forward and inner reverse primers) also appeared to be unsuccessful (only four samples produced a band close to the expected size), but the Zhou *gp60* inner PCR amplified the correctly sized amplicon, which was confirmed to be *C. hominis* IbA10G2.

The failure to amplify *gp60* using the Alves et al., (2003) and Strong et al., (2000) [57, 53] assays was unexpected, especially considering the high degree of conservation for the primer binding sites across the *C. parvum* and *C. hominis gp60* subtypes and the successful amplification of the large 18S rRNA gene fragment, which demonstrates that the DNA quantity and quality was sufficient for amplification within the first round of PCR. The lack of amplification

at other loci is unlikely to be due to PCR inhibition, as spike analysis indicated no inhibition. To investigate this further, a published PCR assay targeting the MIC1 locus from both *C. parvum* and *C. hominis* [52] was also tested and failed to amplify the expected fragment from these samples. The MIC1 gene encodes a thrombospondin-like domain-containing protein, which is secreted in sporozoites prior to host cell attachment and localized to the apical complex after microneme discharge [63]. As secreted proteins often play a critical role in determining virulence and host specificity in host-pathogen relationships, it has been hypothesized that MIC1 may play a role in the differences in host range observed between *C. parvum* and *C. hominis* [52]. Previous analysis of the CryptoDB has identified that both the *gp60* and *MIC1* loci are on chromosome 6 and in close proximity (≈60 kb) [52], and it has previously been reported that these two genes are genetically linked [64]. Given that 3 different *gp60* reverse primers appear to have failed, as well as failure of at least one of the MIC1 primers, it would require the occurrence of multiple individual single nucleotide polymorphisms for the results to be accounted for by point mutations. Alternatively, a truncation or rearrangement on chromosome 6 affecting the 3' end of *gp60* and *MIC1* could affect these PCR assays. To test for any deletions affecting these loci, PCR assays were developed targeting flanking ORFs. The PCR assays targeting two ORFs in the region between *MIC1* and *gp60* (based on the *C. parvum* chromosome 6 map) were positive for some of the samples tested, suggesting that a wholesale deletion is not the cause for the failure to amplify *MIC1* or the entire *gp60*. The other two PCR assays produced equivocal results in the samples, although they yielded strong amplification in the positive controls. The variable sample results may have been due to a combination of the low amount of *Cryptosporidium* DNA present and non-specific amplification from other DNA in the sample extracts. The latter is likely, considering that the positive controls produced a single amplicon, whereas most of the sample extracts yielded multiple fragments of different sizes.

Sequencing of chromosome 6 or the entire genome of this variant *C. hominis* is required to determine the underlying cause for the failure to amplify *MIC1* or the larger *gp60* region. Considering the role of *gp60* in host cell adhesion and the hypothesized role of MIC1 in infection, it is possible that changes or loss of key genes involved in host specificity could explain the success of this particular variant of *C. hominis* in infecting hosts other than humans. If the function of these genes has been altered to better support infection in non-human hosts, then the infectivity of this variant in humans needs to be re-evaluated.

Of the detected species, all but *C. macropodum* have been reported to cause infection in humans at varying frequencies [7, 10]. *Cryptosporidium hominis* and *C. parvum* are responsible for the majority of human infections worldwide [7, 6]. In the present study, the prevalence of the variant *C. hominis* in kangaroo and cattle faecal samples was 2.9% (95% CI: 1.7%-4.7%) and 4.2% (95% CI: 1.6%-9%) respectively, and the prevalence of *C. parvum* in cattle faecal samples was 2.8% (95% CI: 0.8%-7.1%). Both of these parasites have been linked to numerous waterborne outbreaks around the world [7, 1] and although this prevalence is relatively low, both these host species represent a risk of waterborne transmission to humans. A number of previous studies have identified *C. hominis/C. parvum*-like isolates at the 18S rRNA locus in marsupials including bandicoots (*Isoodon obesulus*), brushtail possums (*Trichosurus vulpecula*), eastern grey kangaroos (*Macropus giganteus*) and brush-tailed rock-wallabies (*Petrogale penicillata*) [65, 66, 67]. However, in those studies, despite efforts, the identification of *C. hominis/C. parvum* could not be confirmed at other loci. This may be due to low numbers of oocysts and the multi-copy nature of the 18S rRNA gene, which provides better sensitivity at this locus. Alternatively, failure to confirm identity in these other studies could be due the presence of variants with substantial differences in the diagnostic loci used, causing those PCR assays to fail. Such is the case in the present study, which for the first time has identified a novel *C.*

hominis in kangaroo faecal samples based on analysis of multiple loci (18S rRNA, *Clec*, *MIC1*, *lib13* and *gp60*).

Cryptosporidium cuniculus, the most prevalent species detected here (13.2%), has been previously identified in rabbits, humans and a kangaroo in Australia [14, 20, Sari et al., 2013 unpublished—KF279538, 21]. It was implicated in a waterborne outbreak of cryptosporidiosis in humans in England in 2008 [8, 9] and has been linked to a number of sporadic human cases across the UK [68, 69], Nigeria [70] and France [71]. *Cryptosporidium ubiquitum* was detected in three sheep samples and is a common human pathogen [7], but has not been identified in Australia in the limited typing of Australian human *Cryptosporidium* isolates that has been conducted to date [10], however it has been identified in surface waters in Australia (Monis et al., unpublished).

Subtyping at the *gp60* locus identified the *C. hominis* subtype IbA10G2 in twelve kangaroo and four cattle faecal samples. This is a dominant subtype responsible for *C. hominis*-associated outbreaks of cryptosporidiosis in the United States, Europe and Australia [7, 72, 73, 74]. *Cryptosporidium hominis* has previously been reported in cattle in New Zealand [75], Scotland [76], India [77] and Korea [78]. Subtyping at the *gp60* locus identified IbA10G2 [76, 75], and IdA15G1 [77]. It has been suggested that the IbA10G2 infects cattle naturally in particular circumstances and thus could act as a zoonotic infection source in some instances [76]. Interestingly, the studies that detected IbA10G2 in cattle, used PCR-based assays that only sequenced the 5' end of *gp60*, similar to the assay used in this study, so it is possible that these reports also represent detection of a variant *C. hominis gp60*. This is the first report of the same subtype of *C. hominis* in kangaroos and cattle in the same catchment. In two kangaroo samples, the *C. hominis* IdA15G1 subtype was identified. This is also a common *C. hominis* subtype identified in humans worldwide [28, 79, 80, 81, 74]. The source and human health significance of the novel *C. hominis* detected in kangaroo and cattle samples in the present study is currently unknown. Environmental pollution from human and domestic animal faeces such as contamination of watersheds due to anthropogenic and agricultural activities conducted in the catchment area, in particular livestock farming, could be a potential source for wildlife infections with *C. hominis*. However, further studies are required to better understand the involvement of humans and livestock in the epidemiology of zoonotic *Cryptosporidium* species in wildlife.

The *C. parvum* subtype IIaA18G3R1 was identified in four cattle samples. IIaA18G3R1 is also a common subtype in both humans and cattle worldwide and has been reported widely in both calves and humans in Australia [10]. Subtyping of the single *C. ubiquitum* isolate from sheep identified XIIa. To date six subtype families (XIIa to XIIf) have been identified in *C. ubiquitum* [58]. Of these, XIIa, XIIb, XIIc, and XIId have been found in humans and therefore XIIa is a potentially zoonotic subtype [54] The *C. cuniculus* subtype identified in the present study was VbA23. Two distinct *gp60* subtype families, designated Va and Vb have been identified in *C. cuniculus* [8]. Most cases described in humans relate to clade Va and the first waterborne outbreak was typed as VaA22 [82, 8]. Previous studies in Australia have identified subtype VbA26 from an Eastern grey kangaroo [42], subtypes VbA23R3 and VbA26R4 [14, 20], VbA22R4, VbA24R3 and VbA25R4 [20] in rabbits and subtype VbA25 [42] and VbA27 (Sari et al., 2013 unpublished—KF279538) in a human patient.

Accurate quantification of *Cryptosporidium* oocysts in animal faecal deposits on land is important for estimating catchment *Cryptosporidium* loads. In the present study, oocyst concentration (numbers per gram of faeces—g^{-1}) was also determined for 18 *C. hominis* and 4 *C. parvum* positives using qPCR and for a subset of samples (n = 8) by microscopy. qPCR quantitation was conducted at the *Clec* locus rather than the 18S rRNA locus as the former is unique to *Cryptosporidium* and therefore more specific than the available 18S rRNA qPCR assays.

There was poor correlation between qPCR and microscopy for the 8 samples for which data from both methods were available, with qPCR detecting higher numbers of oocysts than microscopy with the exception of one sample (Eastern grey kangaroo 3). Increased sensitivity of qPCR and the estimation of much higher numbers of oocysts in faecal samples by qPCR versus microscopy has been previously reported [83]. A major limitation of qPCR is that the quantitative data generated are only as accurate as the standards used. A study which compared droplet digital PCR (ddPCR) (which provides absolute quantitation without the need for calibration curves) with qPCR, reported that qPCR overestimated the oocysts counts compared to ddPCR [47]. In the present study, the discrepancy between qPCR and microscopy could be due to a number of different factors; (1) IMS for microscopy and direct DNA extraction from faeces were conducted on different subsamples of each faecal sample and therefore the numbers of oocysts present in the subsamples may differ, (2) microscopy counts intact oocysts whereas qPCR will detect not only oocysts but also sporozoites that have been released from oocysts, other lifecycle stages and any free DNA, therefore qPCR may produce higher counts than microscopy. In the present study, the mean oocysts g^{-1} for kangaroos and cattle that were positive for *C. hominis* was 6,041 (range 26–16,890) and for cattle that were positive for *C. parvum* was 1535(range 936–2,197) as determined by PCR. By microscopy, oocysts counts were available for kangaroo samples only and the mean was 5,643 (range <0.5–11,076). A previous study in WaterNSW catchments, reported mean *Cryptosporidium* oocysts g^{-1} of 40 (range 1–5,988) for adult cattle, 25 for juvenile cattle (range <1–17,467), 23 for adult sheep (range <1–152,474), 49 for juvenile sheep (range <1–641) and 54 for adult kangaroos (range <1–39,423) [84]. The age of the kangaroos and cattle sampled in the present study are unknown, but qPCR quantitation suggests that these were actual infections and not mechanical transmission. However, future studies should include oocyst purification via IMS prior to qPCR for more accurate quantitation. In addition, homogenisation of samples is important when comparing microscopy and qPCR i.e faecal slurries should be made, mixed well and aliquots of that mixture used for both microscopy and qPCR to ensure better consistency between techniques.

It is important to note that of the numbers of oocysts detected in animal faeces in catchments, only a fraction of oocysts may be infectious. For example, a recent study has shown that the infectivity fraction of oocysts within source water samples in South Australian catchments was low (~3.1%) [85]. While it would be expected that oocysts in faecal samples would have much higher infectivity than oocysts in source water, reports suggest that only 50% of oocysts in fresh faeces are infectious, and that temperature and desiccation can rapidly inactivate oocysts in faeces while solar inactivation, predation and temperature will all impact oocyst survival in water [86].

The identification of mostly zoonotic *Cryptosporidium* species in animals inhabiting Sydney catchments indicates that there is a need to diligently monitor *Cryptosporidium* in source waters. Such monitoring is also critical, given the resistance of *Cryptosporidium* oocysts to chlorine [87]. Further studies are essential to confirm the nature of the *C. hominis* variant detected in this study and to determine if it represents an infection risk for humans.

Conclusions

Of the five *Cryptosporidium* species identified in this study, four species are of public health significance. The presence of zoonotic *Cryptosporidium* species in both livestock and wildlife inhabiting drinking water catchments may have implications for management of drinking water sources. Therefore, continued identification of the sources/carriers of human pathogenic strains would be useful to more accurately assess risk.

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Next Generation Sequencing uncovers within-host differences in the genetic diversity of *Cryptosporidium gp60* subtypes

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ABSTRACT

The extent of within-host genetic diversity of parasites has implications for our understanding of the epidemiology, disease severity and evolution of parasite virulence. As with many other species, our understanding of the within-host diversity of the enteric parasite *Cryptosporidium* is changing. The present study compared Sanger and Next Generation Sequencing of glycoprotein 60 (*gp60*) amplicons from *Cryptosporidium hominis* ($n = 11$), *Cryptosporidium parvum* ($n = 22$) and *Cryptosporidium cuniculus* ($n = 8$) DNA samples from Australia and China. Sanger sequencing identified only one *gp60* subtype in each DNA sample: one *C. hominis* subtype (IbA10G2) ($n = 11$), four *C. parvum* subtypes belonging to IIa ($n = 3$) and IId ($n = 19$) and one *C. cuniculus* subtype (VbA23) ($n = 8$). Next Generation Sequencing identified the same subtypes initially identified by Sanger sequencing, but also identified additional *gp60* subtypes in *C. parvum* and *C. cuniculus* but not in *C. hominis*, DNA samples. The number of *C. parvum* and *C. cuniculus* subtypes identified by Next Generation Sequencing within individual DNA samples ranged from two to four, and both *C. parvum* IIa and IId subtype families were identified within the one host in two samples. The finding of the present study has important implications for *Cryptosporidium* transmission tracking as well as vaccine and drug studies.

1. Introduction

Humans and animals often become co-infected with different species and genotypes of the same parasite genus, resulting in within-host parasite interactions (Holmes and Price, 1986; Read and Taylor, 2001; Choisy and de Roode, 2010; Seppälä and Jokela, 2016). Importantly, the presence of co-infecting parasite species/genotypes within a host can potentially modify parasite fitness by allowing them to adapt to different selection pressures and can drive the evolution of parasite virulence and alter host susceptibility to other parasites, infection duration, disease severity, transmission risks, clinical symptoms and consequently treatment and prevention strategies (Vaumourin et al., 2015; Grinberg and Widmer, 2016; Seppälä and Jokela, 2016). Therefore within-host parasite interactions have important repercussions for human or animal health. For instance, parasite co-infections within a single host can result in gene exchange via recombination. This can drive parasite evolution by making the parasites more resistant to drugs. Interactions among co-infecting parasite species, genotypes and subtypes of the same parasite genus can also modify co-evolutionary dynamics between the host and parasites. In addition, parasite interactions can help with maintaining genetic variation in parasite traits such as infectivity and virulence which are crucial components of pathogen fitness and are important to better understand disease dynamics and the changing epidemiology of parasitic diseases (Seppälä et al., 2012; Vaumourin et al., 2015; Seppälä and Jokela, 2016).

Cryptosporidium spp are ubiquitous enteric parasites that infect a broad range of hosts including humans and animals (Xiao, 2010). They are a major contributor to moderate-to-severe diarrhoeal disease in developing countries and are second only to rotavirus as a cause of moderate-to-severe diarrhoea in children younger than 2 years (Kotloff et al., 2013). Of the 31 species currently recognised, *Cryptosporidium parvum* and *Cryptosporidium hominis* account for the majority of human infections and typed outbreaks (Xiao, 2010; Ryan et al., 2017), with the exception of *Cryptosporidium cuniculus* which was responsible for a waterborne outbreak in the UK (Puleston et al., 2014). The parasite is transmitted via the

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faecal-oral route through human to human, animal to human and animal to animal contact, and via contaminated water; therefore hosts are exposed to multiple sources of potentially genetically diverse oocysts (Xiao, 2010; Grinberg and Widmer, 2016). Once ingested, sporozoites excyst from the oocyst, invade the host cells and undergo subsequent rounds of asexual and sexual reproduction.

Currently, the only available drug for human infections (nitazoxanide - Romark Laboratories, Florida, USA), has variable efficacy (Abubakar et al., 2007; Amadi et al., 2009) and an effective vaccine has yet to be developed (Mead, 2014; Ryan et al., 2016). Halofuginone lactate (Halocur; Intervet, New Zealand) is commercially available against cryptosporidiosis in dairy calves, with variable efficacy (Trotz-Williams et al., 2011; Almawly et al., 2013). Therefore, *Cryptosporidium* control currently relies mainly on improved sanitation and understanding its transmission dynamics.

Analysis of the extent of within-host genetic diversity in *Cryptosporidium* has been hampered due to the difficulties in culturing this parasite, with clonal lineages derived from individual sporozoites unavailable (Grinberg and Widmer, 2016). Of the multilocus sequencing typing strategies employed to examine within-host genetic diversity, sequence analysis of the glycoprotein 60 (*gp60*) gene is the most common (Xiao, 2010), as it is the most polymorphic locus in the genome (Abrahamsen et al., 2004). Despite the importance of within-host genetic diversity for our understanding of cryptosporidiosis epidemiology, relatively little is known (Cama et al., 2006; Waldron and Power, 2011; Jeníková et al., 2011; Shrestha et al., 2014; Ramo et al., 2014, 2016). Most studies have relied on conventional PCR and Sanger-based genotyping methods, and automated fragment analysis, however a major limitation of these approaches is their inability to resolve complex DNA mixtures and detect low-abundance intra-isolate variants (Paparini et al., 2015; Grinberg and Widmer, 2016).

Next Generation Sequencing (NGS) of amplicons offers the advantage of massive parallelization of sequencing reactions to more effectively identify low-abundance genotypes in mixed infections. To date, only one study has examined the extent of intra-isolate diversity of *Cryptosporidium* at the *gp60* locus using NGS (Grinberg et al., 2013). In that study, NGS analysis of two *C. parvum* samples from one geographic location (New Zealand) revealed much higher levels of intra-isolate diversity compared with Sanger sequencing. In the present study, we examined intra-host genetic diversity of a much larger number of *Cryptosporidium* samples ($n = 41$) from three different species (*C. hominis*, *C. parvum* and *C. cuniculus*) and from two distinct geographic regions (Australia and China), using both NGS and conventional Sanger sequencing at the *gp60* locus to better understand the epidemiology of this important parasite. Animal faecal samples in Australia were collected from watersheds within the WaterNSW (New South Wales) area of operations and included two dairy farms and faecal samples collected from the surrounding bushland. Faecal samples from China were collected from a cattle breeding centre and two dairy farms, all located in Henan province.

2. Materials and methods

2.1. Sample collection and processing

A total of 41 DNA samples positive for *Cryptosporidium*, belonging to three *Cryptosporidium* spp. (as determined by Sanger sequencing – see Section 2.3), were analysed in the present study; *C. parvum* ($n = 22$) from cattle (*Bos taurus*), *C. hominis* ($n = 11$) from Eastern Grey kangaroos (*Macropus giganteus*), and *C. cuniculus* ($n = 8$) from rabbits (*Oryctolagus cuniculus*).

2.2. DNA isolation

Upon collection, faecal samples were stored at 4 °C until analysed. Following five cycles of freeze–thaw, genomic DNA was extracted from 250 mg of each faecal sample using a Power Soil DNA Kit (MO BIO, Carlsbad, California, USA). Extraction blanks (no faecal sample) were used in each extraction group. Purified DNA was stored at −20 °C prior to PCR. DNA extraction and post-DNA extraction procedures were performed in separate dedicated laboratories.

2.3. Sanger sequencing

All samples were initially identified to species level at the 18S locus using nested PCR amplification and Sanger sequencing of a fragment of the 18S locus as previously described (Silva et al., 2013). Samples were then subtyped at the *gp60* locus using a nested PCR to amplify an approximately 400 bp product using the primers AL3531 (5′-ATAGTCTCCGCTGTATTC-3′) and AL3533 (5′-GAGATATATCTTGGTGCG-3′) for the primary PCR, and AL3532 (5′-TCCGCTGTATTCTCAGCC-3′) and LX0029 (5′-CGAACCACATTA CAAATGAAGT-3′) for the secondary PCR (Sulaiman et al., 2005). Each 25 μl PCR mixture contained 1 μl of genomic DNA, 1× Go Taq PCR buffer (KAPA Biosystems, South Africa), 3.75 mM $MgCl_2$, 400 μM of each dNTP, 0.4 μM of forward and reverse primers and 1 U of Kapa DNA polymerase (MO BIO). The PCR cycling conditions were modified and consisted of an initial denaturation at 94 °C for 3 min and then 40 cycles of 94 °C for 45 s, 54 °C for 45 s and 72 °C for 1 min, followed by a final extension step at 72 °C for 7 min. PCR contamination controls were used including negative controls. PCR setup and DNA handling procedures were performed in separate and dedicated exclusion hoods; PCR and post-PCR procedures were performed in separate dedicated laboratories.

Gel electrophoresis was used to separate the amplified DNA fragments from the secondary PCR products at the *gp60* locus, which were subsequently purified for sequencing using an in-house filter tip method as previously described (Yang et al., 2013). Purified PCR products were sequenced independently in both directions using an ABI Prism™ Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, California, USA) according to the manufacturer's instructions and with a 54 °C annealing temperature. Sanger sequencing chromatogram files were imported into Geneious Pro 8.1.6 (Kearse et al., 2012), and the nucleotide sequences of each gene were analysed and aligned with reference sequences from GenBank using Clustal W (http://www.clustalw.genome.jp).

2.4. Next Generation Sequencing (NGS)

Partial *Cryptosporidium gp60* gene sequences were amplified for NGS on the MiSeq (Illumina) platform using the same assay described for Sanger sequencing (Sulaiman et al., 2005), with the exception that secondary PCR primers were modified to contain MiSeq adapter sequences on the 5′ end, as per standard protocols for the MiSeq platform (Illumina Demonstrated Protocol: Metagenomic Sequencing Library Preparation). PCRs were performed in 25 μl volumes containing PCR buffer (KAPA Biosystems), 2 mM $MgCl_2$, 0.01 mg of BSA (Fisher Biotech, Australia), 1 mM dNTPs (Fisher Biotech), 0.4 μM of each primer and 0.5 U of KAPA Taq DNA Polymerase (KAPA Biosystems). Primary PCRs used 2 μl of DNA as a template and secondary reactions contained 1–2 μl of the primary product as a template. All PCRs contained no-template controls and extraction reagent blank controls. All PCRs were performed with an initial denaturation at 95 °C for 5 min, fol-

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lowed by 35 cycles of 95 °C for 30 s, 50 °C for 30 s and 72 °C for 1 min, and a final extension period at 72 °C for 5 min.

From the resulting *Cryptosporidium gp60* amplicons, sequencing libraries for the MiSeq sequencing platform were produced according to Illumina recommended protocols (Illumina Demonstrated Protocol: Metagenomic Sequencing Library Preparation), with the following amendments. Briefly, purified, uniquely indexed libraries from individual DNA samples were pooled for sequencing in equimolar quantities based on the fluorescent intensity of amplicon libraries after electrophoresis through a 2% agarose gel stained with GelRed (Fisher Biotech) and visualised under UV light. Sequencing was performed on an Illumina MiSeq using 500-cycle V2 chemistry (250 paired-end reads) following the manufacturer's recommendations.

2.5. Bioinformatics analysis

Sequences were processed to retain only reads with perfect AL3532 and LX0029 primer sequences (no mismatches allowed). Primer sequences and distal bases were removed in Geneious Pro 8.1.6 (Kearse et al., 2012) and remaining reads were quality filtered using USEARCH v9.1.13 (Edgar, 2010), retaining only sequences with a <1% expected error rate (96.4% of sequences retained). Singletons, doubletons, and sequences with replicate copies less than 0.01% of the total number of unique sequences per sample (14.8% of quality filtered sequences) were discarded due to their high probability of being generated by sequencing and/or PCR error. Chimeric sequences (<0.08%) were identified and removed using USEARCH v9.1.13 (Edgar, 2010).

All remaining high quality sequences were compared, using BLAST (Altschul et al., 1990), with an in-house reference database containing 131 *Cryptosporidium gp60* sequences from all characterised *C. parvum*, *C. hominis* and *C. cuniculus* subtypes available in GenBank (Supplementary Table S1). For *C. hominis* and *C. parvum*, *gp60* subtypes were only assigned if NGS reads matched identically (100% pairwise identify and query coverage) to only one *gp60* subtype reference sequence. For *C. cuniculus*, all available reference GenBank sequences were 1–3 bp shorter at the 5′ end compared with the NGS reads obtained in the present study. To accommodate this incongruity between query and reference sequences, *C. cuniculus* subtypes were assigned only if NGS reads matched to only one *C. cuniculus gp60* subtype reference sequence with 100% pairwise identify and 99% query coverage.

3. Results

3.1. Sanger sequencing

Sanger sequencing identified one subtype per amplicon with only one *C. hominis* subtype (IbA10G2) identified in all 11 *C. hominis* DNA samples from Eastern Grey kangaroos. Four *C. parvum* subtypes were identified in ruminant-derived DNA samples from Australia and China: IIaA16G2R1 ($n = 3$), IIdA15G1 ($n = 2$), IIdA18G3R1 ($n = 2$), IIdA19G1 ($n = 15$); and only one *C. cuniculus* subtype (VbA23) was identified in all eight DNA samples positive for *C. cuniculus* from rabbits (Table 1). Both IIa and IId *C. parvum* subtype families were identified in Australian samples positive for *C. parvum*, but only the IId *C. parvum* subtype family was identified in samples from China.

3.2. NGS

From the 41 DNA samples, a total of 566,719 high quality NGS reads were obtained after initial quality filtering procedures. For the 11 *C. hominis* DNA samples, there was 100% agreement between Sanger and NGS sequencing with *C. hominis* IbA10G2, the only subtype identified in assigned reads (Table 1). For the 22 *C. parvum* samples, however, although the subtype identified by Sanger was also the main subtype identified by NGS, multiple additional subtypes, ranging from 0.4% to 31% of the total assigned reads, were identified. A total of 11 *C. parvum* subtypes were identified by NGS; IIaA14G2R1 ($n = 3$), IIaA15G2R1 ($n = 3$), IIaA16G2R1 ($n = 3$), IIaA16G3R1 ($n = 2$), IIdA14G1 ($n = 2$), IIdA15G1 ($n = 2$), IIdA17G1 ($n = 7$), IIdA18G1 ($n = 15$), IIdA18G3R1 ($n = 2$), IIdA19G1 ($n = 15$), IIdA20G1 ($n = 12$). The number of *C. parvum* subtypes identified by NGS within individual samples ranged from two to four, with both IIa and IId subtype families identified within the one host in two samples (i.e. AUSC9 and AUSC20) (Table 1).

For *C. cuniculus*, as with *C. parvum*, the subtype identified by Sanger was also identified by NGS, with multiple additional subtypes ranging from 0.4% to 6.7% of the total assigned reads identified. A total of three *C. cuniculus* subtypes were identified by NGS: VbA22 ($n = 8$); VbA23 ($n = 8$); VbA25 ($n = 8$); with all three subtypes identified within individual DNA samples.

The extremely high level of stringency used in identifying the *C. parvum*, *C. hominis*, and *C. cuniculus* subtypes resulted in a high number of unassigned reads that failed to match known reference sequences with 100% pairwise identity and 100% query cover (or 99% for *C. cuniculus*) (Table 1).

4. Discussion

In the present study, the extent of within-host diversity of *gp60* subtypes in three *Cryptosporidium* spp. (*C. hominis*, *C. parvum* and *C. cuniculus*) from two geographic locations (Australia and China) was analysed using Sanger and NGS. Sanger sequencing identified only one *gp60* subtype in each DNA sample (positive for *Cryptosporidium*); NGS identified the same subtype, but also identified additional within-host subtypes for samples positive for *C. parvum* and *C. cuniculus*, but not for *C. hominis*. The direct Sanger sequencing of PCR amplicons employed in the present study is not necessarily the best-suited method for uncovering within-host diversity. Other tools such as sequencing clones and Single Strand Conformational Polymorphism (SSCP) can also be employed to identify minor sequence variants or to determine whether products of a PCR are homogeneous or heterogeneous. All the Australian samples were sourced from water catchments in Sydney (New South Wales), and Sanger-based typing of these samples have been previously discussed (Zahedi et al., 2016). Typing of the samples from China (collected from dairy cattle farms) has also been previously reported (Wang et al., 2011, 2014).

Two *C. parvum* subtype families (IIa and IId) were identified by both Sanger and NGS in ruminant-derived DNA samples from both Australia and China, with four and 11 subtypes identified, respectively. The IIa and IId subtypes are found in both humans and ruminants and are responsible for zoonotic cryptosporidiosis (Xiao, 2010). One *C. cuniculus* subtype family (Vb) was identified by both Sanger and NGS, with one and three subtypes identified in individual samples, respectively. Two *gp60* subtype families (Va and Vb) have been previously identified in *C. cuniculus*, with most human cases of cryptosporidiosis caused by the Va subtype family, including the first waterborne outbreak of cryptosporidiosis with *C. cuniculus* in the UK which was typed as VaA22 (Puleston et al., 2014). Both Sanger and NGS identified only one *C. hominis* subtype (IbA10G2) in all 11 DNA samples from kangaroos. This is a dominant subtype responsible for the majority of *C. hominis*-associated outbreaks worldwide (Xiao, 2010) and the identification of this subtype in kangaroos has previously been independently confirmed (Zahedi et al., 2016).

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Table 1
Comparison of glycoprotein 60 (*gp60*) subtypes identified in *Cryptosporidium* spp. from Australia and China using Sanger and Next Generation Sequencing. Genotypes in bold are the main subtypes identified by Next Generation Sequencing, based on fraction of assigned reads.

Sample code	Host	Country of origin	Sanger *gp60* subtype	Number of NGS sequences	Number and (%) of NGS sequences assigned	NGS *gp60* subtypes
EGK 1	EGK	Australia	*C. hominis* IbA10G2	15,609	7,896 (50.6)	***C. hominis* IbA10G2**
EGK 2	EGK	Australia	*C. hominis* IbA10G2	13,335	6,651(49.9)	***C. hominis* IbA10G2**
EGK 3	EGK	Australia	*C. hominis* IbA10G2	7,845	4,019 (54.2)	***C. hominis* IbA10G2**
EGK 4	EGK	Australia	*C. hominis* IbA10G2	1,963	845 43.0	***C. hominis* IbA10G2**
EGK 5	EGK	Australia	*C. hominis* IbA10G2	1,840	858 (46.6)	***C. hominis* IbA10G2**
EGK 6	EGK	Australia	*C. hominis* IbA10G2	1,869	781 (41.8)	***C. hominis* IbA10G2**
EGK 8	EGK	Australia	*C. hominis* IbA10G2	2,024	900 (44.5)	***C. hominis* IbA10G2**
EGK 9	EGK	Australia	*C. hominis* IbA10G2	2,473	1,156 (46.7)	***C. hominis* IbA10G2**
EGK 10	EGK	Australia	*C. hominis* IbA10G2	12,760	6,291 (51.0)	***C. hominis* IbA10G2**
EGK 11	EGK	Australia	*C. hominis* IbA10G2	12,824	6,536 (51.5)	***C. hominis* IbA10G2**
EGK 12	EGK	Australia	*C. hominis* IbA10G2	12,927	6,663 (48.4)	***C. hominis* IbA10G2**
AUSC 9	Cattle	Australia	*C. parvum* IIdA18G3R1	10,524	4,871 (46.2)	***C. parvum* IIdA18G3R1**
					228 (2.1)	*C. parvum* IIaA16G3R1
AUSC 20	Cattle	Australia	*C. parvum* IIdA18G3R1	17,161	8,223 (47.9)	***C. parvum* IIdA18G3R1**
					399 (2.3)	*C. parvum* IIaA16G3R1
AUSC 21	Cattle	Australia	*C. parvum* IIaA16G2R1	24,600	2,670 (10.8)	***C. parvum* IIaA16G2R1**
					531 (2.1)	*C. parvum* IIaA15G2R1
					102 (0.4)	*C. parvum* IIaA14G2R1
AUSC 22	Cattle	Australia	*C. parvum* IIaA16G2R1	21,155	2,313 (10.9)	***C. parvum* IIaA16G2R1**
					512 (2.4)	*C. parvum* IIaA15G2R1
					91 (0.4)	*C. parvum* IIaA14G2R1
AUSC 24	Cattle	Australia	*C. parvum* IIaA16G2R1	21,838	2,458 (11.2)	***C. parvum* IIaA16G2R1**
					485 (2.2)	*C. parvum* IIaA15G2R1
					95 (0.4)	*C. parvum* IIaA14G2R1
AUSC 25	Rabbit	Australia	*C. cuniculus* VbA23	14,597	4,941 (33.8)	***C. cuniculus* VbA23**
					437 (2.9)	*C. cuniculus* VbA25
					61 (0.4)	*C. cuniculus* VbA22
AUSC 26	Rabbit	Australia	*C. cuniculus* VbA23	9,177	3,654 (39.8)	***C. cuniculus* VbA23**
					349 (3.8)	*C. cuniculus* VbA25
					50 (0.5)	*C. cuniculus* VbA22
AUSC 27	Rabbit	Australia	*C. cuniculus* VbA23	7,340	2,984 (40.6)	***C. cuniculus* VbA23**
					492 (6.7)	*C. cuniculus* VbA25
					58 (0.7)	*C. cuniculus* VbA22
AUSC 28	Rabbit	Australia	*C. cuniculus* VbA23	11,031	4,226 (38.3)	***C. cuniculus* VbA23**
					487 (4.41)	*C. cuniculus* VbA25
					79 (0.7)	*C. cuniculus* VbA22
AUSC 29	Rabbit	Australia	*C. cuniculus* VbA23	10,548	3,863 (36.6)	***C. cuniculus* VbA23**
					401 (3.8)	*C. cuniculus* VbA25
					71 (0.6)	*C. cuniculus* VbA22
AUSC 30	Rabbit	Australia	*C. cuniculus* VbA23	12,066	4,794 (39.7)	***C. cuniculus* VbA23**
					387 (3.2)	*C. cuniculus* VbA25
					80 (0.6)	*C. cuniculus* VbA22
AUSC 31	Rabbit	Australia	*C. cuniculus* VbA23	9,006	3,609 (40)	***C. cuniculus* VbA23**
					312 (3.4)	*C. cuniculus* VbA25
					81 (0.8)	*C. cuniculus* VbA22
AUSC 32	Rabbit	Australia	*C. cuniculus* VbA23	14,987	5,743 (38.3)	***C. cuniculus* VbA23**
					581 (3.8)	*C. cuniculus* VbA25
					156 (1)	*C. cuniculus* VbA22
ChS 1	Sheep	China	*C. parvum* IIdA15G1	15,851	7,577 (47.8)	***C. parvum* IIdA15G1**
					932 (5.8)	*C. parvum* IIdA14G1
ChG 3	Goat	China	*C. parvum* IIdA15G1	23,637	7346 (31)	***C. parvum* IIdA14G1**
					6,681 (28.2)	*C. parvum* IIdA15G1
ChC 6	Cattle	China	*C. parvum* IIdA19G1	22,857	11,169 (48.8)	***C. parvum* IIdA19G1**
					2,430 (10.7)	*C. parvum* IIdA18G1
					359 (1.5)	*C. parvum* IIdA17G1
					171 (0.7)	*C. parvum* IIdA20G1
ChC 7	Cattle	China	*C. parvum* IIdA19G1	17,491	8,103 (46.3)	***C. parvum* IIdA19G1**
					1736 (1)	*C. parvum* IIdA18G1
					271 (1.5)	*C. parvum* IIdA17G1
					130 (0.7)	*C. parvum* IIdA20G1
ChC 8	Cattle	China	*C. parvum* IIdA19G1	22,137	10,857 (49)	***C. parvum* IIdA19G1**
					2,766 (12.4)	*C. parvum* IIdA18G1
					582 (2.6)	*C. parvum* IIdA17G1
					253 (1.1)	*C. parvum* IIdA20G1
ChC 9	Cattle	China	*C. parvum* IIdA19G1	13,843	6,891 (49.7)	***C. parvum* IIdA19G1**
					1,477 (10.6)	*C. parvum* IIdA18G1
					280 (2)	*C. parvum* IIdA17G1
					113 (0.8)	*C. parvum* IIdA20G1
ChC 10	Cattle	China	*C. parvum* IIdA19G1	9,854	3,859 (39.1)	***C. parvum* IIdA19G1**
					632 (6.4)	*C. parvum* IIdA18G1
ChC 11	Cattle	China	*C. parvum* IIdA19G1	16,988	8,543 (50.2)	***C. parvum* IIdA19G1**
					2,150 (12.6)	*C. parvum* IIdA18G1

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Table 1 (*continued*)

Sample code	Host	Country of origin	Sanger *gp60* subtype	Number of NGS sequences	Number and (%) of NGS sequences assigned	NGS *gp60* subtypes
					128 (0.7)	*C. parvum* IIdA20G1
ChC 12	Cattle	China	*C. parvum* IIdA19G1	15,253	7,856 (51.5)	***C. parvum* IIdA19G1**
					1,902 (12.4)	*C. parvum* IIdA18G1
					118 (0.7)	*C. parvum* IIdA20G1
ChC 13	Cattle	China	*C. parvum* IIdA19G1	19,493	8,945 (45.8)	***C. parvum* IIdA19G1**
					2,289 (11.7)	*C. parvum* IIdA18G1
					591 (3)	*C. parvum* IIdA17G1
					176 (0.9)	*C. parvum* IIdA20G1
ChC 14	Cattle	China	*C. parvum* IIdA19G1	11,222	5,873 (52.3)	***C. parvum* IIdA19G1**
					1,238 (11)	*C. parvum* IIdA18G1
					80 (0.7)	*C. parvum* IIdA20G1
ChC 15	Cattle	China	*C. parvum* IIdA19G1	16,251	8,125 (49.9)	***C. parvum* IIdA19G1**
					1,975 (12.1)	*C. parvum* IIdA18G1
					126 (0.7)	*C. parvum* IIdA20G1
ChC 17	Cattle	China	*C. parvum* IIdA19G1	21,536	11,598 (53.8)	***C. parvum* IIdA19G1**
					2,464 (11.4)	*C. parvum* IIdA18G1
					204 (0.9)	*C. parvum* IIdA20G1
ChC 18	Cattle	China	*C. parvum* IIdA19G1	13,933	6,801 (48.8)	***C. parvum* IIdA19G1**
					1,404 (10)	*C. parvum* IIdA18G1
ChC 20	Cattle	China	*C. parvum* IIdA19G1	17,406	7,152 (41.1)	***C. parvum* IIdA19G1**
					1,294 (7.4)	*C. parvum* IIdA18G1
ChC 24	Cattle	China	*C. parvum* IIdA19G1	15,193	8,117 (51.3)	***C. parvum* IIdA19G1**
					1,885 (12.4)	*C. parvum* IIdA18G1
					347 (2.2)	*C. parvum* IIdA17G1
					125 (0.8)	*C. parvum* IIdA20G1
ChC 25	Cattle	China	*C. parvum* IIdA19G1	24,275	11,985 (49.3)	***C. parvum* IIdA19G1**
					2,694 (11.1)	*C. parvum* IIdA18G1
					598 (2.4)	*C. parvum* IIdA17G1
					213 (0.8)	*C. parvum* IIdA20G1

EGK, Eastern Grey kangaroo (*Macropus giganteus*).

The finding of multiple *gp60* subtypes (2–4) in individual hosts in the present study is consistent with the study by Grinberg et al. (2013) which identified 10 unique subtypes within a single *C. parvum* sample through NGS techniques. However, in that study, the number of identified subtypes for a single sample using NGS is much greater than that observed in our study. However, in the study by Grinberg et al. (2013), two of the four least abundant subtypes were only observed once (singletons) out of a total of 1,589 sequence types and the remaining two subtypes fell outside the percentage cut-off used in this present study. Therefore, it is likely that a more stringent analysis would have resulted in a reduced number of subtypes being identified.

There are several limitations of the present study including the possibility that assignment of multiple subtypes may have been due to NGS sequence artefacts. Error rates for Illumina Miseq sequencing have been estimated at an average of 0.9 errors per 100 bases (Loman et al., 2012; Salipante et al., 2014), which is considerably less than for other benchtop sequencers, such as the Ion Torrent (~1.5 errors per 100 bases) (Loman et al., 2012; Salipante et al., 2014). To accommodate for this potential source of error, we employed a very high stringency for assignment to subtype: 100% pairwise identity and 100% query cover (or 99% for *C. cuniculus*). This resulted in a large number of unassigned reads (Table 1). However, at the high stringency level used to assign subtypes, no sequences were equivocally assigned to multiple subtypes, which lends confidence to the data.

It is possible that PCR polymerase slippage artefacts contributed to the number of subtypes detected. However, it does not account for all the diversity. For example, co-occurrence of *C. parvum* IIa and IId in two samples cannot be explained by PCR slippage, as this would have required the occurrence of slippage by multiple trinucleotides in the TCA repeat region repeatedly across samples, which is unlikely, given the stability of imperfect repeat regions (Bacon et al., 2000; Klintschar and Wiegand, 2003). In addition, if PCR polymerase slippage was the main cause of subtype diversity identified, then it would be expected that subtype diversity would also have been seen in *C. hominis*, which was not the case.

It is possible that the lower PCR annealing temperature required to amplify *gp60* sequences for NGS (50 °C) compared with Sanger (54 °C) resulted in non-target sequences being amplified and sequenced alongside *Cryptosporidium gp60* sequences, which would contribute greatly to the number of unassigned sequences in this analysis.

Another limitation of the present study is that both Sanger and NGS were conducted using nested and not single round PCR. Nested PCR approaches have an inherent risk of contamination and have previously been shown to exhibit strong amplification biases (Park and Crowley, 2010). By involving two sequential rounds of amplification, nested PCR may not accurately represent the extent of genetic diversity initially present in the sample, because it introduces a bottleneck in the genetic variation between the first and second round. However, attempts to produce amplicons from single round PCRs were unsuccessful for most of the samples and therefore a nested PCR approach was necessary. This is a well-recognised but inherent problem of *Cryptosporidium* epidemiological analysis, as frequently *Cryptosporidium*-positive faecal samples contain very low numbers of oocysts and high levels of PCR inhibitors, which necessitates a nested PCR approach (Paparini et al., 2015). However, while nested PCR bias may reduce the number of variants detected, in the present study, multiple subtypes were successfully identified in individual samples.

While multiple *gp60* subtypes were identified within *C. parvum* and *C. cuniculus*, only one subtype was identified in DNA samples positive for *C. hominis*, which may reflect their local population structures. Little information is available for *C. cuniculus*, but available data for *C. parvum* and *C. hominis* indicates a flexible reproductive strategy with panmictic (where genetic exchange occurs at random with limited or no sub-structuring), clonal and epidemic population structures (Mallon et al., 2003; Morrison et al., 2008; Tanriverdi et al., 2008; Widmer and Sullivan, 2010; Drumo et al.,

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2012; De Waele et al., 2013; Ramo et al., 2015, 2016; Widmer et al., 2015). The relative contribution of each type of population structure appears to vary between regions and hosts, and may reflect the prevailing ecological transmission dynamics (Mallon et al., 2003; Tanriverdi et al., 2008; Widmer and Sullivan, 2010; Herges et al., 2012; Widmer et al., 2015). The finding of only one *C. hominis* subtype by both Sanger and NGS in the kangaroo-derived DNA samples may reflect a clonal population structure operating locally in kangaroo populations from the main Sydney drinking water catchment. Analysis of the population structure, however, requires analysis of multiple loci which was not conducted in the present study.

Importantly, the identification of only one *C. hominis* subtype by both Sanger and NGS in the kangaroo-derived DNA samples suggests a single, recent introduction of *C. hominis* into kangaroos, which may spill over to infect other hosts in catchments, providing a reservoir for human infection. However, further research is required on a much larger number of samples belonging to different subtypes. In addition, inferences regarding the population structure are complicated by the fact that the rate of mutation of the *gp60* gene remains unknown, multi-locus analysis is required and *Cryptosporidium* within-host genetic diversity may occur both within and between the oocysts (Grinberg et al., 2013; Grinberg and Widmer, 2016).

The extent of within-host genetic diversity at the *gp60* locus, as demonstrated by the present study, may have implications for *Cryptosporidium* vaccine and drug development. For example, vaccine research for *Cryptosporidium* has focused on proteins involved in attachment to, and invasion of, host cells (Mead 2014; Ludington and Ward, 2015). Therefore, immunisation with predominant antigens could result in vaccine failures in some regions where heterogeneous parasite populations dominate (Grinberg and Widmer, 2016). As with malaria, undetected low-level drug-resistant co-infecting species and genotypes of *Cryptosporidium* within the same host could also impact anti-*Cryptosporidium* drug discovery studies and result in unexplained chemotherapy failure (Tyagi et al., 2013; Grinberg and Widmer, 2016). These findings also have implications for our understanding of the epidemiology and transmission dynamics of *Cryptosporidium*, as previous studies have relied on Sanger sequencing, which may not reflect the extent of within-host diversity and result in incorrect assumptions regarding transmission of the parasite. More extensive studies employing NGS approaches on a wider range of samples are important to determine the extent of *Cryptosporidium* within-host genetic diversity and should be an essential prerequisite for vaccine, drug and epidemiological studies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijpara.2017.03.003.

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Appendix 5

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Cryptosporidium species and subtypes in animals inhabiting drinking water catchments in three states across Australia

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ABSTRACT

As part of long-term monitoring of *Cryptosporidium* in water catchments serving Western Australia, New South Wales (Sydney) and Queensland, Australia, we characterised *Cryptosporidium* in a total of 5774 faecal samples from 17 known host species and 7 unknown bird samples, in 11 water catchment areas over a period of 30 months (July 2013 to December 2015). All samples were initially screened for *Cryptosporidium* spp. at the 18S rRNA locus using a quantitative PCR (qPCR). Positives samples were then typed by sequence analysis of an 825 bp fragment of the 18S gene and subtyped at the glycoprotein 60 (*gp60*) locus (832 bp). The overall prevalence of *Cryptosporidium* across the various hosts sampled was 18.3% (1054/5774; 95% CI, 17.3–19.3). Of these, 873 samples produced clean Sanger sequencing chromatograms, and the remaining 181 samples, which initially produced chromatograms suggesting the presence of multiple different sequences, were re-analysed by Next- Generation Sequencing (NGS) to resolve the presence of *Cryptosporidium* and the species composition of potential mixed infections. The overall prevalence of confirmed mixed infection was 1.7% (98/5774), and in the remaining 83 samples, NGS only detected one species of *Cryptosporidium*. Of the 17 *Cryptosporidium* species and four genotypes detected (Sanger sequencing combined with NGS), 13 are capable of infecting humans; *C. parvum, C. hominis, C. ubiquitum, C. cuniculus, C. meleagridis, C. canis, C. felis, C. muris, C. suis, C. scrofarum, C. bovis, C. erinacei* and *C. fayeri*. Oocyst numbers per gram of faeces (g^{-1}) were also determined using qPCR, with medians varying from 6021–61,064 across the three states. The significant findings were the detection of *C. hominis* in cattle and kangaroo faeces and the high prevalence of *C. parvum* in cattle. In addition, two novel *C. fayeri* subtypes (IVaA11G3T1 and IVgA10G1T1R1) and one novel *C. meleagridis* subtype (IIIeA18G2R1) were identified. This is also the first report of *C. erinacei* in Australia. Future work to monitor the prevalence of *Cryptosporidium* species and subtypes in animals in these catchments is warranted.

1. Introduction

Globally, it is estimated that there are between 1.7 and 4.6 billion episodes of diarrhea every year with 2.2 million associated deaths (Keusch et al., 2016; WHO, 2017). The transmission of many gastrointestinal diseases is closely linked to water, yet the true disease burden attributable to water-associated pathogens is currently unknown, largely due to lack of adequate detection and surveillance systems (Ryan et al., 2017). This is particularly the case in countries such as Australia, that have a relatively low level of endemic diarrhoeal disease, which means that even specially designed high-quality epidemiological trials have a limited ability to detect cases of diarrhoea attributable to drinking water (Sinclair et al., 2015). Routine disease surveillance systems are even less

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sensitive and detect only a small fraction of the pathogen infections that occur in the community (O'Toole et al., 2015). For example, a national survey of gastroenteritis in Australia in 2002, suggested a ratio of about 500 community cases to one notified case (Hall et al., 2006).

Cryptosporidium is one of the most prevalent waterborne parasitic infections. From the start of the last century to 2016, there were a total of 905 reported waterborne outbreaks caused by protozoan parasites and of these *Cryptosporidium* accounted for almost 60% (524 outbreaks) (Karanis et al., 2007; Baldursson and Karanis, 2011; Efstratiou et al., 2017). The largest *Cryptosporidium* outbreak occurred in Milwaukee in 1993, which affected 403,000 individuals via contaminated drinking water (MacKenzie et al., 1995), with an estimated illness-associated cost of US$ 96.2 M and 100 deaths (Corso et al., 2003). Rates of waterborne parasitic protozoan outbreaks have been increasing due to increased and improved surveillance. Between 2010 and 2016, 381 outbreaks were reported, nearly half of which (49% −188 outbreaks), were reported in Australia and New Zealand (Efstratiou et al., 2017). However, the true level of waterborne disease in Australia is unknown.

Currently, 34 *Cryptosporidium* species are recognised (Jezkova et al., 2016; Zahedi et al., 2017), of which 17 have been reported in humans worldwide. In Australia, seven *Cryptosporidium* species (*C. hominis, C. parvum, C. meleagridis, C. cuniculus, C. fayeri, C. andersoni* and *C. bovis*) have been reported in humans (Koehler et al., 2014a, b; Zahedi et al., 2016a). However, *C. hominis* and *C. parvum* have been responsible for the majority of human infections throughout the world (Xiao, 2010; Ryan and Power, 2012) and for all waterborne outbreaks typed to date, with the exception of a single outbreak in the UK caused by *C. cuniculus* (Xiao, 2010; Puleston et al., 2014; Efstratiou et al., 2017).

The 2011 Australian Drinking Water Guidelines 2011 (ADWG) contains mostly qualitative information on treatment requirements (https://www.nhmrc.gov.au/guidelines-publications/eh52), but will soon move to a health-based target of 10^{-6} disability adjusted life years (DALYs) per person per year for *Cryptosporidium* (O'Toole et al., 2015). This allows for up to approximately 6% of diarrheal disease caused by *Cryptosporidium* to be associated with consumption of drinking water (O'Toole et al., 2015). To meet this target, data about the prevalence of human-infectious *Cryptosporidium* species in source waters and catchments is important for quantitative microbial risk assessment (QMRA) processes. However, relatively few large-scale longitudinal studies have been undertaken in Australia (e.g. Ryan et al., 2005; Ng et al., 2011a; Nolan et al., 2013; Koehler et al., 2016; Zahedi et al., 2016b) and no studies have compared catchments in different states in Australia. The aim of the present study therefore was to use molecular tools to more accurately determine the prevalence, species and oocyst load of *Cryptosporidium* in Australian water catchments across three states; New South Wales (NSW), Queensland (QLD) and Western Australia (WA).

2. Materials and methods

2.1. Catchment and sample collection in each state

To comply with the ADWG, water utilities employ a risk-based multiple barrier approach with water source/catchment management and protection, being the first barrier, and other barriers at the treatment, storage, and distribution stages of water supply systems. Some drinking water catchments have a relatively low density of development, and little significant anthropogenic activity; however, this is not always the case. With the exception of rodents, which are seldom infected with human-infectious *Cryptosporidium* species (Feng et al., 2007; Foo et al., 2007), the predominant animals in Australian catchments are marsupials (mainly kangaroos), rabbits, sheep and cattle (Ryan and Power, 2012). However, the importance of these host species varies between states and individual catchments. The most abundant hosts were selected on a per catchment basis after consultation with water utility staff from each state. The population size of livestock examined in individual catchments was known and appropriate sample sizes were estimated using Epitools (http://epitools.ausvet.com.au/content.php?page=home). Unfortunately, the size of wildlife populations in these catchments is unknown, which precluded sample size analysis.

Greater Sydney's drinking water catchments cover 16,000 km^2 of land and are managed by WaterNSW. About 30% of catchment land is national park and bushland, but over 60% of the catchments are privately owned. Two catchments were chosen in NSW (Catchment A and Catchment B), for which cattle and sheep grazing is the largest single land use, but horse studs, piggeries, dairies and poultry production are also present. Eastern grey kangaroos (*Macropus giganteus*) and rabbits (*Oryctolagus cuniculus*) are the dominant wildlife species inhabiting these catchments. More than one-quarter of catchment B (2600 km^2) has been protected from most human activities for over 70 years.

Seqwater is one of Australia's largest water businesses with the most geographically spread and diverse asset base of any capital city water authority. South-east QLD's catchments cover more than 12,000 km^2 of land but only 650 km^2 hectares of this land is owned by Seqwater. Three catchment areas were analysed; Catchment A covers an area of 67 km^2 and supplies a large portion of the Sunshine Coast's drinking water. Catchment B is located in the Gold Coast hinterland in South-east QLD and supplies bulk raw water to local irrigators and Seqwater. Catchment C is situated between Brisbane and the Sunshine Coast. Cattle and sheep are the main livestock present in these catchments.

Water Corporation (WC) in Western Australia, is one of the world's largest water utilities servicing an area of over 2.5 million km^2. Six catchments were analysed; Catchment A is used for agriculture and urban development; catchment B is located in the South of WA; catchment C supplies approximately 20 percent of Perth's fresh water; catchment D is located in the southwest of WA; catchment E located ~70 km from metropolitan Perth; and catchment F is located approximately 100 km south of Perth.

2.2. Sample collection and processing

In NSW, animal faecal samples were collected by WaterNSW staff within the WaterNSW area of operations. Samples were collected at monthly intervals over a 30-month period (July, 2013 to December, 2015). A total of 1521 faecal samples were collected from Eastern grey kangaroos (*Macropus giganteus*) (n = 835), beef cattle (n = 243), sheep (n = 217), rabbits (n = 217), horses (n = 5) and pigs (n = 4) (Table 1).

In QLD, animal faecal samples were collected by Seqwater staff over a 14-month period (September, 2014 to November, 2015). A total of 653 faecal samples were collected from cattle (n = 568, of which 216 were dairy cattle and 352 were beef cattle), sheep (n = 9), horses (n = 38), birds (n = 9), pigs (n = 4), rabbits (n = 5), flying foxes (*Pteropus* sp.) (n = 9), feral red deer (*Cervus elaphus*) (n = 6), wild dogs (n = 3), a single goat, and a single wallaby (species unknown) (Table 1).

In WA, a total of 3600 faecal samples were collected from beef cattle (n = 300), sheep (n = 150), Western grey kangaroos (*Macropus fuliginosus*) (n = 2393), rabbits (n = 450), birds (n = 7) and pigs (n = 300) (Table 1).

The animal sources of the faecal samples were determined by visually sighting the animals defecating and with the aid of a scat and tracking manual published for Australian animals (Triggs,

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Table 1
Prevalence of *Cryptosporidium* in different hosts and catchments across three states of Australia; NSW, QLD and WA, as determined by Sanger sequencing.

Catchment	Cattle No +/total no (% prevalence + 95% CI)	Sheep No +/total no (% prevalence + 95% CI)	Kangaroos No +/total no (% prevalence + 95% CI)	Rabbits No +/total no (% prevalence + 95% CI)	Horses No +/total no (% prevalence + 95% CI)	Birds No +/total no (% prevalence + 95% CI)	Pigs No +/total no (% prevalence + 95% CI)	Other No +/total no (% prevalence + 95% CI)
QLD								
Catchment A	48/179 (26.8%- 20.5–33.9)	1/9 (11.1%- 0.3 –48.2)	NC	NC	0/15 (0%- 0–21.8)	2/2 (100%- 15.8 –100)	2/3 (66.7%- 9.4 –99.2)	0/1[a] (0%- 0–97.5)
Catchment B	56/194 (28.9%- 22.6–35.8)	NC	NC	NC	0/16 (0%- 0–26)	2/4 (50%- 6.8 –93.2)	N/C	NC
Catchment C	25/195 (12.8–8.5- 18.3)	NC	NC	0/5 (0%- 0–52.2)	0/7 (0%- 0–41)	2/3 (66.7%- 9.4 –99.2)	0/1 (0%- 0–97.5)	11/19[b] (57.9%- 33.5–79.7)
Total	**129/568 (22.7%- 19.3-26.4)**	**1/9 (11.1%- 0.3- 48.2)**	**NC**	**0/5 (0%- 0-52.2)**	**0/38 (0%- 0-9.3)**	**6/9 (66.7%- 29.9- 92.5)**	**2/4 (50%- 6.8- 93.2)**	**11/20 (55%- 31.5- 76.9)**
NSW								
Catchment A	45/243 (18.5%- 13.8–24)	12/217 (5.5%- 2.9 –9.5)	35/261 (13.4%- 9.5 –18.2)	60/217 (27.6%- 21.8–34.1)	NC	NC	NC	NC
Catchment B	NC	NC	37/574 (6.4%- 4.6 –8.8)	NC	0/5 (0%- 0–52.2)	NC	0/4 (0%- 0–60.2)	NC
Total	**45/243 (18.5%- 13.8-24)**	**12/217 (5.5%- 2.9- 9.5)**	**72/835 (8.6%- 6.8- 10.7)**	**60/217 (27.6%- 21.8-34.1)**	**0/5 (0%- 0-52.2)**	**NC**	**0/4 (0%- 0-60.2)**	**NC**
WA								
Catchment A	NC	NC	14/443 (3.2%- 1.7 –5.2)	4/150 (2.7%- 0.7 –6.7)	NC	5/7 (71.4%- 29 –96.3)	NC	NC
Catchment B	NC	NC	56/600 (9.3%- 7.1 –11.9)	NC	NC	NC	NC	NC
Catchment C	NC	NC	84/450 (18.7%- 15.2–22.6)	23/150 (15.3%- 10 –22.1)	NC	NC	NC	NC
Catchment D	65/300 (21.7%- 17.1–26.8)	17/150 (11.3%- 6.7 –17.5)	29/150 (19.3%- 13.3–26.6)	NC	NC	NC	NC	NC
Catchment E	NC	NC	85/450 (18.9%- 15.4–22.8)	9/150 (6%- 2.8 –11.1)	NC	NC	NC	NC
Catchment F	NC	NC	96/300 (32%- 26.8 –37.6)	NC	NC	NC	48/300 (16%- 12 –20.6)	NC
Total	**65/300 (21.7%- 17.1-26.8)**	**17/150 (11.3%- 6.7-17.5)**	**364/2393 (15.2%- 13.8-16.7)**	**36/450 (8%- 5.7- 10.9)**	**NC**	**5/7 (71.4%- 29- 96.3)**	**48/300 (16%- 12- 20.6)**	**NC**
Overall Prevalence	**239/1111 (21.5%- 19.1-24)**	**30/376 (8%- 5.4- 11.2)**	**436/3228 (13.5%- 12.3-14.7)**	**96/672 (14.3%- 11.7-17.2)**	**0/43 (0%- 0-8.2)**	**11/16 (68.7%- 41.3-89)**	**50/308 (16.2%- 12.3-20.8)**	**11/20 (55%- 31.5- 76.9)**

NC = Not Collected.
[a] Goat sample.
[b] Samples collected opportunistically from wildlife (7/9 flying fox, 0/1 wallaby, 2/6 feral deer, 2/3 wild dog).

2004). All faecal samples were collected off the ground into individual 75 ml faecal collection pots and stored at 4 °C until required, with samples collected in NSW and QLD transported at 4 °C to Murdoch University for analysis.

2.3. DNA isolation and qPCR

Genomic DNA (gDNA) was extracted from 250 mg of each faecal sample using a Power Soil DNA Kit (MO BIO, Carlsbad, California, USA). An extraction reagent blank (no faecal sample) was used in each extraction group. Purified gDNA was stored at −20 °C prior to molecular analyses. All samples were screened for the presence of *Cryptosporidium* at the 18S rRNA locus using a quantitative PCR (qPCR) as previously described (King et al., 2005; Yang et al., 2014) using a *Ct* threshold of <35 cycles. At the 18S locus, *C. macropodum,* a marsupial adapted species of *Cryptosporidium,* was used as a positive control for PCR amplifications of non-marsupial-derived samples. For samples collected from marsupials (kangaroos), *C. parvum* was used as a positive control.

Quantitation was conducted using standards consisting of recombinant plasmids containing partial fragments of the *Cryptosporidium* 18S rRNA, calibrated by droplet digital PCR (ddPCR) as described by Yang et al. (2014). Target copy numbers detected were converted to numbers of oocysts based on the fact that the 18S gene in *Cryptosporidium* has five copies (Le Blancq et al., 1997), and there are four haploid sporozoites per oocyst. Therefore, every 20 copies of 18S detected by qPCR were equivalent to one oocyst.

2.4. PCR amplification at the 18S and gp60 loci by nested PCR

Samples that were positive by qPCR were amplified at the 18S locus using nested primers which produced an approximately 825 bp product as previously described (Xiao et al., 1999). PCR contamination controls were used, including negative controls to detect contamination and separate laboratory areas were used for DNA and PCR mastermix preparation and post-PCR handling.

Samples that were typed as *C. hominis, C. parvum*, *C. cuniculus, C. meleagridis, C. ubiquitum* and *C. fayeri* by Sanger sequencing at the 18S locus were subtyped at the 60 kDa glycoprotein (*gp60*) locus using nested PCR's as previously described (Strong et al., 2000; Glaberman et al., 2001; Peng et al., 2003; Zhou et al., 2003; Power et al., 2009; Li et al., 2014). At the *gp60* locus, for samples which were initially identified as *C. hominis* and *C. parvum* at the 18S locus, *C. cuniculus* was used as a positive control. For samples which were previously identified as *C. cuniculus* at the 18S locus, *C. parvum* was used as a positive control.

2.5. Sanger sequence analysis

The amplified DNA from secondary PCRs were separated by gel

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electrophoresis and purified for sequencing using an in-house filter tip method (Yang et al., 2013). Purified PCR products from both loci were sequenced independently on an ABI Prism™ Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, California) according to the manufacturer's instructions, at 57 °C annealing temperature for the 18S rRNA and a range of different annealing temperatures for different species at *gp60* (56 °C for *C. fayeri*, 58 °C for *C. meleagridis* and 54 °C for the remaining species). Sanger sequences were assigned taxonomy by aligning chromatograms to curated reference sequences from GenBank using 99% sequence identity. Alignments were produced with Clustal W (http://www.clustalw.genome.jp), utilised as a plugin within Geneious Pro 8.1.6 (Kearse et al., 2012) and phylogenetic analysis was conducted using MEGA6 (after selection of the best nucleotide substitution models) (Tamura et al., 2013) (data not shown).

2.6. Next generation sequencing (NGS)

Samples that produced mixed chromatograms by Sanger sequencing (n = 251) were analysed by NGS on the MiSeq (Illumina) platform at the 18S locus using the 18S iF/iR primers (Morgan et al., 1997) as previously described (Paparini et al., 2015). These primers were selected over the longer Xiao et al. (1999) primers used for Sanger sequencing, due to length limitation imposed by the 250 bp paired-end sequencing technology utilised. Briefly, PCR primers were modified to contain MiSeq adapter sequences on the 5' end, as per standard protocols for the MiSeq platform (Illumina Demonstrated Protocol: Metagenomic Sequencing Library Preparation). All PCR amplicons were double purified using the Agencourt AMPure XP Bead PCR purification protocol (Beckman Coulter Genomics, USA) and pooled in approximate equimolar ratios. To minimize laboratory and cross-contamination all DNA handling and PCR-setup procedures were performed within dedicated and physically separated PCR containment hoods that are UV-sterilized between each use. Post-PCR procedures were all performed in a physically separate dedicated laboratory.

Sequencing was performed on an Illumina MiSeq using 500-cycle V2 chemistry (250 paired-end reads) following the manufacturer's recommendations. Two no-template controls and two DNA extraction reagent blank controls were included in the library preparation, and distributed between samples in the PCR plate layout. All no-template and DNA extraction reagent blank controls produced no detectable amplification of *Cryptosporidium* DNA after initial amplicon-generation PCRs or indexing PCRs. This indicated that level of cross contamination between samples, or from the laboratory environment, was below the detection limit of the library preparation procedure and for this reason were not sequenced.

2.7. Statistical analysis

The prevalence of *Cryptosporidium* in each host species was expressed as the percentage of samples positive by qPCR, which were also confirmed by Sanger and/or NGS analysis of the 18S rRNA locus, with 95% confidence intervals calculated assuming a binomial distribution, using the software Quantitative Parasitology 3.0 (Rózsa et al., 2000). DNA extraction efficiency was estimated for each extraction, based on the number of the gene copies/oocysts equivalents measured by ddPCR. Chi-square and non-parametric analyses were performed using IBM SPSS 21.0 (statistical package for the social sciences) for Windows (SPSS inc. Chicago, USA) to determine if there were any associations between the prevalence and concentration of *Cryptosporidium* oocysts at different sampling times and across states.

2.8. Bioinformatics analysis

Paired-end reads (73.42% of basecalls > Q30) were merged and quality filtered with USEARCH v10 (Edgar, 2010), retaining reads with >50 bp merged overlap, < 0.1% expected error, no mismatches in the primer sequences, a minimum length of 200 bp, and a minimum of 100 identical replicate copies. Primer sequences and any distal bases were also removed from all reads. Reads were then denoised and chimera filtered with the UNOISE3 algorithm (Edgar, 2016), to generate 107 zero-radius operational taxonomic units (ZOTUS), that represent unique biologically correct sequences (Edgar, 2016). Remaining high quality *Cryptosporidium* spp. 18S ZOTU sequences were assigned to taxonomic groups by comparing ZOTUs to a curated custom in-house database using BLAST 2.6.0 (Altschul et al., 1990). The reference database contained 63 reliable 18S reference sequences from 63 *Cryptosporidium* species and genotypes extracted from GenBank (Benson et al., 2005). Taxonomy was only assigned if there was a single unambiguous best BLAST hit with >99% pairwise identity over >98% of the query sequence length. This high stringency threshold was based on the minimum pairwise percentage dissimilarity between any two *Cryptosporidium* species/genotypes in the database to unsure unambiguous taxonomic classifications. Of the 107 ZOTUs generated, 41 ZOTUs were not *Cryptosporidium* 18S sequences when compared to GenBank using BLAST. Where possible genus-level taxonomy was assigned when queries hit reference sequences with >99% identity over >98% of the reads, and assigned to no other taxa at the same level. Although non-*Cryptosporidium* ZOTUs were abundant (38.3% of total ZOTUs), they represented a very small proportion of the total reads, with only a median of 580 sequences each.

3. Results

3.1. Overall prevalence of Cryptosporidium as determined by Sanger sequencing and NGS

The overall PCR prevalence of *Cryptosporidium* species in the 5774 faecal samples collected systematically from seven main host species and opportunistically from 10 host species, in addition to seven unknown bird samples, was 18.3% (1054/5774; 95% CI, 17.3–19.3), based on PCR positive samples that were confirmed by Sanger or NGS sequencing.

The overall prevalence in each state based on qPCR positive samples that have been confirmed by Sanger sequencing or NGS was 25.9% (169/653; 95% CI, 22.6–29.4) in QLD, 17.5% (632/3600; 95% CI, 16.3–18.8) in WA and 16.6% (253/1521; 95% CI, 14.8–18.6) in NSW. For ease of analysis and reporting, the prevalence and species detected by Sanger and NGS are discussed separately.

3.2. Prevalence of Cryptosporidium as determined by Sanger sequencing in various hosts and catchments

Of the 873 samples which produced clean Sanger chromatograms, the highest prevalence was detected in birds (68.7%), followed by 21.5% in cattle, 16.2% in pigs, 14.3% in rabbit samples, 13.5% in kangaroos and 8% in sheep. No *Cryptosporidium* was detected in the 43 horse faecal samples screened (Table 1). A high prevalence was also detected in the small numbers of wildlife sampled (Table 1). The overall prevalence of *Cryptosporidium* in each state was 22.8%; 95% CI, 19.7–26.2 (149/653) in QLD, 14.9%; 95% CI, 13.7–16.1 (535/3600) in WA, and 12.4%; 95% CI, 10.8–14.2 (189/1521) in NSW.

The prevalence of *Cryptosporidium* in cattle was not significantly different across the three states; 18.5% in NSW, 21.7% in WA and 22.7% in QLD (29.6% in dairy cattle and 18.5% in beef cattle. The

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prevalence of *Cryptosporidium* was highest in sheep from WA (11.3%), compared to 11.1% in QLD and 5.5% in NSW. Kangaroos were only sampled in NSW (8.6%) and WA (15.2%). *Cryptosporidium* was not detected in the small numbers of rabbits sampled in QLD (n = 5), but was more prevalent in rabbits in NSW (27.6%) than WA (8%). *Cryptosporidium* was detected in two (out of 4) pigs in QLD and in 16% of pigs in WA (Table 1).

3.3. Oocyst load

Oocyst numbers per gram of faeces (g^{-1}) were also determined using qPCR (Table 2; mean, median and range for samples identified as *Cryptosporidium* species by Sanger sequencing and NGS). The highest median concentration of *Cryptosporidium* oocysts was identified in cattle; (31,072 oocysts/g^{-1}), followed by rabbits (27,919 oocysts/g^{-1}), while the lowest median concentration of oocysts was observed among samples collected opportunistically from wildlife in QLD (9063 oocysts/g^{-1}). Overall, there was no significant difference between the median *Cryptosporidium* oocysts per gram of faeces in samples collected from sheep, kangaroos, pigs and birds, which ranged from 10,032 to 26,756 oocysts/g^{-1} (Table 2).

3.4. Cryptosporidium species detected in various hosts at the 18S locus by Sanger sequencing

Clean 18S Sanger sequences were obtained from 873 positives. Of these, a total of 14 species and two genotypes were detected; *C. macropodum* (n = 260), *C. fayeri* (n = 150), *C. parvum* (n = 106), *C. cuniculus* (n = 96), *C. bovis* (n = 60), *C. hominis* (n = 42), *C. ryanae* (n = 41), *C. ubiquitum* (n = 36), *C. scrofarum* (n = 35), *C. suis* (n = 15), *C. muris* (n = 15), *C. galli* (n = 8), *C. meleagridis* (n = 3), *C. canis* (n = 2), *Cryptosporidium* rat genotype I (n = 3) and *C. molnari*-like genotype (n = 1) (Table 3).

In cattle, of the 239 positives, a total of seven species and one genotype were detected; *C. parvum* (n = 106), *C. bovis* (n = 60), *C. ryanae* (n = 41), *C. hominis* (n = 16), *C. muris* (n = 8), *C. ubiquitum* (n = 4), *C. galli* (n = 1) and rat genotype I (n = 3), with *C. ubiquitum*, *C. galli* and rat genotype I only detected in beef cattle. All the sheep were infected with *C. ubiquitum* (30/30) and all the rabbits were infected with *C. cuniculus* (96/96). In kangaroos, *C. hominis* (26/436), *C. macropodum* (260/436) and *C. fayeri* (150/436) were detected. In pigs, *C. suis* (15/50) and *C. scrofarum* (35/50) were detected. Three species were detected in the 11 positives from birds, a *C. molnari-like* genotype in a single shag, *C. galli* (n = 7) and *C. meleagridis* (n = 3). In wildlife, of the 11 positives typed, *C. ubiquitum* was identified in feral deer (n = 2), *C. muris* in flying foxes (n = 7) and *C. canis* from wild dogs (n = 2) (Table 3).

3.5. Cryptosporidium gp60 subtypes detected in various hosts by Sanger sequencing

Of the 89 *C. parvum* cattle isolates subtyped at the *gp60* locus, a total of seven subtypes were identified; IIaA15G2R1 (n = 5), IIaA16G2R1 (n = 5), IIaA17G2R1 (n = 27), IIaA18G3R1 (n = 40), IIaA19G2R1 (n = 1), IIaA19G3R1 (n = 11), and a variant of the IIaA13G1 subtype in five cattle, which exhibited one single nucleotide polymorphism from the only other previous record of this

Table 2
Numbers of *Cryptosporidium* oocysts in positive samples per gram of faeces (g^{-1}) (mean, median with range in parenthesis (determined by qPCR) per host species per catchment across three states of Australia; NSW, QLD and WA.

Catchment	Cattle	Sheep	Kangaroos	Rabbits	Horses	Birds	Pigs	Other
QLD								
Catchment A	36,093, 28,290 (928–122,080)	24,500, NA (only one sample)	NC	NC	ND	11,518, 11,518 (9982–13,054)	16,135, 16,135 (1,2450–19,820)	ND
Catchment B	53,508, 42,803 (1920–182,664)	NC	NC	NC	NC	6,201, 6201 (2622–9780)	NC	NC
Catchment C	44,914, 33,581 (680–228,548)	NC	NC	ND	ND	7,376, 7376 (4720–10,032)	NC	8,832, 9063 (622–19,047)
Total	**45,002, 33,585 (680–228,548)**	**24,500, NA (only one sample)**	**NC**	**ND**	**ND**	**8,365, 9882 (2622–13,054)**	**16,135, 16,135 (19,820–3,2270)**	**8,832, 9063 (622–19,047)**
NSW								
Catchment A	24,685, 22,568 (836–88,014)	29,455, 33,522 (6068–58,892)	9,766, 8044 (26–44,738)	27,510, 27,155 (498–67,233)	NC	NC	NC	NC
Catchment B	NC	NC	11,615, 10,265 (131–41,088)	NC	ND	NC	ND	NC
Total	**24,685, 22,568 (836–88,014)**	**29,454, 33,522 (6068–58,892)**	**10,549, 8735 (26–44,738)**	**27,510, 27,155 (498–67,233)**	**ND**	**NC**	**ND**	**NC**
WA								
Catchment A	NC	NC	15,907, 13,728 (2076–40,210)	64,572, 61,034 (1004–108,706)	NC	38,841, 44,812 (5270–78,433)	NC	NC
Catchment B	NC	NC	19,978, 17,738 (440–63,002)	NC	NC	NC	NC	NC
Catchment C	NC	NC	21,892, 19,507 (698–71,194)	38,336, 38,297 (1004–82,633)	NC	NC	NC	NC
Catchment D	43,418, 37,322 (6014–144,328)	27,281, 19,701 (4492–51,178)	14,420, 12,529 (522–45,490)	NC	NC	NC	NC	NC
Catchment E	NC	NC	19,747, 17,820 (11–66,904)	30,412, 18,595 (13,090–89,160)	NC	NC	NC	NC
Catchment F	NC	NC	23,482, 18,085 (714–275,402)	NC	NC	NC	22,861, 22,062 (873–88,026)	NC
Total	**43,418, 37,322 (6014–144,328)**	**27,281, 19,701 (4492–51,178)**	**24,680, 19,098 (11–275,042)**	**39,175, 36,064 (1004–108,706)**	**NC**	**38,841, 44,812 (5270–78,433)**	**22,861, 22,062 (873–88,026)**	**NC**
Overall	**39,834, 31,072 (680–228,548)**	**28,058, 26,756 (4492–58,892)**	**18,442, 16,018 (11–275,042)**	**32,380, 27,919 (498–108,706)**	**ND**	**22,217, 10,032 (2622–78,433)**	**22,625, 21,164 (873–88,026)**	**8,832, 9063 (622–19,047)**

NA = Not Available.
NC = Not Collected.
ND = Not Detected.

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Table 3
Cryptosporidium species detected by Sanger sequencing in different hosts and catchments across three states of Australia; NSW, QLD and WA.

Host	*C. parvum* No +/total no (% proportion+ 95 CI)	*C. hominis* No +/total no (% proportion + 95 CI)	*C. bovis* No +/total no (% proportion + 95 CI)	*C. ubiquitum* No +/total no (% proportion + 95 CI)	*C. ryanae* No +/total no (% proportion + 95 CI)	*C. suis* No +/total no (% proportion + 95 CI)	*C. scrofarum* No +/total no (% proportion + 95 CI)	*C. cuniculus* No +/total no (% proportion + 95 CI)	*C. macropodum* No +/total no (% proportion + 95 CI)	Other No +/total no (% proportion + 95 CI)
QLD										
Cattle	55/129 (42.6%-34 −51.6)	7/129 (5.4%- 2.2 −10.9)	26/129 (20.2%- 13.6 −28.1)	4/129 (3.1%- 0.9 −7.7)	29/129 (22.5%- 15.6 −30.7)	ND	ND	ND	ND	8/129[a] (6.2 %- 2.7 −11.9)
Sheep	ND	ND	ND	1/1 (100%- 2.5 −100)	ND	ND	ND	ND	ND	ND
Horses	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Birds	ND	ND	ND	ND	ND	ND	ND	ND	ND	6/6[b] (100%- 54.1 −100)
Pigs	ND	ND	ND	ND	ND	ND	2/2 (100%- 15.8 −100)	ND	ND	ND
Wildlife	ND	ND	ND	2/11[c](18.2%- 2.3 −51.8)	ND	ND	ND	ND	ND	9/11[d] (81.8%- 48.2 −97.7)
Total	**55/129 (42.6%- 34 −51.6)**	**7/129 (5.4%- 2.2 −10.9)**	**26/129 (20.2%- 13.6 −28.1)**	**7/141 (5%- 2−10)**	**29/129 (22.5%- 15.6 −30.7)**	**ND**	**2/2 (100%- 15.8 −100)**	**ND**	**ND**	**23/146 (15.7%- 10.3 −22.7)**
NSW										
Cattle	19/45 (42.2%- 27.7 −57.8)	6/45 (13.3%- (5.1 −26.8)	18/45 (40%- 25.7 −55.7)	N/D	1/45 (2.2%- 0.1 −11.8)	ND	ND	ND	ND	1/45[e] (2.2%- 0.1 −11.8)
Sheep	N/D	ND	ND	12/12 (100%- 73.5 −100)	ND	ND	ND	ND	ND	ND
Kangaroos	N/D	26/72 (36.1%- 25.1 −48.3)	ND	ND	ND	ND	ND	ND	44/72 (61.1%- 48.9 −72.4)	2/72[f] (2.8%- 0.3 −9.7)
Rabbits	N/D	ND	ND	ND	ND	ND	ND	60/60 (100%- 94 −100)	ND	ND
Total	**19/45 (42.2%- 27.7 −57.8)**	**32/117 (27.5%- 19.5 −36.4)**	**18/45 (40%- 25.7 −55.7)**	**12/12 (100%- 73.5 −100)**	**1/45 (2.2%- 0.1 −11.8)**	**ND**	**ND**	**60/60 (100%- 94 −100)**	**44/72 (61.1%- 48.9 −72.4)**	**3/117 (2.6%- 0.5 −7.3)**
WA										
Cattle	32/65 (49.2%- 36.6 −61.9)	3/65 (4.62%- 1 −12.9)	16/65 (24.6%- 14.8 −36.9)	ND	11/65 (24.6%- 14.8 −36.9)	ND	ND	ND	ND	3/65[g] (4.6%- 1 −12.9)
Sheep	ND	ND	ND	17/17 (100%- 80.5 −100)	ND	ND	ND	ND	ND	ND
Kangaroos	ND	ND	ND	ND	ND	ND	ND	ND	216/364 (59.3%- 54.1−64.4)	148/364[f] (40.7%- 35.6−45.9)
Rabbits	ND	ND	ND	ND	ND	ND	ND	36/36 (100%- 90.3 −100)	ND	ND
Pigs	ND	ND	ND	ND	ND	15/48 (31.2%- 18.7 −46.3)	33/48 (75%- 60.4 −86.4)	ND	ND	ND
Birds	ND	ND	ND	ND	ND	ND	ND	ND	ND	5/5[h] (100%-47.8-100)
Total	**32/65 (49.2%- 36.6 −61.9)**	**3/65 (4.62%- 1 −12.9)**	**16/65 (24.6%- 14.8 −36.9)**	**17/17 (100%- 80.5 −100)**	**11/65 (24.6%- 14.8 −36.9)**	**15/48 (31.2%- 18.7 −46.3)**	**33/48 (75%- 60.4 −86.4)**	**36/36 (100%- 90.3 −100)**	**216/364 (59.3%- 54.1−64.4)**	**156/434 (35.9%- 31.4−40.7)**

ND = Not Detected.
[a] *Cryptosporidium muris* (n = 8).
[b] *Cryptosporidium molnari* (n = 1, Shag), *Cryptosporidium gall*i (n = 5; 2 from an ibis, 1 from a goose, 1 from a boiler chicken, 1 from a swallow).
[c] *Cryptosporidium ubiquitum* from feral deer (n = 2).
[d] *Cryptosporidium muris* from flying fox (n = 7), *Cryptosporidium canis* from wild dog (n = 2).
[e] *Cryptosporidium galli*.
[f] *Cryptosporidium fayeri*.
[g] *Cryptosporidium* rat genotype I (n = 3).
[h] *Cryptosporidium galli* (n = 2), *Cryptosporidium meleagridis* (n = 3).

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subtype (JX471005). Two *C. hominis* subtypes were identified; IbA10G2 (of which 11 were identified in cattle and 23 in kangaroos) and IdA15G1 (two in kangaroos and three in cattle). All the *C. ubiquitum* typed (n = 28) belonged to subtype family XIIa and all the *C. meleagridis* (n = 3) were identified as a novel subtype; IIIeA18G2R1. Six *C. cuniculus* subtypes were identified; VbA18 (n = 12), VbA23 (n = 46), VbA25 (n = 16), VbA26 (n = 8), VbA28 (n = 2) and VbA29 (n = 5). Three *C. fayeri* subtypes were identified; IVfA12G1T1 (n = 23) and two novel subtypes; IVaA11G3T1 (n = 16) and IVgA10G1T1R1 (n = 81) (Table 6). The novel subtypes (IIaA13G1, IIIeA18G2R1, IVaA11G3T1 and IVgA10G1T1R1) identified in the present study have been submitted to GenBank under accession numbers MG516789, MG516778, MG516791 and MG516790. All other nucleotide sequences reported in this paper are available in the GenBank database under accession numbers MG516739 to MG516774 (18S) and MG516775 to MG516798 (*gp60*).

3.6. Prevalence and species of Cryptosporidium identified by NGS

A total of 251 samples produced mixed Sanger chromatograms and were re-analysed by NGS. However, of these, only 181 were assigned to *Cryptosporidium* species and the remaining 70 samples were discarded due to unassigned reads or failure to pass quality filtering. Of these 181 samples, which produced mixed chromatograms by Sanger, 83 samples did not exhibit mixed infections via NGS, i.e. only one species/genotype was detected (Supplementary Table 1). This suggests that some of the mixed chromatograms identified by Sanger, were due to co-amplification of *Cryptosporidium* with non-specific contaminants. Therefore, for the scope of the present study, the prevalence of mixed infections is reported based on only those samples which were assigned to multiple *Cryptosporidium* species by NGS (n = 98).

Therefore, the overall prevalence of mixed infections was 1.7% (98/5774; 95% CI, 1.4–2.1) (Table 4 and supplementary Table 1). These mixed infections were detected in faecal samples collected from cattle, kangaroos, rabbits, wild pigs and two feral deer. Apart from 10% overall prevalence among samples collected opportunistically from wildlife, the highest prevalence of mixed infections was detected in kangaroos (2.8%), followed by 1.7% in cattle, 0.9% in rabbits and 0.3% in pigs (Table 4). Overall, a total of 15 species and four genotypes of *Cryptosporidium* were detected by NGS; *C. bovis*, *C. cuniculus*, *C. fayeri*, *C. felis*, *C. galli*, *C. hominis*, *C. macropodum*, *C. meleagridis*, *C. muris*, *C. parvum*, *C. rayane*, *C. scrofarum*, *C. suis*, *C. erinacei, C. ubiquitum*, kangaroo genotype I, rat genotype I, rat genotype II and rat genotype III (Table 5). The number of species identified in individual hosts ranged from 1 to 6 species. However, *C. parvum*, *C. hominis*, *C. cuniculus*, *C. muris*, *C. fayeri*, *C. macropodum*, *C. suis*, *C. galli,* kangaroo genotype I and rat genotype III were the most abundant species identified in samples with mixed infections and therefore, were considered for prevalence and statistical analysis in this study. A total of 39 cattle samples, of which 32 were from beef cattle (11 from QLD, 19 from NSW and two from WA), and

Table 4
Prevalence of mixed infection with different species of *Cryptosporidium* identified by NGS in samples which produced mixed chromatograms by Sanger-sequencing across three states of Australia; NSW, QLD and WA.

Catchment	Cattle No +/total no (% prevalence + 95 CI)	Sheep No +/total no (% prevalence + 95 CI)	Kangaroos No +/total no (% prevalence + 95 CI)	Rabbits No +/total no (% prevalence + 95 CI)	Horses No +/total no (% prevalence + 95 CI)	Birds No +/total no (% prevalence + 95 CI)	Pigs No +/total no (% prevalence + 95 CI)	Other No +/total no (% prevalence + 95 CI)
QLD								
Catchment A	5/179 (2.8%- 0.9–6.4)	0/9 (0%- 0–33.6)	NC	NC	0/15 (0%- 0–21.8)	0/2 (0%- 0–84.2)	0/3 (0%- 0–70.8)	0/1 (0%- 0–97.5)
Catchment B	2/194 (1%- 0.1–3.7)	NC	NC	NC	0/16 (0%- 0–26)	0/4 (0%- 0–60.2)	N/C	NC
Catchment C	5/195 (2.6- 0.8-5.9)	NC	NC	0/5 (0%- 0–52.2)	0/7 (0%- 0–41)	0/3 (0%- 0–70.8)	0/1 (0%- 0–97.5)	2[a]/19 (10.5%- 1.3–33.1)
Total	**12/568 (2.1%- 1.1-3.7)**	**0/9 (0%- 0-33.6)**	**NC**	**0/5 (0%- 0-52.2)**	**0/38 (0%- 0-9.3)**	**0/9 (0%- 0-33.6)**	**0/4 (0%- 0-60.2)**	**2/20 (10%- 1.2-31.7)**
NSW								
Catchment A	5/243 (2.1%- 0.7–4.7)	0/217 (0%- 0–1.7)	23/261 (8.8%- 5.7–12.9)	0/217 (0%- 0–1.7)	NC	NC	NC	NC
Catchment B	NC	NC	6/574 (1%- 0.4–2.3)	NC	0/5 (0%- 0–52.2)	NC	0/4 (0%- 0–60.2)	NC
Total	**5/243 (2.1%- 0.7-4.7)**	**0/217 (0%- 0-1.7)**	**29/835 (3.5%- 2.3-4.9)**	**0/217 (0%- 0-1.7)**	**0/5 (0%- 0-52.2)**	**NC**	**0/4 (0%- 0-60.2)**	**NC**
WA								
Catchment A	NC	NC	3/443 (0.7%- 0.1–2)	1/150 (0.7%- 0–3.7)	NC	0/7 (0%- 0–41)	NC	NC
Catchment B	NC	NC	12/600 (2%- 1–3.5)	NC	NC	NC	NC	NC
Catchment C	NC	NC	11/450 (2.4%- 1.2–4.3)	3/150 (2%- 0.4–5.7)	NC	NC	NC	NC
Catchment D	2/300 (0.6%- 0.1–2.4)	0/150 (0%- 0–2.4)	3/150 (2%- 0.4–5.7)	NC	NC	NC	NC	NC
Catchment E	NC	NC	7/450 (1.6%- 0.6–3.2)	2/150 (1.3%- 0.2–4.7)	NC	NC	NC	NC
Catchment F	NC	NC	5/300 (1.7%- 0.5–3.8)	NC	NC	NC	1/300 (0.3%- 0–1.8)	NC
Total	**2/300 (0.6%- 0.1-2.4)**	**0/150 (0%- 0-2.4)**	**41/2393 (1.7%-1.2-2.3)**	**6/450 (1.3%- 0.5-2.9)**	**NC**	**0/7 (0%- 0–41)**	**1/300 (0.3%- 0 -1.8)**	**NC**
Overall Prevalence	**19/1111 (1.7%- 1-2.7)**	**0/376 (0%- 0–1)**	**70/3228 (2.8%-1.7-2.7)**	**6/672 (0.9%- 0.3-1.9)**	**0/43 (0%- 0-8.2)**	**0/16 (0%- 0-20.6)**	**1/308 (0.3%- 0-1.8)**	**2/20 (10%- 1.2-31.7)**

[a] Feral deer.

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Table 5
Most abundant *Cryptosporidium* species detected by NGS in different hosts and catchments across three states of Australia; NSW, QLD and WA.

Host	*C. parvum* No +/total no (% proportion+ 95 CI)	*C. hominis* No +/total no (% proportion + 95 CI)	*C. bovis* No +/total no (% proportion + 95 CI)	*C. ubiquitum* No +/total no (% proportion + 95 CI)	*C. ryanae* No +/total no (% proportion + 95 CI)	*C. suis* No +/total no (% proportion + 95 CI)	*C. scrofarum* No +/total no (% proportion + 95 CI)	*C. cuniculus* No +/total no (% proportion + 95 CI)	*C. macropodum* No +/total no (% proportion + 95 CI)	Other No +/total no (% proportion + 95 CI)
QLD										
Cattle	8/18 (44.4%- 21.5 −69.2)	ND	ND	ND	ND	ND	ND	ND	ND	10/18[a] (55.6%- 30.8 −78.5)
Sheep	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Horses	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Birds	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Pigs	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Wildlife	ND	ND	ND	ND	ND	ND	ND	ND	ND	2/2[b] (100%- 15.8 −100)
Total	**8/18 (44.4%- 21.5-69.2)**	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	**12/20 (60%- 36.1-80.9)**
NSW										
Cattle	6/19 (31.6%- 12.6 −56.6)	3/19 (15.8%- 3.4 −39.6)	ND	N/D	ND	ND	ND	ND	ND	10/19[c] (52.6%- 28.9 −75.6)
Sheep	N/D	ND	ND	ND	ND	ND	ND	ND	ND	ND
Kangaroos	24/45 (53.3%- 37.9 −68.3)	17/45 (37.8%- 23.8 −53.5)	ND	ND	ND	ND	ND	ND	2/45 (4.4%- 0.5 −15.1)	2/45[d] (4.4%- 0.5 −15.1)
Rabbits	N/D	ND	ND	ND	ND	ND	ND	ND	ND	ND
Total	**30/64 (46.9%-34.3-60)**	**20/64 (31.2%-20.2-44.1)**	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**	**2/45 (4.4%- 0.5-15.1)**	**12/64 (18.7%-10.1-30.5)**
WA										
Cattle	2/2 (100%- 15.8 −100)	ND	ND	ND	ND	ND	ND	ND	ND	ND
Sheep	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Kangaroos	28/81 (34.6%-24.3-46)	31/81 (38.3%- 27.7 −29.7)	ND	ND	ND	ND	ND	ND	15/81 (18.5%- 10.8 −28.7)	7/81[e] (5.6%- 3.5 −17)
Rabbits	ND	ND	ND	ND	ND	ND	ND	7/7 (100%-59-100)	ND	ND
Pigs	ND	ND	ND	ND	ND	7/7 (100%-59-100)	ND	ND	ND	ND
Birds	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Total	**30/83 (30.1%-25.9-47.4)**	**31/81 (38.3%-27.7-29.7)**	**ND**	**ND**	**ND**	**7/7 (100%-59 −100)**	**ND**	**7/7 (100%-59 −100)**	**15/81 (18.5%-10.8-28.7)**	**7/81 (5.6%- 3.5-17)**

ND = Not Detected.
[a] *Cryptosporidium muris* (n = 4), *Cryptosporidium galli* (n = 5), rat genotype III (n = 1).
[b] *Cryptosporidium muris* (n = 2, feral deer).
[c] *Cryptosporidium muris* (n = 10).
[d] *Cryptosporidium galli*.
[e] *Cryptosporidium fayeri* (n = 5), *Cryptosporidium galli* (n = 1), kangaroo genotype I (n = 1).

Table 6
gp60 subtypes detected in different hosts and catchments across three states of Australia; NSW, QLD and WA.

Host	QLD						NSW					WA					Overall number
	Cattle	Sheep	Birds	Pigs	Wildlife	**Total**	Cattle	Sheep	Kangaroo	Rabbits	**Total**	Cattle	Sheep	Kangaroo	Rabbits	Pigs	Birds	**Total**	
C. parvum IIaA13G1	ND	ND	ND	ND	ND	**ND**	ND	ND	ND	ND	**ND**	5	ND	ND	ND	ND	ND	**5**	**5**
C. parvum IIaA15G2R1	ND	ND	ND	ND	ND	**ND**	ND	ND	ND	ND	**ND**	5	ND	ND	ND	ND	ND	**5**	**5**
C. parvum IIaA16G2R1	ND	ND	ND	ND	ND	**ND**	5	ND	ND	ND	**5**	ND	ND	ND	ND	ND	ND	**ND**	**5**
C. parvum IIaA17G2R1	13	ND	ND	ND	ND	**13**	ND	ND	ND	ND	**ND**	14	ND	ND	ND	ND	ND	**14**	**27**
C. parvum IIaA18G3R1	24	ND	ND	ND	ND	**24**	9	ND	ND	ND	**9**	7	ND	ND	ND	ND	ND	**7**	**40**
C. parvum IIaA19G2R1	ND	ND	ND	ND	ND	**ND**	1	ND	ND	ND	**1**	ND	ND	ND	ND	ND	ND	**ND**	**1**
C. parvum IIaA19G3R1	8	ND	ND	ND	ND	**8**	3	ND	ND	ND	**3**	ND	ND	ND	ND	ND	ND	**ND**	**11**
C. hominis IbA10G2	7	ND	ND	ND	ND	**7**	4	ND	23	ND	**27**	ND	ND	ND	ND	ND	ND	**ND**	**34**
C. hominis IdA15G1	ND	ND	ND	ND	ND	**ND**	ND	ND	2	ND	**2**	3	ND	ND	ND	ND	ND	**3**	**5**
C. ubiquitum XIIa	4	1	ND	ND	2[a]	**7**	ND	10	ND	ND	**10**	ND	11	ND	ND	ND	ND	**11**	**28**
C. cuniculus VbA18	ND	ND	ND	ND	ND	**ND**	ND	ND	ND	ND	**ND**	ND	ND	ND	9	ND	ND	**12**	**12**
C. cuniculus VbA23	ND	ND	ND	ND	ND	**ND**	ND	ND	ND	37	**37**	ND	ND	ND	9	ND	ND	**9**	**46**
C. cuniculus VbA25	ND	ND	ND	ND	ND	**ND**	ND	ND	ND	16	**16**	ND	ND	ND	ND	ND	ND	**ND**	**16**
C. cuniculus VbA26	ND	ND	ND	ND	ND	**ND**	ND	ND	ND	ND	**ND**	ND	ND	ND	8	ND	ND	**8**	**8**
C. cuniculus VbA28	ND	ND	ND	ND	ND	**ND**	ND	ND	ND	2	**2**	ND	ND	ND	ND	ND	ND	**ND**	**2**
C. cuniculus VbA29	ND	ND	ND	ND	ND	**ND**	ND	ND	ND	5	**5**	ND	ND	ND	ND	ND	ND	**ND**	**5**
C. meleagridis IIIeA18G2R1	ND	ND	ND	ND	ND	**ND**	ND	ND	ND	ND	**ND**	ND	ND	ND	ND	ND	3	**3**	**3**
C. fayeri IVgA10G1T1R1	ND	ND	ND	ND	ND	**ND**	ND	ND	ND	2	**2**	ND	ND	79	ND	ND	ND	**79**	**81**
C. fayeri IVaA11G3T1	ND	ND	ND	ND	ND	**ND**	ND	ND	ND	ND	**ND**	ND	ND	16	ND	ND	ND	**16**	**16**
C. fayeri IVfA12G1T1	ND	ND	ND	ND	ND	**ND**	ND	ND	ND	ND	**ND**	ND	ND	23	ND	ND	ND	**23**	**23**

ND = Not Detected.
[a] Feral deer.

seven from dairy cattle from QLD), produced mixed chromatograms by Sanger sequencing. NGS analysis identified mixed infections and the four most abundant species and one most abundant *Cryptosporidium* genotype in individual samples were; *C. parvum* (n = 16), *C. hominis* (n = 3), *C. muris* (n = 14), *C. galli* (n = 5) and rat genotype III (n = 1). In kangaroos, *C. parvum* (n = 52), *C. hominis* (n = 48), *C. macropodum* (n = 17), *C. fayeri* (n = 5), *C. galli* (n = 3) and kangaroo genotype I (n = 1) were identified. Re-analysis of all seven faecal samples from wild pigs, all seven rabbit samples and two faecal samples collected from feral deer by NGS, identified *C. suis*, *C. cuniculus* and *C. muris* as the most abundant species, respectively (Table 5).

4. Discussion

Relatively few large-scale studies of *Cryptosporidium* species in animals inhabiting drinking water catchments have been conducted. The present study is the largest single published study conducted to date globally and analysed *Cryptosporidium* species and subtypes in 5774 animal faecal samples from catchments across three states of Australia; NSW, QLD and WA. As such, it provides a unique perspective on the epidemiology of *Cryptosporidium* in animals inhabiting water catchments across Australia. In the present study, the overall prevalence of *Cryptosporidium* was 18.3% (1054/5774; 95% CI, 17.3–19.3), with a prevalence of 25.9% (169/653, 95% CI; 22.6–29.4) in QLD, 17.6% (632/3,600, 95% CI; 16.3–18.8) in WA and 16.6% (253/1,521, 95% CI; 14.8–18.6) in NSW. Previous studies in Australia have reported *Cryptosporidium* in animal faecal samples from catchments across Australia at varying prevalence; 5–25.8% in NSW (Power et al., 2004, 2005; Cox et al., 2005; Ryan et al., 2005; Ng et al., 2011b; Zahedi et al., 2016b), 1.6–2.8% in Victoria (Cinque et al., 2008; Nolan et al., 2013; Koehler et al., 2014a, 2016) and 6.7–16% in WA (McCarthy et al., 2008; Ferguson, 2010). All of these, with the exception of one study (Cox et al., 2005), have conducted genetic characterisation. The largest study was conducted in Melbourne drinking water catchments and screened 4256 wildlife faecal samples between 2011 and 2015 (Koehler et al., 2016) and overall between 2009 and 2015, analysed 6265 samples (Nolan et al., 2013; Koehler et al., 2016) and reported a prevalence of 2.8% (56/2009) and 1.6% (69/4256), respectively (Nolan et al., 2013; Koehler et al., 2016). The reason for the much lower prevalence of *Cryptosporidium* in Melbourne catchments is unclear, but it has been suggested that the low prevalence could be due to animal culls (resulting in lower density of animals), changing water levels of the reservoirs and the end of a nine-year drought (Koehler et al., 2016).

Several studies have examined the transport of oocysts from the site of deposition in catchments into surface waters used for producing drinking water (Davies et al., 2004; Atwill et al., 2006b; Ferguson et al., 2007; Curriero et al., 2011; Khaldi et al., 2011). However, in Australia, little published information is available on the prevalence of *Cryptosporidium* in source water. Analysis of *Cryptosporidium* monitoring data across Australia in source water over a period of 11–18 years by Water Research Australia (WaterRA), revealed that detection of total (i.e. presumptive) *Cryptosporidium* ranged from ~20% of samples from South Australia and WA, to ~15% in Melbourne and Canberra, and 2–7% in NSW (Deere et al., 2014), suggesting that significant numbers of oocysts in faecal samples deposited in catchments are transported into source waters due to rainfall run-off. Another study in South Australia, reported a significant increase in oocyst concentrations after a rainfall event (Swaffer et al., 2014). Transport of oocysts into drinking water will be affected by climate change, as it is expected to result in less winter rainfall but more extreme precipitation events during summer (Sterk et al., 2016). Initial modelling of the

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impact of climate change on runoff of *Cryptosporidium* from land to surface water suggests a complex relationship that will require site-specific analysis (Sterk et al., 2016), and highlights the importance of continued monitoring of *Cryptosporidium* in catchments.

In the present study, the prevalence of mixed infection assigned to *Cryptosporidium* spp. in faecal samples collected from animals inhabiting 11 catchments across three states in Australia was 1.7% (98/5774; 95% CI, 1.4–2.1). This is the first study to apply NGS in a large-scale study to determine the prevalence of *Cryptosporidium* species in animal faecal samples with mixed *Cryptosporidium* infections, with up to 6 species identified in a single host. Interestingly *C. parvum* was not detected by Sanger sequencing in kangaroos, yet *C. parvum* and *C. hominis* were among the most abundant species detected in co-infections in cattle and kangaroos by NGS (Table 5 and Supplementary Table 1). This has important implications for catchment management, as animals may be shedding human-infectious species intermittently or in lesser abundance than a dominant species, but if analysed by Sanger sequencing, these species may not be detected. In the present study, NGS was used only on samples that produced mixed chromatograms via Sanger sequencing due to cost constraints. Future studies should apply NGS to type all *Cryptosporidium* positives from catchments.

Because NGS technologies are highly sensitive, allowing for the detection of low abundance sequences in complex DNA mixtures, the presence of cross-contamination between samples, or contamination from the laboratory environment has an increased potential to influence the taxonomic composition of samples. In addition, sequences that occur in very low abundances are likely to be caused by sequencing error, as errors are introduced randomly and are not reproduced, and are likely to be unique. Therefore, there is an intrinsic distrust of sequences or taxa that are present in low abundances in samples, such as some of the mixed *Cryptosporidium* infections detected in the present study.

However, no amplified *Cryptosporidium* DNA was detected in no-template or extraction reagent blank PCR controls after both initial amplicon-generation PCR or the indexing PCR, indicating that any cross contamination, or contamination from the laboratory, was below the detectable limit for the library preparation process. In addition, sequences with less than 100 identical replicate copies were excluded from the dataset in an attempt to mitigate sequencing error. Many taxa in the present study, such as *C. erinacei, C. ubiquitum*, *C. scrofarum, C. ryanae*, *C. meleagridis*, and *C. bovis* are only present in low abundances, and therefore their presence cannot be attributed to cross contamination from highly infected samples.

Analysis of the oocyst load per gram of faeces (g^{-1}) revealed that the highest median concentration of *Cryptosporidium* oocysts was shed by cattle; 31,072 oocyst/g^{-1}, followed by rabbits (27,919 oocyst/g^{-1}), sheep (26,756 oocyst/g^{-1}), pigs (21,164 oocyst/g^{-1}), kangaroos (16,018 oocyst/g^{-1}) and birds (10,032 oocyst/g^{-1}). These values are much higher than a previous study, which examined a range of animal faeces in Sydney catchments and reported that the range of oocyst shedding concentration for cattle was <1–17,467 g^{-1}, with medians of 0.5–23 oocysts g^{-1} for adult and juvenile cattle respectively, for sheep, a range of 1–152,474 g^{-1} with medians of 148 and 275 g^{-1} for adults and juveniles respectively, <1-770 g^{-1} for pigs with a median of 0.5 g^{-1} and 1–39,423 g^{-1} for kangaroos with a median of 0.5 g^{-1} (Davies et al., 2003). Studies in the US reported that feedlot cattle shed 7.7×10^4 to 2.3×10^5 and 1.3 to 3.6 oocysts g^{-1} respectively (Hoar et al., 2000; Atwill et al., 2006a). This translates to 1.7×10^5 and $1.4–2.8 \times 10^4$ oocysts/animal per day (Hoar et al., 2000; Atwill et al., 2006a). A limitation of these studies, is that oocyst shedding is intermittent (Xiao and Herd, 1994) and recovery rates from faecal samples and across animal types can be highly variable. For example, recovery rates ranging from 14 to 70% for adult cattle faeces, 0–83% for calf faeces, 4–48% for sheep faeces, 40–73% for kangaroo faeces, and 3–24% for pig faeces have been reported (Davies et al., 2003). A more recent study based on qPCR, reported a range of 63–7.9×10^6 and a median of 3.2×10^4 g^{-1} for oocysts in sheep faeces across three states (WA, NSW and South Australia) (Yang et al., 2014). Other studies have reported that neonatal calves can excrete up to 30 billion oocysts or more over a 1–2 week period (Kuczynska and Shelton, 1999) and that even apparently healthy animals can shed high numbers of oocysts (>5×10^6 oocysts g^{-1}) (Chalmers and Giles, 2010). This coupled with the very low infectious dose (10–100 oocysts) of *Cryptosporidium* (DuPont et al., 1995), has resulted in very significant numbers of oocysts entering drinking water supplies resulting in outbreaks e.g. the waterborne outbreak of cryptosporidiosis in humans in England in 2008 caused by *C. cuniculus* (Puleston et al., 2014).

In the present study, oocyst numbers (g^{-1}) were determined directly by qPCR using droplet digital PCR (ddPCR) calibrated standards, which obviates the need for recovery rate calculations and has the advantage of providing more accurate quantitation (Yang et al., 2014). Unfortunately, as the population size of animal hosts are unknown in all the catchments, it is not possible to calculate catchment loading of oocysts. This is clearly a knowledge gap that needs to be addressed.

In the present study, a total of 17 *Cryptosporidium* species and four genotypes were detected (Sanger sequencing combined with NGS). Of these, 13 are infectious to humans; *C. parvum, C. hominis* and *C. meleagridis* are the most common species in humans in Australia (Ryan and Power, 2012), *C. ubiquitum* and *C. cuniculus* are considered emerging human pathogens (Puleston et al., 2014; Koehler et al., 2014a; Li et al., 2015), there have been numerous reports of *C. canis*, *C. felis*, *C. muris*, *C. suis, C. erinacei* and *C. scrofarum* in humans (cf. Ryan et al., 2017), two reports of *C. bovis* (Khan et al., 2010; Ng et al., 2012) and one report of *C. fayeri* in humans (Waldron et al., 2010). This is the first report of *C. erinacei* in Australia. It was detected in cattle and kangaroo faecal samples by NGS only, where it accounted for 2–32% of reads (Supplementary Table 1). As with the prevalence of oocysts in source water, very little is known about the species of *Cryptosporidium* in source water in Australia, but a study in South Australia identified *C. parvum, C. muris, C. ubiquitum, C. ryanae, C. bovis, C. cuniculus* (subtypes Va and Vb), *C. fayeri, C. canis*, rat genotype and mouse genotype II (Swaffer et al., 2014; King et al., 2015). Little is known however about the prevalence of *Cryptosporidium* species in source water in other states and future studies in this area are needed.

In cattle, the prevalence of *Cryptosporidium* was high (22.3%–26.3%) across three states and *C. parvum* was the dominant species ranging from 39.1% to 50.7% of samples positive for *Cryptosporidium* in cattle in each state, followed by *C. bovis* (17.6%–28.1%), *C. muris* (8.1%–15.6%) *C. hominis* (4.7–14.1%), *C. ubiquitum* (2.7%) and *C. ryanae* (1.6%–19.7%). *Cryptosporidium andersoni* was not detected. Most of the cattle sampled were adult cattle and therefore the high prevalence of *C. parvum* is surprising, as other studies have suggested that *C. parvum* dominates in pre-weaned calves but that *C. bovis, C. ryanae* and *C. andersoni* dominate in older cattle (Santín et al., 2008). This highlights the importance of site-specific analysis for accurate QMRA analysis. The *C. parvum gp60* subtypes identified (IIaA15G2R1, IIaA16G2R1, IIaA17G2R1, IIaA18G3R1, IIaA19G2R1, IIaA19G3R1 and IIaA13G1) are commonly identified subtypes in humans and animals worldwide (Xiao, 2010; Feng et al., 2013), with the exception of subtype IIaA13G1, which has previously only been detected in a single human patient from WA (Ng-Hublin et al., 2013).

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Cryptosporidium hominis was detected in cattle faecal samples across all three states at a prevalence ranging from 4.5 to 14.1%. Although *C. hominis* predominately infects humans, it has been previously reported in cattle in Australia (Zahedi et al., 2016b), China (Chen and Huang, 2012; Zhang et al., 2018), Kenya (Kang'ethe et al., 2012), Korea (Park et al., 2006), Malawi (Banda et al., 2009), New Zealand (Abeywardena et al., 2012), and Scotland (Smith et al., 2005). However, there is no molecular evidence confirming transmission of *C. hominis* between cattle and humans, and therefore more studies should be conducted to fully elucidate the transmission dynamics of *C. hominis* in cattle. In the present study, two *C. hominis* subtypes were detected in cattle; IbA10G2 and IdA15G1. Subtype IbA10G2 is a dominant subtype responsible for *C. hominis*-associated outbreaks of cryptosporidiosis worldwide (Xiao, 2010). Subtype IdA15G1 was identified in three cattle isolates from WA and has been detected in humans from Victoria with a history of gastrointestinal disorders (Koehler et al., 2013). It is also the dominant subtype infecting Aboriginal people in WA (Ng-Hublin et al., 2017).

Cryptosporidium macropodum, which is currently considered non-zoonotic, was the dominant species in kangaroos (49.2% of samples positive for *Cryptosporidium* in kangaroos), followed by *C. fayeri* (27.5%). Three *C. fayeri gp60* subtypes were identified; IVfA12G1T1 and two novel subtypes; IVaA11G3T1 and IVgA10G1T1R1. The former subtype has previously been reported in kangaroos (Power et al., 2009), but this is the first report of IVaA11G3T1 and IVgA10G1T1R1 in marsupials. The subtype identified in the first human patient infected with *C. fayeri* was IVaA9G4T1R1 (Waldron et al., 2010) and has previously been identified in eastern grey kangaroos from NSW catchments (Power et al., 2009).

In addition to *C. macropodum* and *C. fayeri, C. hominis, C. parvum*, *C. galli* and kangaroo genotype I were also detected in kangaroos from NSW and WA in 13.2%, 9.2%, 0.5% and 0.2% of positives, respectively. Unfortunately, no faecal samples from kangaroos were collected from QLD catchments and given the identification of *C. hominis* in kangaroos from NSW, future studies in catchment areas of QLD should include kangaroos. In NSW catchments, a previous study reported an overall prevalence of 5% for *Cryptosporidium* from 952 animal faecal samples and 3.6% (21/576) in kangaroos (Zahedi et al., 2016b). The present study includes these samples and extends the analysis to include a total of 1521 samples from NSW with an overall prevalence of 16.6% (95% CI, 14.8–18.6) and a prevalence of 14% (95% CI, 11.7–16.6) in kangaroos in NSW. The prevalence of *Cryptosporidium* species in Eastern grey kangaroo faecal samples in Sydney catchments has been analysed in several studies. The first genetic study (2000–2002), reported a prevalence of 6.7% (239/3557) (Power et al., 2005) and identified only *C. macropodum* and *C. fayeri* in the populations (Power et al., 2004). The second major study (2006–2008), identified a prevalence of 16.9% (27/160) and identified *C. macropodum* (n = 2), *C. parvum* (n = 6), *C. hominis* (n = 18) and a *C. parvum*-like isolate (n = 1) (Ng et al., 2011b). However, the finding of *C. hominis* and *C. parvum* in the kangaroo faecal samples in that study could not be confirmed at additional loci, presumably due to the low levels of oocysts in the samples. In the present study, *C. hominis* was identified by Sanger analysis and NGS in 5.2% (43/835; 3.8–6.9) of kangaroos screened in NSW and in the previous related study by Zahedi et al. (2016b), the median numbers of *C. hominis*/g^{-1} was 4831 with a range of 26–16,890 g^{-1} (Zahedi et al., 2016b), indicating that significant numbers of oocysts were present in some samples. Another recent study also analysed these kangaroo-derived *C. hominis* isolates using both Sanger and NGS (Zahedi et al., 2017). In that study, unlike *C. parvum* isolates, in which additional within-host *gp60* subtype diversity was identified by NGS, only one *C. hominis* subtype was identified by both Sanger and NGS in the kangaroo-derived DNA samples, suggesting a single, recent introduction of *C. hominis* into kangaroos (Zahedi et al., 2017). The *C. hominis* in the kangaroos may have come from spill-back from humans in the catchments, which may have also have spilled-over to infect cattle in the catchments. The lack of identification of *C. hominis* in kangaroos in NSW catchments prior to 2011 tends to support this. However, only a small fraction of samples were typed in those studies and it is not possible to determine if even the same kangaroo populations were analysed in the previous studies and therefore it is impossible to draw any real inferences. Collection site coordinates of *C. hominis* positive kangaroo and cattle samples in NSW indicated that there was a geographical overlap between areas from which six cattle and nine kangaroo *C. hominis* positives (including both subtypes IbA10G2 and IdA15G1) were collected (S-34.61278, E150.58498). *Cryptosporidium galli* (a common bird parasite) and kangaroo genotype I, previously only reported in western grey kangaroos in WA (Yang et al., 2011) were also detected in kangaroos, but neither are considered zoonotic.

Despite being the third most common *Cryptosporidium* species detected in humans in Australia (Ryan and Power, 2012), *C. meleagridis* was only detected in three bird isolates from WA, however very low numbers of faecal samples were collected from birds from WA (n = 7) and QLD (n = 9) and no samples were collected from NSW. The *C. meleagridis gp60* subtype detected was IIIeA18G2R1, which has not been previously reported.

Cryptosporidium cuniculus was not detected in the five samples that were collected from QLD, but was detected at an overall prevalence of 27.6% (60/217) in rabbits in NSW and 9.6% (43/450; 95% CI, 7–12.7%) in rabbits in WA. This species has been previously identified in rabbits, humans and a kangaroo in Australia (Nolan et al., 2010, 2013; KF279538; Koehler et al., 2014a). It has also been linked to a number of sporadic human cases across the UK (Chalmers et al., 2011; Elwin et al., 2012), Nigeria (Molloy et al., 2010) and France (ANOFEL, 2010) and was implicated in a waterborne outbreak of cryptosporidiosis in humans in England in 2008 (Chalmers et al., 2009; Puleston et al., 2014). Two distinct *gp60* subtype families, designated Va and Vb have been identified in *C. cuniculus* (Chalmers et al., 2009). In the present study, all six subtypes belonged to the Vb subtype family (VbA18, VbA23, VbA25, VbA26, VbA28 and VbA29). Most cases described in humans relate to Va and the first waterborne outbreak was typed as VaA22 (Robinson et al., 2008; Chalmers et al., 2009), but a VbA25 variant and VbA27 have been reported in human patients in Australia (KF279538; Koehler et al., 2014b).

Cryptosporidium ubiquitum was the only species detected in sheep by Sanger sequencing (5.5%, 11.1% and 11.3% prevalence across NSW, QLD and WA respectively). It was also detected 33.3% of feral deer faecal samples in QLD. *Cryptosporidium ubiquitum* has not been identified in Australia in the limited typing of Australian human *Cryptosporidium* isolates that has been conducted to date (Ryan and Power, 2012), however it has been identified in surface waters in Australia (Monis et al., unpublished). Subtyping identified all *C. ubiquitum* positives as subtype XIIa, which has been found in humans and therefore XIIa is a potentially zoonotic subtype (Li et al., 2014).

In North America, both wildlife and domestic animals contribute to contamination of *Cryptosporidium* spp. in drinking water catchments, with prevalence ranging from 6 to 20.5% (Feng et al., 2007; Starkey et al., 2007; Ziegler et al., 2007a, b; Jellison et al., 2009; Szonyi et al., 2010). In those studies, the majority of *Cryptosporidium* spp. detected in wildlife species were host adapted (Feng et al., 2007; Ziegler et al., 2007a; Jellison et al., 2009), while some species such as *C. hominis*-like, *C. parvum*, *C. ubiquitum* and *C. meleagridis* occasionally detected in some hosts such as eastern

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gray squirrels, eastern chipmunks, beavers, woodchucks, raccoons, red-backed voles, deer, geese, and deer mice (Feng et al., 2007; Ziegler et al., 2007a; Jellison et al., 2009). In the present study, the majority of samples collected from wildlife were from kangaroos and rabbits and the most significant difference between the findings of the present study and studies conducted in North America, is the identification of *C. hominis* and *C. parvum* in wildlife (kangaroo) populations. In North American catchments, *C. parvum, C. bovis, C. ryanae* and *C. andersoni* are the most commonly reported *Cryptosporidium* in cattle faeces (Starkey et al., 2007; Szonyi et al., 2010). With the exception of *C. andersoni*, these species were also detected in cattle faeces in the present study, along with *C. hominis.* Further studies are required to understand the source and human health significance of *C. hominis* in both wildlife and livestock in drinking water catchments.

5. Conclusions

- The present study has provided an extensive analysis of *Cryptosporidium* species and subtypes from 5774 faecal samples collected from various host species in 11 water catchments across three states of Australia (QLD, NSW and WA).
- The overall prevalence of *Cryptosporidium* was 18.3% and of the 17 *Cryptosporidium* species and 4 genotypes identified, 13 species are of public health significance.
- The significant findings were the detection of a *C. hominis* in cattle and kangaroos and the high prevalence of *C. parvum* in cattle. In addition, two novel *C. fayeri* subtypes (IVaA11G3T1 and IVgA10G1T1R1), one novel *C. meleagridis* subtype (IIIeA18G2R1) were identified and *C. erinacei* was reported for the first time in Australia
- The identification of zoonotic *Cryptosporidium* species in both livestock and wildlife has implications for management of drinking water sources.
- Continued identification of the sources/carriers of human pathogenic strains as well as accurate assessments of the population size of various hosts in catchments is therefore essential for accurate risk assessment and optimal catchment management.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.watres.2018.02.005.

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Appendix 6

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Profiling the diversity of *Cryptosporidium* species and genotypes in wastewater treatment plants in Australia using next generation sequencing

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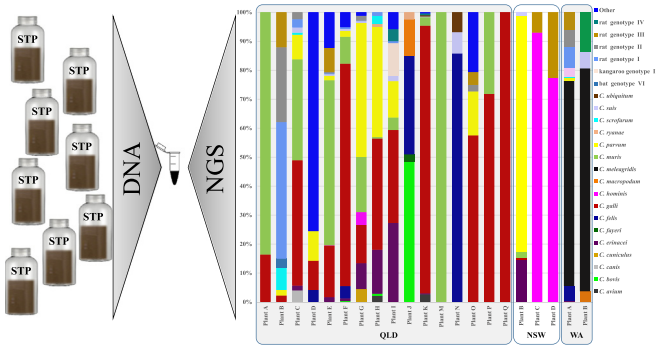
HIGHLIGHTS

- NGS analysis of *Cryptosporidium* spp. in raw wastewater samples ($n = 730$)
- Detection of large diversity of *Cryptosporidium* spp. and genotypes in wastewater
- Identification of *C. hominis*, *C. parvum* and *C. meleagridis* in untreated wastewater
- Potential contribution of livestock, wildlife and birds to wastewater contamination

GRAPHICAL ABSTRACT



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ABSTRACT

Wastewater recycling is an increasingly popular option in worldwide to reduce pressure on water supplies due to population growth and climate change. *Cryptosporidium* spp. are among the most common parasites found in wastewater and understanding the prevalence of human-infectious species is essential for accurate quantitative microbial risk assessment (QMRA) and cost-effective management of wastewater. The present study conducted next generation sequencing (NGS) to determine the prevalence and diversity of *Cryptosporidium* species in 730 raw influent samples from 25 Australian wastewater treatment plants (WWTPs) across three states: New South Wales (NSW), Queensland (QLD) and Western Australia (WA), between 2014 and 2015. All samples were screened for the presence of *Cryptosporidium* at the 18S rRNA (18S) locus using quantitative PCR (qPCR), oocyst numbers were determined directly from the qPCR data using DNA standards calibrated by droplet digital PCR, and positives were characterized using NGS of 18S amplicons. Positives were also screened using *C. parvum* and *C. hominis* specific qPCRs. The overall *Cryptosporidium* prevalence was 11.4% (83/730): 14.3% (3/21) in NSW; 10.8% (51/470) in QLD; and 12.1% (29/239) in WA. A total of 17 *Cryptosporidium* species and six genotypes were detected by NGS. In NSW, *C. hominis* and *Cryptosporidium* rat genotype III were the most prevalent species (9.5% each). In QLD, *C. galli*, *C. muris and C. parvum* were the three most prevalent species (7.7%, 5.7%, and 4.5%,

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respectively), while in WA, *C. meleagridis* was the most prevalent species (6.3%). The oocyst load/Litre ranged from 70 to 18,055 oocysts/L (overall mean of 3426 oocysts/L: 4746 oocysts/L in NSW; 3578 oocysts/L in QLD; and 3292 oocysts/L in WA). NGS-based profiling demonstrated that *Cryptosporidium* is prevalent in the raw influent across Australia and revealed a large diversity of *Cryptosporidium* species and genotypes, which indicates the potential contribution of livestock, wildlife and birds to wastewater contamination.

1. Introduction

Australia is the driest of the world's inhabited continents, with the lowest percentage of rainfall as run-off and the lowest amount of water in rivers (Anonymous, 2004). Drinking water resources are under considerable strain as a result of major shifts in long-term climate change, and climate predictions for all Australian States and Territories suggest increasing temperatures, a decline in average rainfall, but increasing severity and frequency of storm events (Garnaut Review, 2008). Consequently, there is increasing pressure for more efficient use of water resources, both in urban and rural environments (Toze, 2006a). Recycling wastewater will help address these challenges and is a prominent option among the various alternative sources of water in both developing and developed countries (Miller, 2006; Mekala and Davidson, 2016). However, infection with pathogenic microorganisms is a major risk factor (Rodriguez-Manzano et al., 2012) and therefore water destined for reuse must be fit for purpose (Toze, 2006b).

The waterborne parasite *Cryptosporidium* represents an important public health concern for water utilities, as it is a major cause of diarrhoea and there is neither a vaccine nor an effective treatment (Ryan et al., 2016; Zahedi et al., 2016a). *Cryptosporidium* is particularly suited to waterborne transmission as the oocyst stage is highly resistant to chlorine disinfection and can penetrate and survive routine water and wastewater treatment systems (King and Monis, 2007; King et al., 2017; Ryan et al., 2017a). The parasite has been responsible for numerous large-scale waterborne outbreaks worldwide (Efstratiou et al., 2017) and is highly prevalent in wastewater (Amorós et al., 2016; Ma et al., 2016).

Cryptosporidium species are currently monitored in wastewater using standard detection methodologies (i.e. fluorescence microscopy using EPA method 1623 - USEPA, 2012), however, this method cannot discriminate between different *Cryptosporidium* species. Of the 37 recognised *Cryptosporidium* species, *C. hominis* and *C. parvum* are the dominant species that infect humans (Ryan et al., 2016; Zahedi et al., 2017a; Čondlová et al., 2018; Kváč et al., 2018). As not all species of *Cryptosporidium* are infectious to humans (Ryan et al., 2016), understanding the diversity of *Cryptosporidium* in wastewater is crucial for more accurate quantitative microbial risk assessment (QMRA), for proper management of wastewater and its recycling. Due to the complex composition, abundance, and distribution patterns of *Cryptosporidium* species present in wastewater samples, molecular techniques such as conventional PCR and Sanger sequencing-based genotyping methods are unable to resolve complex DNA mixtures due to mixed sequencing chromatograms and are also unable to detect low abundance species or variants of *Cryptosporidium* (which typically appear as a "bumpy baseline" in Sanger chromatograms) (Murray et al., 2015; Paparini et al., 2015; Grinberg and Widmer, 2016).

Next-generation sequencing (NGS) technologies have allowed the comprehensive characterization and deep coverage of microbial community structure and diversity in environmental samples such as soil, water, the atmosphere and other environments (Cruaud et al., 2014). NGS is also more sensitive for the detection of less abundant species within microbial communities (Salipante et al., 2013). Recently, NGS approaches have been described that examine the composition and diversity of microbial communities (Shanks et al., 2013; Ma et al., 2015; Newton et al., 2015; Xu et al., 2017), adenovirus (Ogorzaly et al., 2015), norovirus (Prevost et al., 2015), astrovirus (Brinkman et al., 2013) and protists (Maritz et al., 2017) in sewage. However, to the best of the authors' knowledge, to date no large scale longitudinal studies have been undertaken to investigate the composition and diversity of *Cryptosporidium* species in wastewater using high-throughput amplicon NGS. As the costs of NGS continue to decrease and the bioinformatics analysis of data continues to improve, NGS screening of wastewater samples has become more feasible (Muir et al., 2016).

Therefore, the aim of the present study was to use NGS, for the first time on a large scale, to more accurately determine the prevalence and composition of *Cryptosporidium* species in Australian WWTPs across three states: New South Wales (NSW), Queensland (QLD) and Western Australia (WA).

2. Materials and methods

2.1. Study sites and sample collection

In NSW, WWTP samples (250 mL raw influent) were collected on a monthly interval over five months (April 2015 to August 2015). A total of 21 WWTP samples were collected from four wastewater plants within the WaterNSW area of operations (greater Sydney) (Table 1). In QLD, a total of 470 WWTP samples (250 mL raw influent) were collected on fortnightly intervals from WWTP sites ($n = 19$) across south east Queensland (Table 1) over a year (January 2014 to January 2015). In WA, a total of 239 WWTP samples (250 mL raw influent) were collected from two treatment plants on weekly intervals from December 2014 to December 2015 (Table 1). All raw influent WWTP samples were collected into individual 250 mL collection pots and stored at 4 °C until required and samples collected in NSW and QLD were shipped to Murdoch University for analysis.

2.2. Sample processing and DNA isolation

All 250 mL WWTP samples were transferred to 50 mL centrifuge tubes, and evenly weighed tubes ($n = 5$) were prepared from the same samples. These samples were centrifuged at 10,000 ×*g* for 20 min and pellets from the same samples were mixed together again. DNA was extracted from aseptically separated 250 mg aliquots of each sample (pellet), using a Power Soil DNA Kit (MO BIO, Carlsbad, California, USA) (Walden et al., 2017). An extraction blank (no WWTP sample) and a positive extraction control (a *Cryptosporidium* positive faecal sample from a kangaroo), was included in each extraction batch, as a process control for extraction efficiency. Purified DNA was stored at −20 °C prior to molecular analyses.

2.3. qPCR and oocyst enumeration

All WWTP sample extracts were screened for the presence of *Cryptosporidium* at the 18S rRNA (18S) locus using a quantitative PCR (qPCR) as previously described (King et al., 2005; Yang et al., 2014). A spike analysis of the 18S qPCR assay (addition of 0.5 μL of positive control DNA into test samples) was conducted on randomly selected negative samples from each group of DNA extractions, to determine if negative results were due to PCR inhibition by comparing the cycle threshold (Ct) values of the spike and the positive control (both with same

concentration of DNA). In addition, *Cryptosporidium* oocyst concentrations in each sample (oocyst numbers per litre) were determined directly from the qPCR data using DNA standards calibrated by droplet digital PCR (ddPCR) (QX100™ droplet digital PCR system, Bio-Rad), which has the advantage of providing more accurate quantitation (Yang et al., 2014). Briefly, target copy numbers of the 18S gene detected in individual samples were converted to estimates of oocyst numbers based on the fact that the 18S gene in *Cryptosporidium* has five copies per haploid sporozoite (Le Blancq et al., 1997; Abrahamsen et al., 2004), and there are four haploid sporozoites per oocyst. Therefore, every 20 copies of 18S detected by qPCR were equivalent to one oocyst. To estimate oocyst density per litre, oocyst numbers detected per 250 mg aliquots of each sample (pellet) were extrapolated to the corresponding total pellet weight extracted from each 250 mL wastewater sample, and then multiplied by four.

2.4. Next generation sequencing

Samples that were positive by qPCR were analysed by NGS on the MiSeq (Illumina) platform at the 18S locus using the 18S iF/iR primers (Morgan et al., 1997) that were modified to contain MiSeq adapter sequences on the 5′ and 3′ end as previously described (Paparini et al., 2015). The library was prepared as per standard protocols for the MiSeq platform (Illumina Demonstrated Protocol: 16S Metagenomic Sequencing Library Preparation) with the following modifications: all PCR amplicons (uniquely indexed per sample) were double purified using the Agencourt AMPure XP Bead PCR purification protocol (Beckman Coulter Genomics, USA) and pooled in approximate equimolar ratios (based on gel electrophoresis). Sequencing was performed on an Illumina MiSeq using 500-cycle V2 chemistry (250 bp paired-end reads) following the manufacturer's recommendations. Two no-template controls and two DNA extraction reagent blank controls were included in the library preparation and distributed between samples in the PCR plate layout. All no-template and extraction reagent blank controls produced no detectable amplification of *Cryptosporidium* DNA throughout the library preparation. This indicated that level of cross contamination between samples, or from the laboratory environment, was below the detection limit of the library preparation procedure and for this reason were not sequenced. We have also previously sequenced extraction blanks and no-template controls from other NGS studies in our laboratory, and after quality filtering, <10 reads were detected in those samples.

2.5. Species-specific PCR for detection and enumeration of C. hominis and C. parvum

All WWTP samples positive for *Cryptosporidium* spp. by qPCR at the 18S locus were also screened and enumerated independently using primers and species-specific minor groove binder (MGB) probes to a unique *Cryptosporidium* specific gene (Clec) that codes for a novel mucin-like glycoprotein that contains a C-type lectin domain to confirm the presence/absence of *C. hominis* and *C. parvum* as described by Yang et al. (2013).

2.6. Statistical analysis

The overall prevalence of *Cryptosporidium* in samples collected from each WWTP was expressed as the percentage of samples positive by combined qPCR and NGS, with 95% confidence intervals calculated assuming a binomial distribution, using the software Quantitative Parasitology 3.0 (Rózsa et al., 2000). DNA extraction efficiency was estimated for each extraction, based on the number of the gene copies/oocyst equivalents measured by ddPCR. Odds ratios (OR) and their 95% confidence intervals were used to measure the strength of association of season (risk factor) with the occurrence of the *Cryptosporidium* species in WWTP samples. Chi-square and non-parametric analyses were performed using IBM SPSS 21.0 (statistical package for the social sciences) for Windows (SPSS Inc. Chicago, USA) to determine if there were any associations between the prevalence and concentration of *Cryptosporidium* oocysts at different sampling seasons and across states.

2.7. Bioinformatics analysis

Illumina MiSeq sequencing resulted in 1,068,270,250 bp paired-end reads with 78% of the basecalls >Q30. Paired-end reads were merged and quality filtered with USEARCH v9.2 (Edgar, 2010), retaining reads with >50 bp merged overlap, <0.1% expected error, no mismatches in the primer sequences, a minimum length of 200 bp, and a minimum of 100 identical replicate copies as previously described (Zahedi et al., 2017b). Primer sequences and any distal bases were also removed from all reads. Reads were then denoised and chimera filtered with the UNOISE3 algorithm (Edgar, 2016) to generate 169 zero-radius operational taxonomic units (ZOTUs) that represent unique biologically correct sequences (Edgar, 2016). *Cryptosporidium* 18S ZOTU sequences were assigned taxonomy by comparing ZOTUs to a curated custom database containing 63 reliable 18S reference sequences from 35 *Cryptosporidium* species and 28 genotypes extracted from GenBank using the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990). Taxonomy was only assigned if there was a single unambiguous BLAST hit with >99% pairwise identity over >98% of the query ZOTU sequence. Of the 169 ZOTUs generated, 62 did not match any known *Cryptosporidium* species or genotypes. These non-specific ZOTUs were compared to GenBank using BLAST, and where possible, taxonomy was assigned when queries hit reference sequences with >99% identity over >98% of the query reads and matched to no other taxa at the same level. Many of these non-specific ZOTUs were assigned to uncultured eukaryotes or benign waterborne fungi, algae and dinoflagellates (Supplementary Table 1). Although abundant at the ZOTU level, these non-specific sequences represented a very small proportion of the total reads per sample (mean 0.71%).

3. Results

3.1. Overall prevalence of Cryptosporidium in WWTP samples

In the present study, a total of 730 WWTP samples from 25 WWTPs across three states in Australia (NSW, QLD and WA) were screened using qPCR, and the composition of *Cryptosporidium* species in positive samples was determined by NGS. Results were tabulated as the prevalence of the most abundant single species (determined by NGS), detected per sample (Table 2) and the prevalence of all *Cryptosporidium* species detected across all samples regardless of their abundance (Table 3). Overall, *Cryptosporidium* was detected in 11.4% (83/730; 95% CI, 9.2–13.9) of WWTP samples collected across three states. (Table 4 and Supplementary Table 1). This comprises a prevalence of 14.3% (3/21; 95% CI, 3–36.3) in NSW, 10.8% (51/470; 95% CI, 8.2–14) in QLD and 12.1% (29/239; 95% CI, 8.3–17) in WA. However, there was no significant difference between the prevalence in different states ($p > 0.05$). In general, across the three states, samples collected in summer were 1.9 times more likely to have *Cryptosporidium* than samples collected during winter months (Odds ratio = 1.9; 95% CI, 1.2–3.4), but there was no statistical difference between samples collected in spring, winter and autumn ($p > 0.05$). For NSW, samples were only collected for autumn and winter.

In QLD, the prevalence of *Cryptosporidium* peaked at 17.5% (18/103; 95% CI, 10.7–26.2) during summer months (averaged over two partial summers; 2014 and 2015), when the samples were 2.3 times more likely to have *Cryptosporidium* than samples collected during winter months (averaged over winter 2014 and 2015) (Odds ratio = 2.3; 95% CI, 1.2–5.2). There was no significant difference between the prevalence in spring, autumn and winter ($p > 0.05$). Unlike QLD, WA had the highest prevalence of *Cryptosporidium* in WWTP samples collected

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Table 1
Wastewater treatment plants (WWTPs) included in the present study.

	Type of plant	Rural/urban plant	Source of sewage	Size of community served	Trade waste received/not received?	Nature of trade waste	Any storm water intrusion	Animal presence/activity around the plant
QLD								
Plant A	Trickle filter	Rural	Domestic/some commercial	9000	Not received	NA	Yes	Yes (cattle in neighbouring properties)
Plant B	Lagoon	Rural	Domestic/some commercial	500	Not received	NA	Yes	Yes (cattle in neighbouring field/wildlife, kangaroo/koalas)
Plant C	Extended activated sludge	Rural	Domestic/some commercial	1000	Not received	NA	Yes	Yes (bats)
Plant D	Activated sludge	Rural	Principally domestic	36,000	Small portion is trade waste	Industrial trade waste	Yes	No
Plant E	Activated sludge	Urban	Principally domestic	45,000	Significant portion is trade waste	Chemical trade waste/tannery waste	Yes	WWTP adjoined to scrub area with possible animal activity, i.e. kangaroos etc. but no linkage to plant inflow.
Plant F	Activated sludge	Rural	Principally domestic	1000	Small portion is trade waste	Restaurant waste	Yes	WWTP in rural area with cattle grazing adjacent but no linkage to plant inflow.
Plant G	Activated sludge	Rural	Principally domestic	105,000	Small portion is trade waste	Industrial trade waste	Yes	WWTP adjoined to scrub area with possible animal activity, i.e. kangaroos etc. but no linkage to plant inflow.
Plant H	Activated sludge	Rural	Principally domestic	12,500	Insignificant portion is trade waste	Rendering plant that pre-treats waste before sending waste to WWTP	Yes	WWTP adjoined to scrub area with possible animal activity, i.e. kangaroos etc. but no linkage to plant inflow.
Plant I	Activated sludge	Rural	Principally domestic	2400	Insignificant portion is trade waste	Industrial waste	Yes	WWTP in rural area with cattle grazing adjacent but no linkage to plant inflow.
Plant J	Activated sludge	Urban	Principally domestic	118,000	Significant portion is trade waste	Restaurant waste	Yes	No
Plant K	Activated sludge	Urban	Principally domestic	60,000	Small portion is trade waste	Seafood waste	Yes	No
Plant L	Activated sludge	Rural	Principally domestic	126,000	Significant portion is trade waste	Industrial trade waste	Yes	No
Plant M	Activated sludge	Rural	Decommissioned (August 2014)	Decommissioned	Decommissioned	NA	Yes	N/A
Plant N	Activated sludge	Rural	Principally domestic	22,000	Small portion is trade waste	Industrial trade waste	Yes	WWTP adjoined to scrub area with possible animal activity, i.e. kangaroos etc. but no linkage to plant inflow.
Plant O	Facultative lagoons	Rural	Principally domestic	300	Small portion is trade waste	Restaurant waste	Yes	WWTP adjoined to scrub area with

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								possible animal activity, i.e. kangaroos etc. but no linkage to plant inflow.
Plant P	Activated sludge	Rural	Principally domestic	43,000	Significant portion is trade waste	Food manufacturing waste	Yes	No
Plant Q	Activated sludge	Rural	Principally domestic	47,000	Small portion is trade waste	Restaurant waste	Yes	WWTP adjoined to scrub area with possible animal activity, i.e. kangaroos etc. but no linkage to plant inflow.
Plant R	Activated sludge	Urban	Principally domestic	5000	Small portion is trade waste	Restaurant waste	Yes	No
Plant S	Activated sludge	Rural	Principally domestic	26,000	Small portion is trade waste	Restaurant waste	Yes	WWTP adjoined to scrub area with possible animal activity, i.e. kangaroos etc. but no linkage to plant inflow.
NSW								
Plant A	Oxidation ditch (Pasveer), sludge lagoons	Urban	Domestic and industrial	2000	Received	Septic tank waste, network waste (food prep, accommodation, vehicle workshop)	Yes	Yes (native wildlife (wombats, kangaroos and birds))
Plant B	IDEA tank, oxidation ditch (Pasveer) (not in operation), sludge drying/lagoons	Urban	Domestic and industrial	5400	Received	Septic tank waste, network waste (food prep, accommodation, vehicle workshop)	Yes	Yes (Native wildlife (wombats, kangaroos and birds))
Plant C	IDEA tank, sludge drying/lagoons.	Urban	Domestic and industrial	9000	Received	Septic tank waste, network waste (food prep, accommodation, vehicle workshop)	Yes	Yes (native wildlife (wombats, kangaroos and birds))
Plant D	IDAL aeration, oxidation ditch (Pasveer), sludge drying/lagoons	Urban	Domestic and industrial	14,600	Received	Septic tank waste, network waste (food prep, accommodation, vehicle workshop)	Yes	Yes (native wildlife (wombats, kangaroos and birds))
WA								
Plant A	Activated sludge	Urban	Principally domestic (but also receives industrial waste through the sewer network)	75,000	Received	Septage and grease trap waste, abattoir and farm waste (through third party tankers)	Yes	Limited (foxes, feral cats, birds and snakes)
Plant B	Pond system	Urban	Domestic	5000	Not received	NA	Yes	Cattle, kangaroos and birds (turtles snakes and birds live in the ponds)

NA = Not Available.
IDEA = Intermittently Decanted Extended Aeration.
IDAL = Intermittently Decanted Aerated Lagoons.

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Table 2
Prevalence of the most abundant *Cryptosporidium* species detected by NGS of individual wastewater treatment plant (WWTP) samples across three states of Australia; NSW, QLD and WA (based on a single species that was the most abundant species detected in each sample).

Host	*C. parvum* No +/total no (% proportion + 95% CI)	*C. hominis* No +/total no (% proportion + 95% CI)	*C. bovis* No +/total no (% proportion + 95% CI)	*C. muris* No +/total no (% proportion + 95% CI)	*C. erinacei* No +/total no (% proportion + 95% CI)	*C. meleagridis* No +/total no (% proportion + 95% CI)	*C. galli* No +/total no (% proportion + 95% CI)	*C. canis* No +/total no (% proportion + 95% CI)	*C. felis* No +/total no (% proportion + 95% CI)	*C. suis* No +/total no (% proportion + 95% CI)	*C. macropodum* No +/total no (% proportion + 95% CI)	Other No +/total no (% proportion + 95% CI)
QLD												
Plant A	ND	ND	ND	1/25 (4%, 0.1–20.4)	ND	ND	1/25 (4%, 0.1–20.4)	ND	ND	ND	ND	ND
Plant B	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	2[a]/20 (10%, 1.2–31.7)
Plant C	ND	ND	ND	ND	ND	ND	3/26 (11.5%, 2.4–30.2)	1/26 (3.8%, 0.1–19.6)	ND	ND	ND	ND
Plant D	1/41 (2.4%, 0.1–12.9)	ND	ND	ND	ND	ND	ND	ND	1/41 (2.4%, 0.1–12.9)	ND	ND	ND
Plant E	ND	ND	ND	2/41 (4.9%, 0.6–16.5)	1/41 (2.4%, 0.1–12.9)	ND	7/41 (17%, 7.2–32.1)	ND	ND	ND	ND	ND
Plant F	ND	ND	1/40 (2.5%, 0.1–13.2)	1/40 (2.5%, 0.1–13.2)	ND	ND	4/40 (10%, 2.8–23.7)	ND	1/40 (2.5%, 0.1–13.2)	ND	ND	ND
Plant G	2/41 (4.9%, 0.6–16.5)	ND	ND	1/41 (2.4%, 0.1–12.9)	ND	ND	2/41 (4.9%, 0.6–16.5)	ND	ND	ND	ND	ND
Plant H	1/41 (2.4%- 0.1–12.9)	ND	ND	ND	1/41 (2.4%, 0.1–12.9)	ND	2/41 (4.9%, 0.6–16.5)	ND	ND	ND	ND	ND
Plant I	ND	ND	ND	ND	ND	ND	1/41 (2.4%, 0.1–12.9)	ND	ND	ND	ND	ND
Plant J	ND	ND	1/41 (2.4%, 0.1–12.9)	ND	ND	ND	ND	ND	ND	ND	3/41 (7.3%, 1.5–19.9)	ND
Plant K	ND	ND	ND	ND	ND	ND	5/41 (12.2%, 4.1–26.2)	ND	ND	ND	ND	ND
Plant L	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Plant M	ND	ND	ND	1/7 (14.3%, 0.4–57.9)	ND	ND	ND	ND	ND	ND	ND	ND
Plant N	ND	ND	ND	ND	ND	ND	ND	ND	1/4 (25%, 0.6–80.6)	ND	ND	ND
Plant O	ND	ND	ND	ND	ND	ND	1/4 (25%, 0.6–80.6)	ND	ND	ND	ND	ND
Plant P	ND	ND	ND	ND	ND	ND	1/4 (25%, 0.6–80.6)	ND	ND	ND	ND	ND
Plant Q	ND	ND	ND	ND	ND	ND	1/4 (25%, 0.6–80.6)	ND	ND	ND	ND	ND
Plant R	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Plant S	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Total	4/123 (3.2%, 0.9–8.1)	ND	2/81 (2.5%, 0.3–8.6)	6/154 (3.9, 1.4–8.3)	2/81 (2.5%, 0.3–8.6)	ND	28/308 (9.1%, 6.1–12.9)	1/26 (3.8%, 0.1–19.6)	3/85 (3.5%, 0.7–10)	ND	3/41 (7.3%, 1.5–19.9)	2/20 (10%, 1.2–31.7)
NSW												
Plant A	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Plant B	1/5 (20%, 0.5–71.6)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Plant C	ND	1/5 (20%, 0.5–71.6)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Plant D	ND	1/6 (16.7%, 0.4–64.1)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Total	1/5 (20%, 0.5–71.6)	2/11 (18.2%, 2.3–51.8)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
WA												
Plant A	3/146 (2.1%, 0.4–5.9)	ND	ND	ND	ND	7/146 (4.8%, 1.9–9.6)	ND	ND	5/146 (3.4%, 1.1–7.8)	ND	ND	6[b]/146 (4.1%, 1.5–8.7)
Plant B	ND	ND	ND	ND	ND	6/93 (6.5%, 2.4–13.5)	ND	ND	ND	1/93 (1.1%, 0–5.8)	1/93 (1.1%, 0–5.8)	3[c]/93 (3.2%, 0.7–9.1)
Total	3/146 (2.1%, 0.4–5.9)	ND	ND	ND	ND	13/239 (5.4%, 2.9–9.1)	ND	ND	5/146 (3.4%, 1.1–7.8)	1/93 (1.1%, 0–5.8)	1/93 (1.1%, 0–5.8)	9/239 (3.8%, 1.7–7)

ND = not detected.
[a] Rat genotype I ($n = 1$), rat genotype II ($n = 1$).
[b] Rat genotype I ($n = 2$), rat genotype II ($n = 2$), rat genotype III ($n = 1$), kangaroo genotype I ($n = 1$).
[c] Kangaroo genotype I ($n = 3$).

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Table 3
Prevalence of all *Cryptosporidium* species/genotypes detected by NGS in wastewater treatment plant (WWTP) samples across three states of Australia; NSW, QLD and WA (regardless of abundance).

Cryptosporidium spp.	No+/total no (% proportion + 95% CI)		
	NSW	QLD	WA
C. hominis	**2/21 (9.5%; 95% CI, 1.2–30.4)**	**2/470 (0.4%; 95% CI, 0.1–1.5)**	**ND**
C. parvum	**1/21 (4.8%; 95% CI, 0.1–23.8)**	**21/470 (4.5%; 95% CI, 2.8–6.7)**	**3/239 (1.3%; 95% CI, 0.3–3.6)**
C. avium	ND	2/470 (0.4%; 95% CI, 0.1–1.5)	ND
C. bovis	ND	5/470 (1.1%; 95% CI, 0.3–2.5)	ND
C. canis	ND	1/470 (0.2%; 95% CI, 0–1.2)	ND
C. cuniculus	ND	1/470 (0.2%; 95% CI, 0–1.2)	ND
C. erinacei	1/21 (4.8%; 95% CI, 0.1–23.8)	14/470 (3.0%; 95% CI, 1.6–4.9)	3/239 (1.3%; 95% CI, 0.3–3.6)
C. fayeri	ND	1/470 (0.2%; 95% CI, 0–1.2)	ND
C. felis	ND	4/470 (0.8%; 95% CI, 0.2–2.2)	5/239 (2.1%; 95% CI, 0.7–4.8)
C. galli	1/21 (4.8%; 95% CI, 0.1–23.8)	36/470 (7.7%; 95% CI, 5.4–10.4)	ND
C. macropodum	ND	3/470 (0.6%; 95% CI, 0.1–1.9)	1/239 (0.4%; 95% CI, 0–2.3)
C. meleagridis	ND	ND	14/239 (5.9%; 95% CI, 3.2–9.6)
C. muris	1/21 (4.8%; 95% CI, 0.1–23.8)	27/470 (5.7%; 95% CI, 3.8–8.2)	1/239 (0.4%; 95% CI, 0–2.3)
C. ryanae	ND	3/470 (0.6%; 95% CI, 0.1–1.9)	ND
C. scrofarum	ND	3/470 (0.6%; 95% CI, 0.1–1.9)	2/239 (0.8%; 95% CI, 0.1–3)
C. suis	1/21 (4.8%; 95% CI, 0.1–23.8)	11/470 (2.3%; 95% CI, 1.2–4.1)	1/239 (0.4%; 95% CI, 0–2.3)
C. ubiquitum	ND	2/470 (0.4%; 95% CI, 0.1–1.5)	ND
Bat genotype VI	ND	1/470 (0.2%; 95% CI, 0–1.2)	ND
Kangaroo genotype I	ND	1/470 (0.2%; 95% CI, 0–1.2)	4/239 (1.7%; 95% CI, 0.5–4.2)
Rat genotype I	ND	9/470 (1.9%; 95% CI, 0.91–3.6)	2/239 (0.8%; 95% CI, 0.1–3)
Rat genotype II	ND	5/470 (1.1%; 95% CI, 0.3–2.5)	2/239 (0.8%; 95% CI, 0.1–3)
Rat genotype III	2/21 (9.5%; 95% CI, 1.2–30.4)	4/470 (0.8%; 95% CI, 0.2–2.2)	2/239 (0.8%; 95% CI, 0.1–3)
Rat genotype IV	ND	2/470 (0.4%; 95% CI, 0.1–1.5)	ND

ND = not detected.
The bold data indicates the most two common species of *Cryptosporidium* reported in humans in Australia, accounting for >95% of human infections.

during spring (16.8%; 95% CI, 8.3–28.5), while there was no significant difference between the prevalence in summer, autumn and winter ($p > 0.05$). Although the prevalence of different species peaked at different times (Supplementary Table 1), in WA (Plant A), there was a winter peak in both *C. parvum* and rate genotype 1 and a spring peak for *C. felis*, and in plant B, there was a summer peak for *C. suis* (Supplementary Table 1).

3.2. Prevalence of all Cryptosporidium species/genotypes as determined by NGS (regardless of abundance)

A total of 17 *Cryptosporidium* species and six genotypes were detected by NGS (Table 3, and Supplementary Table 1). *Cryptosporidium hominis* and rat genotype III were the most prevalent species detected in wastewater samples collected from NSW (9.5% each, 2/21; 95% CI, 1.2–30.4). In addition to *C. hominis* and rat genotype III, *C. parvum* was detected in one NSW sample only (4.8%, 1/21; 95% CI; 0.1–23.8) and *C. erinacei*, *C. galli*, *C. muris* and *C. suis* were also detected in the same sample in low abundance (Table 3 and Supplementary Table 1). In QLD, of the 51 WWTP samples positive for *Cryptosporidium*, NGS detected more than one *Cryptosporidium* species/genotype in 42 samples, ranging from two to eight species in individual samples, whereas in nine samples, only one *Cryptosporidium* species/genotype was identified. In general, the prevalence of different *Cryptosporidium* species/genotypes detected by NGS in WWTP samples across QLD ranged from 0.2% to 7.7% (Table 3). *Cryptosporidium galli* (7.7%), *C. muris* (5.7%) and *C. parvum* (4.5%) were the three most prevalent (and abundant) species detected in WWTP samples from QLD, followed by *C. erinacei* (3.0%), *C. suis* (2.3%) and rat genotype I (1.9%) (Table 3), and were significantly more prevalent than all other species detected in samples from QLD ($p < 0.05$) (Table 3). Unlike QLD, the majority of samples positive for *Cryptosporidium* in WA (22/29) contained only one species/genotype of *Cryptosporidium* (75.9%; 95% CI, 56.5–89.7), and only seven samples were identified with mixed *Cryptosporidium* species present (24.1%; 95% CI, 10.3–43.5). *Cryptosporidium meleagridis* was detected in 5.9% (15/239; 95% CI, 3.2–9.6) of wastewater samples collected from WA, and was significantly more prevalent than any other species detected ($p < 0.05$). However, there was no significant difference between the prevalence of other *Cryptosporidium* species detected in WA samples ($p > 0.05$). *Cryptosporidium C. parvum* (1.3%), *C. erinacei* (1.3%), *C. scrofarum* (0.8%) and *C. muris* (0.4%) were detected at a low prevalence only in samples with mixed *Cryptosporidium* species/genotypes in WA (Table 3 and Supplementary Table 1).

3.3. Abundance and diversity of all Cryptosporidium reads determined by NGS

Overall, the highest number of reads (sequences) across the 83 WWTP samples positive for *Cryptosporidium* was assigned to *C. galli* (22.8% of all sequences analysed). This was followed by *C. meleagridis* (15.7%), *C. muris* (11.9%), *C. felis* (8.7%), *C. parvum* (6.8%), kangaroo genotype I (JF316651) (4.9%), *C. macropodum* (4.5%), rat genotype I (3.9%), rat genotype II (2.9%), *C. hominis* (2.5%), *C. erinacei* (2.5%), rat genotype III (2.1%), *C. suis* (1.7%), *C. bovis* (0.8%), *C. scrofarum* (0.5%), *C. canis* (0.4%), *C. fayeri* (0.3%), *C. cuniculus* (0.2%), *C. avium* (0.2%), *C. ubiquitum* (0.1%), *C. ryanae* (0.1%), rat genotype IV (0.1%) and bat genotype VI (0.1%) (Supplementary Table 1 and Fig. 1). There were also a small proportion of NGS sequences (6.1%), across 11 samples, that were not assigned to any *Cryptosporidium* species or genotypes (Supplementary Table 1 and Fig. 1). In general, at the individual sample level across the three states, the number of species identified in individual wastewater samples ranged from one to eight species.

3.4. Additional confirmation of presence/absence and enumeration of C. hominis and C. parvum in WWTP samples using a species-specific qPCR

Of 83 WWTP samples positive for *Cryptosporidium* spp. NGS detected *C. parvum* and *C. hominis* in 25 and four samples, respectively, including two samples that contained both species (QLD-E76 and QLD-G115) (Table 3, Table 5 and Supplementary Table 1). A *C. parvum* species-specific qPCR assay confirmed the presence of *C. parvum* in 20/25 samples, but failed to amplify the remaining five samples, which were previously identified by NGS to contain *C. parvum* sequences in low abundance, ranging from 113 to 535 reads (Table 5). The occurrence of *C. hominis* in 3/4 WWTP samples was also confirmed a *C. hominis*-

Table 4
Seasonal prevalence, the mean and median *Cryptosporidium* oocyst concentration in positive samples per litre (mean, median with range in parenthesis (determined by qPCR) per season across three states of Australia; NSW, QLD and WA).

State	Summer		Autumn		Winter		Spring		Overall	
	No +/total no (% proportion + 95% CI)	Oocysts/L mean, median and range	No +/total no (% proportion + 95% CI)	Oocysts/L mean, median and range	No +/total no (% proportion + 95% CI)	Oocysts/L mean, median and range	No +/total no (% proportion + 95% CI)	Oocysts/L mean, median and range	No +/total no (% proportion + 95% CI)	Oocysts/L mean, median and range
QLD	18/103 (17.5%, 10.7–26.2)	5966, 3821 (192–18,055)	11/105 (10.5%, 5.3–18)	2583, 1974 (203–8134)	10/131 (7.6%, 3.7–13.6)	2323, 1131 (172–14,602)	12/131 (9.2%, 4.8–15.5)	1953, 1107 (70–6301)	51/470 (10.8%, 8.2–14)	3578, 1619 (70–18,055)
NSW	NC	NC	1/17 (5.9%, 0.1–28.7)	1428, NA (only one sample)	2/4 (50%, 6.8–93.2)	6405, 6405 (two samples)	NC	NC	3/21 (14.3%, 3–36.3)	4746, 4373 (1428-8438)
WA	9/60 (15%, 7.1–26.6)	1632, 1590 (327–2842)	4/60 (6.8%, 1.8–16.2)	1217, 936 (599–2398)	6/59 (10.2%, 3.8–20.8)	2107, 1928 (1105-3326)	10/60 (16.8%, 8.3–28.5)	6326, 3805 (2267-16,812)	29/239 (12.1%, 8.3–17)	3292, 2398 (327–16,812)
Overall	27/163 (16.6%, 11.2–23.2)	4521, 2191 (192–18,055)	16/174 (9.2%, 5.3–14.5)	2170, 1260 (203–8134)	18/202 (8.9%, 5.4–13.7)	2704, 1627 (172–14,602)	22/191 (11.5%, 7.4–16.9)	3941, 2823 (70–16,812)	83/730 (11.4%, 9.2–13.9)	3426, 1828 (70–18,055)

NC = not collected.

specific qPCR, with no *C. hominis* amplification in a single sample which was previously confirmed by NGS to contain *C. hominis* in low abundance (QLD-E76) (Table 5 and Supplementary Table 1). The concentration of *C. hominis* and *C. parvum* oocysts per litre in these samples ranged from 386 to 3294 and from 14 to 6314, respectively (Table 5). The absence of *C. hominis* and *C. parvum* in the remaining samples ($n = 56$) was confirmed by the *C. hominis* and *C. parvum* species-specific qPCR assays (Table 5).

3.5. Enumeration of Cryptosporidium oocysts in wastewater samples using qPCR

Cryptosporidium oocyst concentration per litre was estimated using qPCR standards calibrated by ddPCR at the 18S locus (Table 4). Overall, the oocyst load per litre in samples collected across the three states ranged from 70 to 18,055 oocysts/L and the mean was 3426 oocysts/L (Table 4). The mean *Cryptosporidium* oocyst concentration in samples collected from WWTPs in NSW was the highest among the states (4746 oocysts/L). However, due to the low number of samples collected from NSW ($n = 21$), compared to 470 from QLD and 239 from WA, statistical analysis of oocyst load was only conducted for QLD and WA to avoid potential bias in the analysis.

The mean number of oocysts per litre in samples collected from the two WWTPs in WA over four seasons was 3292 oocysts/L (ranging from 327 to 16,812), while the mean *Cryptosporidium* oocyst concentration in these samples peaked during spring 2015 at 6326 oocysts/L (ranging from 2267 to 16,812). This corresponded with a peak of prevalence at this time (spring 2015) (16.8%; 95% CI, 8.3–28.5) (Table 4).

Compared to WA, the overall mean *Cryptosporidium* oocyst concentration in WWTP samples from QLD was relatively higher (3578 oocysts/L, ranging from 70 to 18,055). Seasonal mean concentrations (averaged over the two same seasons in 2014 and 2015) were 1953 oocysts/L in spring, 2323 oocysts/L in winter, 2583 oocysts/L in autumn and 5966 oocysts/L in summer. This also corresponded with a peak prevalence of 17.5% (95% CI, 10.7–26.2) during summer (averaged over summer 2014, 2015 and 2016) (Table 4).

4. Discussion

The present study has demonstrated the utility of NGS in detecting mixtures of *Cryptosporidium* species and genotypes in sewage and has shown that they are frequently present but variable and diverse in space, time and composition. The overall prevalence of *Cryptosporidium* in WWTP samples across Australia was 11.4% (83/730). Previous studies have reported prevalence ranging from 6.4% to 100% (Xiao et al., 2001; Ward et al., 2002; Zhou et al., 2003; Hanninen et al., 2005; Cantusio Neto et al., 2006; Hashimoto et al., 2006; Hirata and Hashimoto, 2006; Ottoson et al., 2006; Robertson et al., 2006; Castro-Hermida et al., 2008; Feng et al., 2009; Dungeni and Momba, 2010; Liu et al., 2011; Ajonina et al., 2012; Ben Ayed et al., 2012; Li et al., 2012; Gallas-Lindemann et al., 2013, 2016; Hachich et al., 2013; Spanakos et al., 2015; Amorós et al., 2016; Hatam-Nahavandi et al., 2016; Ulloa-Stanojlović et al., 2016; ; Huang et al., 2017; Imre et al., 2017; Ramo et al., 2017; Santos and Daniel, 2017). However, to the best of the authors' knowledge, in Australia little published information is available on the prevalence and composition of *Cryptosporidium* species in wastewater (King et al., 2015, 2017).

In the present study, a total of 17 *Cryptosporidium* species and six genotypes were detected by NGS. This is higher than the diversity reported in previous studies due to the ability of NGS to detect mixtures of sequences in low abundance. Wastewater treatment networks however, rarely contain only domestic wastewater; they often also contain wastewater from industrial sources and can be influenced by environmental water sources, such as stormwater or groundwater (Pandey et al., 2014). In addition, wild animals may directly contribute to contamination of sewage, such as rodents in the sewer networks or birds

Appendix 6

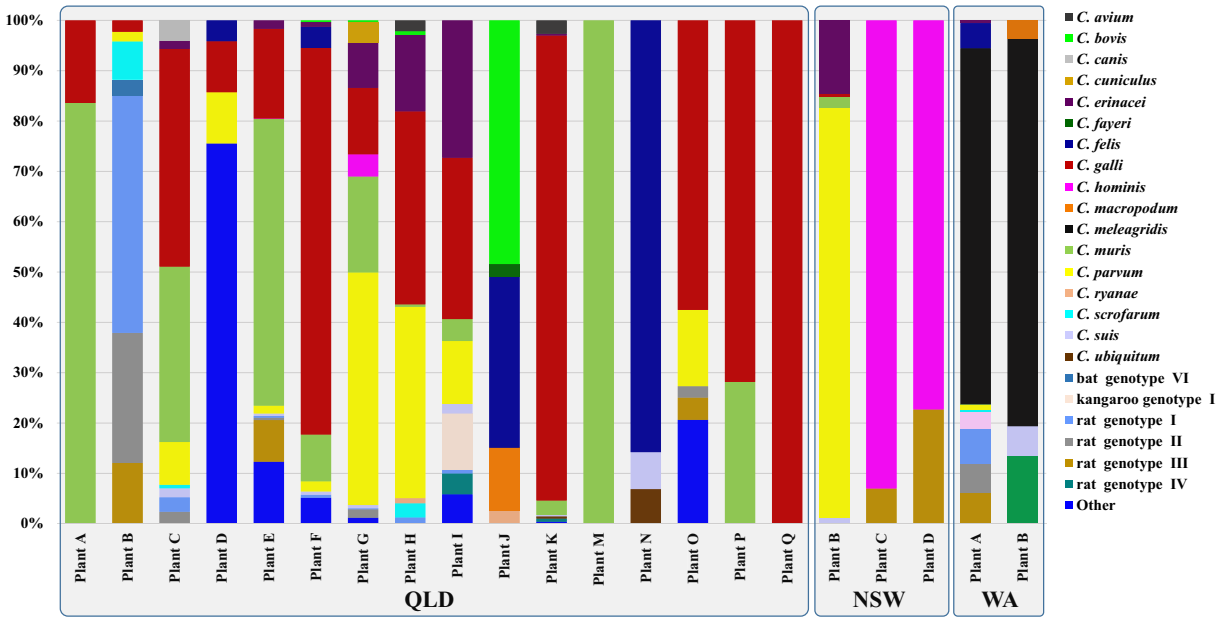

Fig. 1. Percent composition of 18S sequences from *Cryptosporidium* species detected in wastewater treatment plant samples from NSW, QLD and WA.

present at wastewater treatment plants. Therefore, the presence of a variety of *Cryptosporidium* species from livestock, wildlife and birds in sewage samples may be attributed to other sources such as stormwater or industrial waste from animal processing.

In the present study, of 83 WWTP samples positive for *Cryptosporidium* spp., NGS detected *C. hominis* and *C. parvum* in only 27 samples (32.5%; 95% CI, 22.6–43.7), of which two samples contained both species. A *C. hominis* and *C. parvum* species-specific qPCR provided further support for the lack of *C. hominis* and *C. parvum* in the majority of samples, although it failed to detect *C. hominis* and *C. parvum* in one and five samples, respectively, which were mainly samples with low numbers of *C. parvum*/*C. hominis* reads (109–535 reads) by NGS. The qPCR assay has been tested extensively on human faecal samples and has been shown to have an analytical sensitivity of 1 oocyst/μL of DNA extract (Yang et al., 2013). This is the first time we have applied the qPCR to WWTP samples and no inhibition was observed.

In NSW, the dominant species detected were *C. hominis* and rat genotype III, whereas in QLD, *C. galli*, *C. muris* and *C. parvum* were the three most prevalent species, while in WA, *C. meleagridis* was the most prevalent species. Of these, *C. parvum* and *C. hominis* are the most common species reported in humans in Australia, accounting for >95% of human infections, with *C. meleagridis* the third most common species reported and usually accounting for 1–2% of notifications (Ryan and Power, 2012; Ng-Hublin et al., 2017). There have been numerous reports of *C. muris* in humans in other countries (cf. Ryan et al., 2017b). Other *Cryptosporidium* species with zoonotic potential, which were detected at a low prevalence in WWTP samples in the present study included *C. bovis*, *C. canis*, *C. cuniculus*, *C. erinacei*, *C. felis*, *C. scrofarum*, *C. suis* and *C. ubiquitum*. Nevertheless, caution is required when extrapolating any molecular data from WWTP samples to determine host sources of wastewater contamination by *Cryptosporidium*, as there are many potential input sources other than humans into wastewater networks. Unlike faecal material, there is no direct relationship between *Cryptosporidium* oocysts from wastewater samples and any potential host species, and an understanding of existing host-parasite interactions, parasite epidemiology and sources of faecal inputs into the wastewater network is required (Castro-Hermida et al., 2008).

A number of studies across the world have reported *C. hominis* (the predominant species in humans) among the most prevalent species detected in wastewater; Australia (King et al., 2015), Brazil and Peru (Ulloa-Stanojlović et al., 2016), China (Feng et al., 2009; Li et al., 2012; Huang et al., 2017), Japan (Hashimoto et al., 2006; Hirata and Hashimoto, 2006), Switzerland and Germany (Ward et al., 2002), the USA (Xiao et al., 2001; Zhou et al., 2003) and Tunisia (Ben Ayed et al., 2012). In addition to anthroponotic sources of *C. hominis*, several studies in Australia have previously identified *C. hominis* in Australian cattle and wildlife including bandicoots (*Isoodon obesulus*), brushtail possums (*Trichosurus vulpecula*), eastern grey kangaroos (*Macropus giganteus*) and brush-tailed rock-wallabies (*Petrogale penicillata*) (Hill et al., 2008; Ng et al., 2011; Dowle et al., 2013; Vermeulen et al., 2015; Zahedi et al., 2016b; Zahedi et al., 2018). To date there is no conclusive molecular or epidemiological evidence linking contamination of wastewater by animals with the occurrence of *C. hominis* in raw wastewater or in human populations in Australia and further research is required in this area. In the present study, *C. hominis* was detected in NSW in plants C and D which received mainly septic tank waste and accounted for 93.0% and 77.3% of all *Cryptosporidium* species detected in plants C and D respectively, suggesting humans were the source. In NSW, *C. hominis* was detected in plants E and G. Plant E received a significant portion of trade waste and the *C. hominis* detected accounted for only 4.4% of all *Cryptosporidium* species identified, while plant G received mostly human waste and the *C. hominis* detected accounted for 26.6% all *Cryptosporidium* species identified.

In Europe, several studies have reported that *C. parvum* is the dominant species in wastewater (Hanninen et al., 2005; Spanakos et al., 2015; Imre et al., 2017; Ramo et al., 2017), while some studies in China, Iran, Tunisia and the USA have reported that livestock associated species such as *C. andersoni* and *C. xiaoi* dominate (Xiao et al., 2001; Liu et al., 2011; Ben Ayed et al., 2012; Hatam-Nahavandi et al., 2016). In the present study, *C. andersoni* and *C. xiaoi* were not detected in WWTPs across three states in Australia, however *C. parvum* was the third most prevalent species identified in QLD samples and was detected in a single sample and three samples from NSW and WA, respectively. *Cryptosporidium parvum* has been identified widely in both calves and humans in Australia (Ryan and Power, 2012) with reported prevalences for *C. parvum* in humans in Australia ranging from ~24% in Victoria (Jex et al., 2007; Koehler et al., 2013) to 17–19.8% in WA (Morgan et al., 1998; Ng et al., 2010) and 46.8% in NSW (Waldron et al., 2009). There are no published reports on the prevalence of *C. parvum* in the human population in QLD, which is a knowledge gap that needs to be addressed.

Appendix 6

Table 5
C. hominis and *C. parvum* detected by NGS and species specific qPCR in individual wastewater treatment plant (WWTP) samples positive for *Cryptosporidium* spp. across three states of Australia; NSW, QLD and WA.

State	Plant	Sample	NGS		*C. hominis* and *C. parvum* oocysts numbers determined by species specific qPCR (MGB probes)	
			No of reads assigned to *C. hominis*	No of reads assigned to *C. parvum*	*C. hominis* oocysts/L	*C. parvum* oocysts/L
QLD	Plant A	QLD-A7	0	0	ND	ND
		QLD-A24	0	0	ND	ND
	Plant B	QLD-B2	0	0	ND	ND
		QLD-B3	**0**	**109**	**ND**	**61**
	Plant C	**QLD-C2**	**0**	**779**	**ND**	**470**
		QLD-C3	**0**	**246**	**ND**	**117**
		QLD-C19	**0**	**910**	**ND**	**482**
		QLD-C25	**0**	**133**	**ND**	**50[a]**
	Plant D	**QLD-D104**	**0**	**1181**	**ND**	**448**
		QLD-D140	0	0	ND	ND
	Plant E	**QLD-E18**	**0**	**562**	**ND**	**82[a]**
		QLD-E34	0	0	ND	ND
		QLD-E76	**109**	**918**	**76[a]**	**361**
		QLD-E195	0	0	ND	ND
		QLD-E222	0	0	ND	ND
		QLD-E258	0	0	ND	ND
		QLD-E303	0	0	ND	ND
		QLD-E357	0	0	ND	ND
		QLD-E375	**0**	**265**	**ND**	**47**
		QLD-E393	0	0	ND	ND
	Plant F	QLD-F1	0	0	ND	ND
		QLD-F33	**0**	**140**	**ND**	**2834**
		QLD-F84	**0**	**126**	**ND**	**51**
		QLD-F130	**0**	**189**	**ND**	**32**
		QLD-F157	0	0	ND	ND
		QLD-F319	0	0	ND	ND
		QLD-F382	**0**	**140**	**ND**	**38[a]**
	Plant G	**QLD-G53**	**0**	**113**	**ND**	**292[a]**
		QLD-G115	**2382**	**4336**	**386**	**812**
		QLD-G304	**0**	**20,571**	**ND**	**954**
		QLD-G331	0	0	ND	ND
		QLD-G340	0	0	ND	ND
	Plant H	**QLD-H8**	**0**	**18,529**	**ND**	**6314**
		QLD-H179	**0**	**1638**	**ND**	**922**
		QLD-H197	**0**	**4674**	**ND**	**1528**
		QLD-H386	0	0	ND	ND
	Plant I	**QLD-I41**	**0**	**1873**	**ND**	**476**
	Plant J	QLD-J15	0	0	ND	ND
		QLD-J47	0	0	ND	ND
		QLD-J354	0	0	ND	ND
		QLD-J363	0	0	ND	ND
	Plant K	QLD-K71	0	0	ND	ND
		QLD-K119	0	0	ND	ND
		QLD-K281	0	0	ND	ND
		QLD-K380	0	0	ND	ND
		QLD-K389	0	0	ND	ND
	Plant M	QLD-M60	0	0	ND	ND
	Plant N	QLD-N35	0	0	ND	ND
	Plant O	**QLD-O54**	**0**	**991**	**ND**	**14**
	Plant P	QLD-P63	0	0	ND	ND
	Plant Q	QLD-Q11	0	0	ND	ND
NSW	Plant B	**NSW-B2**	**0**	**20,347**	**ND**	**1380**
	Plant C	**NSW-C20**	**9227**	**0**	**2998**	**ND**
	Plant D	**NSW-D21**	**3082**	**0**	**3294**	**ND**
WA	Plant A	WA-A5	0	0	ND	ND
		WA-A8	0	0	ND	ND
		WA-A13	0	0	ND	ND
		WA-A16	0	0	ND	ND
		WA-A24	0	0	ND	ND
		WA-A37	0	0	ND	ND
		WA-A40	0	0	ND	ND
		WA-A55	0	0	ND	ND
		WA-A65	**0**	**884**	**ND**	**92**
		WA-A66	**0**	**2563**	**ND**	**214**
		WA-A68	**0**	**535**	**ND**	**51[a]**
		WA-A78	0	0	ND	ND
		WA-A79	0	0	ND	ND
		WA-A80	0	0	ND	ND
		WA-A81	0	0	ND	ND
		WA-A82	0	0	ND	ND
		WA-A88	0	0	ND	ND

Appendix 6

Table 5 (*continued*)

State	Plant	Sample	NGS		*C. hominis* and *C. parvum* oocysts numbers determined by species specific qPCR (MGB probes)	
			No of reads assigned to *C. hominis*	No of reads assigned to *C. parvum*	*C. hominis* oocysts/L	*C. parvum* oocysts/L
		WA-A91	0	0	ND	ND
	Plant B	WA-B2	0	0	ND	ND
		WA-B4	0	0	ND	ND
		WA-B12	0	0	ND	ND
		WA-B13	0	0	ND	ND
		WA-B14	0	0	ND	ND
		WA-B19	0	0	ND	ND
		WA-B28	0	0	ND	ND
		WA-B30	0	0	ND	ND
		WA-B41	0	0	ND	ND
		WA-B42	0	0	ND	ND
		WA-B45	0	0	ND	ND

ND = not detected.
WWTP samples in which NGS detected *C. parvum* and/or *C. hominis* are in bold.
[a] For these samples, the *C. hominis* and *C. parvum* species-specific qPCR assay failed and oocyst/L is reported based on 18S qPCR and the percentage of NGS reads attributed to *C. hominis* and *C. parvum*.

Considering that most WWTPs in Australia are well fenced-off and protected, with minimal animal access, the predominance of *C. parvum* in wastewater in QLD may indicate that human sewage was the source of *C. parvum* or that it came from a combination of anthroponotic contributions and industry waste from abattoirs. In many of the QLD plants, a significant proportion was "trade waste" some of which may have come from abattoirs, however it was not possible to obtain further information on the sources of the trade waste. In WA, *C. parvum* was detected in plant A, which received both human and abattoir waste. In NSW, the single WWTP (Plant B) that was positive for *C. parvum* received waste predominately from septic tanks, suggesting an anthroponotic source. It is also important to remember that previous studies that reported Australian prevalence data for *Cryptosporidium* were from clinical samples, which in many cases were dominated by samples from the major metropolitan areas. Based on the population sizes for at least some of the WWTPs in the present study, most of the "urban sites" are more likely to be regional centres, so may have a different pattern of *Cryptosporidium* prevalence and species composition compared with major urban centres.

Cryptosporidium meleagridis is a common parasite of humans in Australia (Ryan and Power, 2012) and also infects a wide range of birds (Zahedi et al., 2016a), with many overlapping *C. meleagridis* subtypes found in both birds and humans; suggesting both anthroponotic and zoonotic transmission (Silverlas et al., 2012). This is evidenced by the fact that *C. meleagridis* is commonly reported in wastewater worldwide (Hashimoto et al., 2006; Hirata and Hashimoto, 2006; Feng et al., 2009; Li et al., 2012; Huang et al., 2017). In the present study, *C. meleagridis* was the most prevalent species detected in WWTP samples collected from WA and in many cases was the only species detected (Supplementary Table 1). However, it was not detected in NSW or QLD. Although a variety of bird species are commonly seen at WWTPs in Australia, particularly around lagoons and clarifiers (secondary and tertiary treatment), the raw sewage entries to most WWTPs are covered, and not exposed and accessible to birds and animals. Some of the *C. meleagridis* detected in WWTPs in WA could have been originated from humans, however, further investigation revealed that the raw influent samples were taken directly from the distribution chamber located just before the primary ponds, which was only covered with a layer of mesh, providing easy access to bird contamination. Alternatively, industrial sources of wastewater from poultry farms could also be a major contributor. The predominance of the bird-specific *C. galli* in WWTP samples from QLD also confirms the potential role birds may play in contamination of wastewater by *Cryptosporidium*, but currently data on the contribution of poultry farms to WWTP in both WA and QLD is lacking and is an important knowledge gap. To date, there has only been one report of *C. galli* in wastewater (Ramo et al., 2017), however, *C. baileyi*, another avian *Cryptosporidium* species, has been reported in several studies from China (Feng et al., 2009; Li et al., 2012; Huang et al., 2017). It is possible that the high levels of *C. meleagridis* and *C. galli* detected in WA and QLD respectively, were due to contamination in our laboratory. However, this is unlikely as neither species were included as controls on the same Illumina MiSeq run and quality filtering removed all reads <100. The high number of *C. meleagridis* reads in WA (107 to 58,246 reads/sample) and *C. galli* reads in QLD (129 to 32,164 reads/sample) supports their validity. In addition, if it was due to gross contamination, then both species would be randomly distributed across all samples, with mixtures of both species in some samples.

Two emerging human-pathogenic *Cryptosporidium* species, including *C. ubiquitum* ($n = 2$) and *C. cuniculus* ($n = 1$), were also found in wastewater samples from QLD at a lower frequency and abundance than other major species. *Cryptosporidium cuniculus* is a common parasite of rabbits and has been reported in source water in South Australia (Swaffer et al., 2018) and linked to several sporadic human cases in Australia (Nolan et al., 2010, 2013; Sari et al., 2013 unpublished - KF279538; Koehler et al., 2014), the UK (Chalmers et al., 2011; Elwin et al., 2012), Nigeria (Molloy et al., 2010) and France (ANOFEL, 2010). To date there are no published reports of *C. cuniculus* detected in WWTP samples in Australia, however, it has been previously reported from WWTPs in Brazil, Peru and China (Li et al., 2012; Ulloa-Stanojlović et al., 2016). Mainly infecting small ruminants, *C. ubiquitum* has been identified in a broad range of hosts including humans and wildlife (in particular rodents) with a wide geographic distribution across the world (Zahedi et al., 2016a). It has also been frequently reported from source water, stormwater runoff, stream sediment and wastewater across the world (Xiao et al., 2000; Nolan et al., 2013; Li et al., 2014). In Australia, *C. ubiquitum* has not been detected in the studies conducted to type *Cryptosporidium* isolates from humans (Ryan and Power, 2012); however, it has been identified in source water in Australia (Swaffer et al., 2018). More recently, the identification of similar *C. ubiquitum* subtypes in humans and in wastewater samples from China, Tunisia and the USA strengthens the hypothesis that sheep and wild rodents are a source of *C. ubiquitum* transmission to humans through contamination of untreated drinking water (Zhou et al., 2003; Liu et al., 2011; Ben Ayed et al., 2012; Li et al., 2014; Huang et al., 2017).

In the present study *C. muris*, a predominantly a rodent species of *Cryptosporidium*, was sporadically identified in wastewater samples from NSW and WA and was the second most prevalent species detected in QLD. There have been numerous reports of *C. muris* in humans and wastewater (Xiao et al., 2001; Ward et al., 2002; Zhou et al., 2003;

Feng et al., 2009; Ben Ayed et al., 2012; Ryan and Power, 2012; Li et al., 2014; Spanakos et al., 2015; Huang et al., 2017), suggesting both human contribution as well as faecal contamination by rodents in wastewater distribution systems. However, as the frequency of detection of *C. muris* in humans is low (1–3%) (Wang et al., 2012), rodents are the more likely source. The identification of other rodent *Cryptosporidium* genotypes (rat genotypes I–IV) across all states in the present study, also supports this hypothesis.

Factors that influence oocyst density in wastewater are the incidence of cryptosporidiosis in the community (i.e. number of infected humans and animals in the community served by the WWTP), the intensity of infection (oocyst shedding), the size of the community (population), seasonality and dilution by other waste entering the WWTP (Domenech et al., 2017; King et al., 2017). In the present study, oocyst numbers per litre of sewage across the three states were estimated and ranged from 70 to 18,055 oocysts/L (mean = 3426 oocysts/L). This is similar to a previous study of WWTPs across South Australia and Victoria, with oocyst densities ranging from 3 to 21,335 oocysts/L with a mean density of 2355 oocysts/L (King et al., 2017). It is difficult, however, to compare across different studies using different methodologies. Worldwide, mean densities of between 10 and >700 oocysts/L have been commonly reported (Ajonina et al., 2012; Tonani et al., 2013; Nasser, 2016; Xiao et al., 2018) with a mean of 60,000 oocysts/L reported in one study (Cantusio Neto et al., 2006). The somewhat higher number of oocysts detected in the present study compared to other studies may be due to the fact that the oocyst concentrations were determined directly from total DNA extracted from WWTP samples by qPCR (using ddPCR calibrated standards), which may have overestimated the oocyst concentration, as DNA from lysed (and therefore no longer viable) oocysts would also have been detected. Previous studies have purified oocysts from WWTP samples and counted intact oocysts using USEPA method 1623, however, recovery efficiencies from wastewater samples can be highly variable, ranging from 5.5 ± 1.3% to as high as 85% (Nasser, 2016). The DNA extraction efficiency in the present study is unknown.

Estimation of *Cryptosporidium* risk from wastewater requires an evaluation of the efficiency of oocyst removal and inactivation along the treatment process and the reduction in the levels of oocysts (and their infectivity) in final treated effluent compared with oocyst counts in raw sewage (Xiao et al., 2018). Guideline values have traditionally set $\log_{10}$ removal targets based on end-use application (King et al., 2017), but these guidelines still do not incorporate the potential for inactivation of oocysts throughout the treatment process. A limitation of the present study is that samples were only taken from influent raw wastewater, and oocyst numbers were not investigated across the treatment train including the final effluent. Another limitation is that the viability/infectivity of oocysts detected in WWTP samples was not analysed. A recent study developed an integrated assay to determine oocyst density and infectivity from a single-sample concentrate (King et al., 2017), which will allow for improved QMRA analysis, as only analysing total oocyst numbers in raw sewage could result in an overestimation or underestimation of the *Cryptosporidium* risk in treated water. Finally, in the present study, the weather on sampling days (and preceding days) was not taken into account in the study design and future studies should include this data to better understand the effects of storm water intrusion for all the plants studied.

Conclusions: The current study has demonstrated that *Cryptosporidium* is prevalent in the raw influent of wastewater treatment facilities across Australia. NGS was central to unravelling the large diversity of *Cryptosporidium* species and genotypes in these samples and revealed the potential contribution of livestock, wildlife and birds (in addition to humans), to wastewater contamination. While human waste is a major contributor to WWTPs, the data from the present study suggests that abattoirs and poultry processing plants etc., could also be major contributors to wastewater treatment facilities. NGS analysis of the vertebrate species contributing to the wastewater will also help with determining the origin of the *Cryptosporidium* species detected in wastewater samples, and clearly further research is required to better understand the sources of *Cryptosporidium* in Australian wastewater. Comparisons between the results of the present study with previous studies which used Sanger sequencing are difficult, but as NGS becomes more widely used as the method of choice for typing pathogens in wastewater in the future, comparisons will become much more relevant and meaningful across studies.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2018.07.024.

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DE GRUYTER

CASE REPORT

First report of *Cryptosporidium parvum* in a dromedary camel calf from Western Australia

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Abstract

Cryptosporidium is an important enteric parasite that can contribute large numbers of infectious oocysts to drinking water catchments. As a result of its resistance to disinfectants including chlorine, it has been responsible for numerous waterborne outbreaks of gastroenteritis. Wildlife and livestock play an important role in the transmission of *Cryptosporidium* in the environment. Studies conducted outside Australia have indicated that camels may also play a role in the transmission of zoonotic species of *Cryptosporidium*. Despite Australia being home to the world's largest camel herd, nothing is known about the prevalence and species of *Cryptosporidium* infecting camels in this country. In the present study, *C. parvum* was identified by PCR amplification and sequencing of a formalin-fixed intestinal tissue specimen from a one-week old dromedary camel (*Camelus dromedarius*). Subtyping analysis at the glycoprotein 60 (*gp60*) locus identified *C. parvum* subtype IIaA17G2R1, which is a common zoonotic subtype reported in humans and animals worldwide. Histopathological findings also confirmed the presence of large numbers of variably-sized (1–3 μm in diameter) circular basophilic protozoa – consistent with *Cryptosporidium* spp.– adherent to the mucosal surface and occasionally free within the lumen. Further analysis of the prevalence and species of *Cryptosporidium* in camel populations across Australia are essential to better understand their potential for contamination of drinking water catchments.

Keywords

Cryptosporidium, camel, 18S, actin, *gp60*

Introduction

Cryptosporidium spp. are ubiquitous enteric parasites with a wide range of vertebrate hosts including humans (Hunter *et al*. 2007; Xiao 2010; Ryan *et al*. 2014). Transmission is via direct faecal-oral contact and via contamination of food and water with numerous waterborne outbreaks reported globally (Baldursson and Karanis 2011; Efstratiou *et al*. 2017). *Cryptosporidium* is particularly suited to waterborne transmission as the environmental stage, the oocysts, are shed in large quantities, have a low infectious dose (1–10 oocysts) and are highly resistant to disinfectants including chlorine treatment of community water supplies (Baldursson and Karanis 2011; Efstratiou *et al*. 2017). To date, 34 valid *Cryptosporidium* species have been described, of which more than 20 species have been reported in humans, with *C. hominis* and *C. parvum* responsible for the majority of human infections (Jezkova *et al*. 2016; Zahedi *et al*., 2017).

Wildlife and livestock can contribute large numbers of *Cryptosporidium* oocysts to the environment, which may be transported to surface water and contaminate drinking water sources (Ryan *et al*. 2014; Zahedi *et al*. 2016). Therefore, wild and domestic animals play an important role in the epidemiology and transmission dynamics of zoonotic *Cryptosporidium* species (Appelbee *et al*. 2005; Ziegler *et al*. 2007; Ryan *et al*. 2014). However, very little is known about the range of species and genotypes of *Cryptosporidium* in camelids, particularly domesticated dromedary (*Camelus dromedaries)* and bactrian (*Camelus bactrianus*) camels, and wild bactrian (*Camelus ferus*) camels worldwide. The few

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studies that have investigated *Cryptosporidium* in camelids to date have identified *C. andersoni*, *C. muris*, *C. parvum* and *C. ubiquitum* in bactrian camels, llamas (*Lama glama*) and alpacas (*Vicugna pacos*) (Starkey *et al*. 2007; Wang *et al*. 2008; Gómez-Couso *et al*. 2012; Wang *et al*. 2012; Liu *et al*. 2014; Robertson *et al*. 2014). Reports of cryptosporidiosis in dromedary camels have all been based on microscopy and no molecular data is available (Razavi *et al*. 2009; Nazifi *et al*. 2010; Sazmand *et al*. 2011; Yakhchali and Moradi 2012).

Both dromedary and bactrian camels have a global population of over 27 million (FAO 2014), with the majority of camels in Australia being dromedary camels (Edwards *et al*. 2008). Dromedary camels were imported into Australia in the 19th century for transport and explorations, and are well adapted to arid and semi-arid areas of Australia including Western Australia, the Northern Territory, western Queensland and northern South Australia. With no natural predators, their populations have flourished rapidly resulting in the Australian government introducing a management (culling) program in 2009 (Brown 2004; Edwards *et al*. 2004, 2008). Currently, the feral dromedary camel population in Australia is the world's largest camel herd, estimated at 300,000 (Tan *et al*. 2016), however in-depth information about diseases in these camels is scarce (Brown 2004) and to date, no molecular data is available for *Cryptosporidium* spp. infecting camels in Australia. The present study is the first report of a *Cryptosporidium* species from a dromedary camel calf referred to the Animal Hospital at Murdoch University, Western Australia.

Materials and Methods

Clinical record

A fresh carcass of a one-week old 45 kg female dromedary camel calf in lean body condition was submitted to the Murdoch University Pathology Department for post-mortem examination. The calf was born from a feral dromedary camel that had been brought into a farm two weeks prior with other dromedary camels. No abnormalities were seen for the first five days after birth, and the calf was treated for endo- and ectoparasites on day five as part of the standard treatment before the new camels were introduced to the rest of the herd. On day six, the calf went lame, and the following morning the calf was found in lateral recumbency, poorly responsive, with diarrhoea, and was flaccid and dehydrated by the afternoon, and therefore was referred to the Animal Hospital at Murdoch University, Perth, Western Australia. After a thorough examination by a veterinarian, the animal was euthanised due to a poor prognosis. A post-mortem examination was immediately performed and revealed the entire mucosal surface of the intestinal tract to be necrotic with half a dozen mucosal ulcers within the mid-jejunum. Sections of multiple organs, including the intestinal tract, were placed in a 10% buffered formalin solution for histopathology.

Histopathology

Sections of intestinal tissue were fixed in formalin for 24 h then routinely processed for histopathological diagnosis. 2 µm-thick sections were cut and manually stained with a Giemsa stain for microscopic assessment. For the staining, microscope slides containing cut intestinal tissue sections were acidified with 10% acetic acid for 15 s. The slides were then placed in a solution of 0.75 g Giemsa powder, 65 ml methanol and 65 ml glycerol, which was diluted 1:10 with water, and heated to 50°C for 1 min. The jar containing the slides was then microwaved on a low setting for 30 seconds. The slides were then rinsed with distilled water and cover-slipped. Two veterinary pathologists performed the microscopic assessment, including a diplomat of the American College of Veterinary Pathologists (A.L.W.).

DNA isolation

Following five cycles of freeze-thaw, genomic DNA (gDNA) was extracted from ≤ 25 mg sections of the formalin-fixed intestinal tissue using a QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions (Paraffin removal steps were excluded). An extraction blank negative control (no faecal sample) was included. Purified DNA was stored in –20°C before being processed by PCR. DNA extraction and post-DNA extraction procedures were performed in separate laboratories.

PCR amplification of the 18S rRNA gene

Extracted DNA was amplified at the 18S locus using primers which produced a 611 bp product as previously described (Silva *et al*. 2013) with minor modifications; the annealing temperature was increased to 57°C for 30 s and the number of cycles was increased from 39 to 45 cycles for both primary and secondary reactions. Each 25 µl PCR mixture contained 1 µl of gDNA, 1x KAPA Taq buffer (KAPA Biosystems, South Africa), 3.75 mM $MgCl_2$, 400 µM of each dNTP, 0.4 µM of forward and reverse primers and 1 U/reaction KAPA Taq DNA polymerase (KAPA Biosystems, South Africa). PCR contamination controls used included no-template controls.

PCR amplification of the actin gene

An ~818 bp fragment was amplified at the actin locus as previously described (Ng *et al*. 2006), with the following modifications; denaturation time was increased from 30 s to 45 s, annealing time from 20 s to 30 s and extension time from 40 s to 1 min. Both positive and no-template PCR controls were included in the reaction to validate the PCR.

PCR amplification of the *gp60* gene

As *C. parvum* was identified at both 18S and actin loci, subtyping was conducted using a nested PCR at the 60 kDa gly-

coprotein (*gp60*) locus as previously described (Zhou *et al*. 2003).

Sequence analysis

The amplified DNA from secondary PCR products were separated by gel electrophoresis and were purified for sequencing using an in-house filter tip method (Yang *et al*. 2013). Purified PCR products were sequenced independently using an ABI Prism™ Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, California, USA) according to the manufacturer's instructions, with annealing temperatures of 57°C, 58°C and 54°C for the 18S rRNA, actin and *gp60* loci, respectively. Sanger sequencing chromatogram files were imported into Geneious Pro 8.1.6 (Kearse *et al*. 2012), edited, analysed and aligned with reference sequences from GenBank using Clustal W (http://www.clustalw.genome.jp).

Results

Histopathological findings

Six sections of small intestine were evaluated. Villi were occasionally fused, with blunted tips. There were large numbers of variably-sized (1-3 µm in diameter) circular basophilic protozoa adherent to the mucosal surface and also occasionally free within the lumen (Fig 1). The lamina propria was expanded by moderate to large numbers of neutrophils, lymphocytes, plasma cells and eosinophils, and scattered throughout the sections within the superficial lamina propria were necrotic cells with hypereosinophilic cytoplasm and pyknotic or karyorrhectic nuclei. Blood vessels were congested. Based on these pathological findings, severe, multifocal to coalescing lymphoplasmacytic, neutrophilic and eosinophilic enteritis with multifocal necrosis and intralesional protozoa (consistent with *Cryptosporidium* spp.) was reported.

Cryptosporidium species detected in a camel calf

Based on the sequence analysis at the 18S and actin loci, *C. parvum* was identified in this camel calf. At the actin locus, the isolate exhibited a single nucleotide polymorphism (SNP) from a reference *C. parvum* isolate (AF382337) across 787 bp of the sequence, while across 591 bp at the 18S locus, two SNPs were present when compared to the reference *C. parvum* isolate (AY204230).

Subtyping analysis across 548 bp of *gp60* sequence identified *C. parvum* subtype IIaA17G2R1, with three SNPs from a reference isolate (JF727798) from a human patient in New South Wales, Australia. Nucleotide sequences reported in this paper are available in the GenBank database under accession numbers MG738816, MG738817 and MG738818.

Discussion

Neonatal calf diarrhea is a major issue of camel production industry and can be attributed to nutritional issues as well as a large spectrum of pathogenic agents including bacteria, viruses and parasites (Al-Ruwaili *et al.* 2012; Muktar *et al*. 2015). In the present study, histopathological damage due to the observed infection with *Cryptosporidium* spp., was most likely responsible for the diarrhoea, recumbency and dehydration noted clinically. However, contributing factors and pathogens cannot be ruled out. Further tests are required to confirm the presence and pathogenicity of other infectious agents.

To the best of authors' knowledge, this is the first case of cryptosporidiosis reported from dromedary camels in Australia, for which molecular characterisation was conducted.

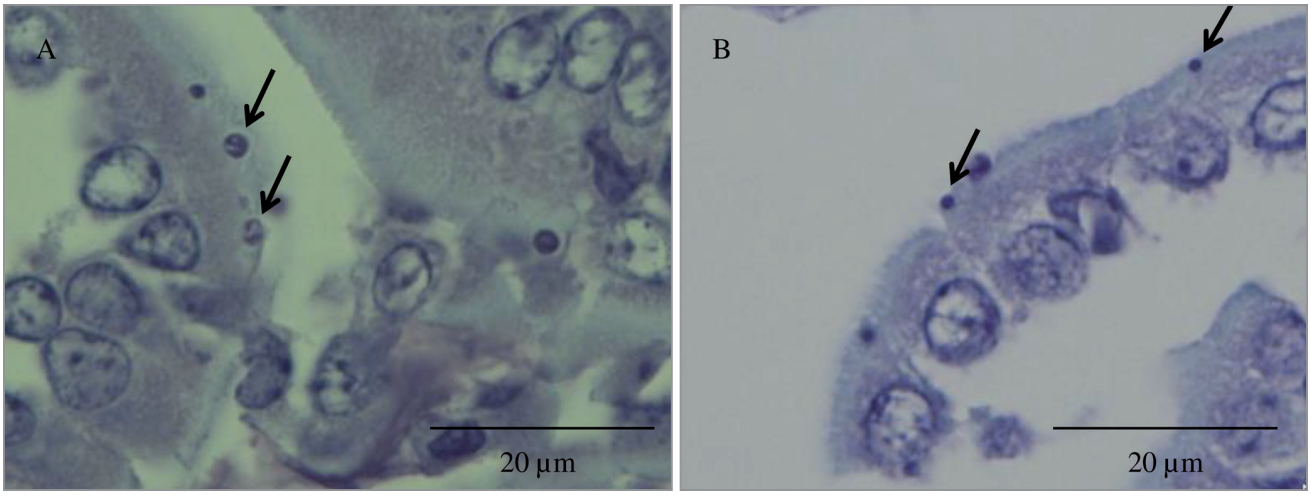

Fig. 1 Small intestine, Giemsa stain, x1000 magnification, depicting various *Cryptosporidium* developmental stages attached to the brush border of villous enterocytes. *Cryptosporidium* spp. meronts (**A**-arrows) are slightly larger than the densely-stained spherical trophozoites (**B**-arrows)

Cryptosporidium parvum is the most commonly reported zoonotic species of *Cryptosporidium* infecting humans and is also the most common species detected in cattle (*Bos taurus*) in Australia (Ryan *et al*. 2014). While there are no previous reports of this species from camelids in Australia, studies have reported *C. parvum* in alpacas (*Vicugna pacos*) from the USA and Peru (Starkey *et al*. 2007; Gómez-Couso *et al*. 2012) and in camels (*C. dromedarius*) from Iran (based on morphology only) (Razavi *et al*. 2009).

In the present study, the *C. parvum* subtype IIaA17G2R1 was identified, which is a common zoonotic subtype reported in both humans and animals worldwide (Xiao *et al*. 2007; Broglia *et al*. 2008; Wielinga *et al*. 2008; Mi *et al*. 2014; Certad *et al*. 2015) and has been reported widely in both cattle and humans in Australia (Nolan *et al*. 2009; Ng *et al*. 2011; Waldron *et al*. 2011). Although most feral camels live in remote arid and semi-arid areas of central and western Australia (Saalfeld and Edwards 2010), they still have access to drinkable water sources (Pople and McLeod 2010), which may also be used by humans.

Recent periods of drought in Australia have resulted in feral camels entering remote communities in search of water and extensively damaging water infrastructure such as bathrooms, bores, taps and tanks, with the potential to contaminate remote communities' drinking water sources. Similarly, during droughts, areas close to remote waterholes, rock-holes, soaks and springs become refuges for camels, where they can easily contaminate these water sources. A study by Brim-Box *et al*. (2010) reported that faecal contamination of a waterhole used as drinking water for people travelling through the Petermann and Katiti Aboriginal Land trusts in the Uluru-Kata Tjuta National Park, Northern Territory, was most likely caused by feral camels that were recorded and monitored at the site, especially during periods of low rainfall. Therefore, camel populations in Australia may contribute to the zoonotic transmission of *C. parvum* via contamination of the environment, in particular water sources for remote communities. These findings indicate the need for further characterisation of the prevalence, intensity of infection and species of *Cryptosporidium* in camel populations across Australia.

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

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Research paper

Prevalence of *Cryptosporidium* species and subtypes in paediatric oncology and non-oncology patients with diarrhoea in Jordan

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ABSTRACT

Cryptosporidiosis is a protozoan parasitic disease which affects human and animals worldwide. In adult immunocompetent individuals, cryptosporidiosis usually results in acute and self-limited diarrhoea; however, it can cause life threatening diarrhoea in children and immunocompromised individuals. In the present study, we compared the prevalence of *Cryptosporidium* species and *gp60* subtypes amongst paediatric oncology patients with diarrhoea (n = 160) from King Hussein Medical Centre for Cancer in Jordan, and non-oncology paediatric patients with diarrhoea (n = 137) from Al-Mafraq paediatric hospital. Microscopy results using modified acid fast staining identified a significantly ($p \leq 0.05$) higher prevalence of *Cryptosporidium* in paediatric oncology patients with diarrhoea (14.4% - 23/160), compared to non-oncology paediatric patients with diarrhoea only (5.1% - 7/137). With the exception of one sample, all microscopy-positive samples (n = 29) and an additional 3/30 microscopy-negative controls were typed to species and subtype level at the 18S and *gp60* loci, respectively. All *Cryptosporidium* positives were typed as *C. parvum*. Of the 22 typed *Cryptosporidium* positives from the paediatric oncology patients, 21 were subtyped as IIaA17G2R1 and one as IIaA16G2R1 *C. parvum* subtypes. The 7 typed positives from the paediatric patients from Al-Mafraq hospital were subtyped as IIaA17G2R1 (n = 5) and IIaA16G2R1 (n = 2). The 3 additional positives from the 30 microscopy negative control samples were subtyped as IIaA17G2R1. The high prevalence of the IIaA17G2R1 subtype, particularly amongst oncology patients, suggests that an outbreak of cryptosporidiosis may have been occurring in oncology patients during the collection period (April to December, 2016). New therapies for cryptosporidiosis in immunocompromised patients are urgently required.

1. Introduction

Cryptosporidium species are intracellular protozoan parasites that infect a wide range of hosts including humans, domestic and wild animals (Ryan et al., 2014; Zahedi et al., 2016). World-wide, human cryptosporidiosis is mainly caused by two species of *Cryptosporidium* (*C. hominis* and *C. parvum*); although numerous species of *Cryptosporidium* have been reported in humans, including *C. meleagridis*, *C. felis*, *C. canis*, *C. cuniculus*, *C. ubiquitum*, *C. viatorum*, *C. suis*, *C. scrofarom*, *C. viatorum*, *C. tyzerri*, *C. xiaoi*, *C. fayeri*, *C. muris*, and *C. andersoni* (Xiao, 2010; Ryan et al., 2016).

Cryptosporidium infection can result in acute diarrhoea, nausea, vomiting and weight loss, which is usually self-limiting (Ryan et al., 2016). In infants, cryptosporidiosis can be more serious and can lead to malnutrition, growth retardation and impairment in cognitive function (Shrivastava et al., 2017). Similarly, patients with some type of immunocompromised condition have an increased probability of acquiring cryptosporidiosis, which can manifest as severe protracted diarrhoea, chronic malabsorption, failure to thrive, malnutrition and increased mortality (Assefa et al., 2009; Idris et al., 2010; Domenech et al., 2011; Kurniawan et al., 2013; Marcos and Gotuzzo, 2013; Valenzuela et al., 2014; Nsagha et al., 2016).

Immunosuppression and diarrhoea are well-recognised side-effects of cancer treatment, yet relatively few studies have been conducted examining the prevalence of *Cryptosporidium* in cancer patients (Botero et al., 2003; Tamer et al., 2008; Al-Qobati et al., 2012; Hassanein et al., 2012; Sulżyc-Bielicka et al., 2012; García-Elorriaga et al., 2013). In Jordan, the molecular epidemiology of cryptosporidiosis is poorly understood and to date, only two genotyping studies have been conducted (Hijjawi et al., 2010; Hijjawi et al., 2016). The aim of the present study was to investigate the prevalence of *Cryptosporidium* species and subtypes in paediatric oncology patients with diarrhoea and paediatric patients with diarrhoea only, to better understand the epidemiology and management of cryptosporidiosis in these patients.

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Table 1
Demographic data, *Cryptosporidium* species and subtypes for the 23 microscopy-positive faecal samples, which were collected from April until December 2016 from paediatric oncology patients attending the King Hussein cancer centre.

Locality	Collection date (day/month)	Age (in years)	Sex	Clinical symptoms	Type of cancer	Species (18S)	*gp60* subtype
Amman	4/4	5	M	Diarrhoea/watery	Leukemia	*C. parvum*	IIaA17G2R1
Zarqa	11/4	3	M	Diarrhoea/mucus	Leukemia	*C. parvum*	IIaA17G2R1
Irbid	11/4	2	F	Diarrhoea/watery	Leukemia	*C. parvum*	IIaA17G2R1
Al-Mafraq	24/4	5	M	Diarrhoea/watery	NA	*C. parvum*	IIaA17G2R1
Amman	2/5	5	M	Diarrhoea/watery	Neuroblastoma	*C. parvum*	IIaA17G2R1
Amman	16/5	2	F	Diarrhoea/mucus	Lymphoma	*C. parvum*	IIaA17G2R1
Amman	30/5	5	M	Diarrhoea/mucus	Leukemia	*C. parvum*	IIaA17G2R1
Amman	30/5	1	F	Soft stool	Brain cancer	*C. parvum*	IIaA16G2R1
Amman	6/6	2	F	Diarrhoea/watery	Leukemia	*C. parvum*	IIaA17G2R1
Zarqa	20/6	3	M	Diarrhoea/mucus	Bone cancer	*C. parvum*	IIaA17G2R1
Zarqa	4/7	4	F	Diarrhoea/watery	Leukemia	*C. parvum*	IIaA17G2R1
Zarqa	18/7	4	F	Diarrhoea/watery	Leukemia	*C. parvum*	IIaA17G2R1
Zarqa	1/8	3	F	Diarrhoea/mucus	NA	*C. parvum*	IIaA17G2R1
Amman	15/8	2	M	Diarrhoea/watery	Leukemia	*C. parvum*	IIaA17G2R1
Jarash	15/8	1.5	M	Diarrhoea/watery	NA	*C. parvum*	IIaA17G2R1
Jarash	29/8	2	M	Diarrhoea/mucus	Leukemia	No amplification	–
Amman	5/9	1	M	Soft stool	NA	*C. parvum*	IIaA17G2R1
Zarqa	5/9	1	F	Diarrhoea/watery	NA	*C. parvum*	IIaA17G2R1
Amman	12/9	5	M	Diarrhoea/watery	Leukemia	*C. parvum*	IIaA17G2R1
Asalt	26/9	5	F	Soft stool	Lymphoma	*C. parvum*	IIaA17G2R1
Zarqa	3/10	4	M	Diarrhoea/watery	Leukemia	*C. parvum*	IIaA17G2R1
Zarqa	17/10	2	M	Soft stool	NA	*C. parvum*	IIaA17G2R1
Amman	17/10	1	M	Diarrhoea/mucus	Leukemia	*C. parvum*	IIaA17G2R1

NA: not available.

2. Materials and methods

2.1. Cryptosporidium isolates

A total of 297 fresh diarrheic stool specimens were collected from two groups of children (aged 1–5 years old) from April until December, 2016; including paediatric oncology patients with diarrhoea at the King Hussein Medical Centre for Cancer (n = 160) and paediatric patients with diarrhoea only at the Al-Mafraq paediatric hospital (n = 137). The samples from King Hussein Medical Centre for Cancer were collected from children undergoing chemotherapy for different types of cancer (mainly leukemia) and who resided in different parts of Jordan but regularly visited the centre for treatment. The children who were referred to the Al-Mafraq paediatric hospital, were mainly from Al-Mafraq city and nearby villages. In addition, a further 30 samples were collected from the same two hospitals (18 samples from the King Hussein Medical Centre for Cancer and 12 from Al-Mafraq hospital), from children with diarrhoea, but which were negative by microscopy for *Cryptosporidium* oocysts, in order to serve as a negative control to compare the sensitivity of microscopy and PCR.

Ethical approval to conduct the study was obtained from the King Hussein Medical Centre for Cancer and Al-Mafraq paediatric hospital and issued by the Institutional Review Board at Hashemite University (Ethics permit number 150/1313/18). A signed consent form was obtained from parents or guardians of each child, who voluntarily participated after a clear explanation of the research objectives. Demographic data regarding age, gender, residency, medical history and duration of symptoms were obtained via questionnaire from the guardian of each child (Ethics permit number 150/1313/18).

2.2. Microscopy, DNA extraction and typing

Microscopy was performed on all faecal samples using a routine modified acid fast staining procedure. Briefly, a smear was prepared using 1 to 2 drops of the collected stool specimen, fixed with absolute methanol for 30 s, stained with carbol fuchsin for 3 min, rinsed briefly with tap water before being stained with methylene blue. The stained dried slides were labelled and examined at 100 × oil immersion lens under a light microscope (Olympus CH40/RF200, Japan) for the presence of *Cryptosporidium* oocysts.

For subsequent DNA extraction, 1 to 2 g of the individually collected fresh stool specimen from each child were fixed in 2.5% potassium dichromate and left at room temperature for 2–6 months. Before DNA extraction, the stool samples were washed three times in 10% PBS buffer and centrifuged at 2000 × *g* for 2–3 min in order to remove the potassium dichromate. Total DNA was extracted using a QIAmp DNA Stool Kit (Qiagen, Germany), following the recommendation of the supplier after 5 cycles of freezing and thawing. The extracted DNA was stored at − 80 °C until further molecular characterization. All samples which were positive for *Cryptosporidium* by microscopy (n = 30) and the 30 samples that were *Cryptosporidium* negative by microscopy (negative controls) were initially screened at the 18S locus and identified to species level using nested PCR amplification and Sanger sequencing as previously described (Xiao et al., 1999). Samples were then subtyped at the glycoprotein 60 (*gp60*) locus using a nested PCR to amplify a ~400 bp product using the primers AL3531 (5′-ATAGTCTCCGCTGTATTC-3′) and AL3533 (5-GAGATATATCTTGGTGCG-3) for the primary PCR, and AL3532 (5′-TCCGCTGTATTCTCAGCC-3′) and LX0029 (5′-CGAACCACATTACAAATGAAGT-3′) for the secondary PCR (Sulaiman et al., 2005).

2.3. Statistical analysis

The prevalence of *Cryptosporidium* in faecal samples collected from each group was expressed as the percentage of samples positive by microscopy, with 95% confidence intervals calculated assuming a binomial distribution, using the software Quantitative Parasitology 3.0 (Rózsa et al., 2000). Fisher's exact test was performed using SPSS 22 for Windows (SPSS Inc. Chicago, USA), to determine if there was a statistical difference in the prevalence of *Cryptosporidium* in paediatric oncology patients with diarrhoea and paediatric patients with diarrhoea only.

3. Results

3.1. Prevalence by microscopy and demographic data

Microscopic screening using modified acid fast staining of the 297

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Table 2
Demographic data, *Cryptosporidium* species and subtypes for the 7 microscopy-positive faecal samples from non-oncology paediatric patients with diarrhoea attending the Al-Mafraq paediatric hospital (all were from the Al-Mafraq area and surrounding villages).

Age (in years)	Collection date (day/month)	Sex	Clinical symptoms	Species (18S)	*gp60* subtype
4	6/4	F	Soft stool	*C. parvum*	IIaA17G2R1
3	20/4	F	Diarrhoea/ mucus	*C. parvum*	IIaA17G2R1
3	11/5	M	Diarrhoea/ watery	*C. parvum*	IIaA16G2R1
1	15/6	M	Diarrhoea/ watery	*C. parvum*	IIaA16G2R1
5	29/6	M	Soft stool	*C. parvum*	IIaA17G2R1
4	6/7	F	Diarrhoea/ watery	*C. parvum*	IIaA17G2R1
3	24/8	M	Soft stool	*C. parvum*	IIaA17G2R1

stool specimens from the recruited children in the present study identified a prevalence of 14.4% (23/160) – 95% CI 9.3–20.8, in paediatric oncology patients with diarrhoea and 5.1% (7/137) – 95% CI, 2.1–10.2 for non-oncology paediatric patients with diarrhoea. This difference was significant ($p < 0.05$). The overall prevalence of *Cryptosporidium* across the two groups was 10.1% (30/297) 95% CI 6.9–14.1.

The majority of *Cryptosporidium*-positive paediatric oncology patients (82.6% - 19/23) were experiencing diarrhoea, whereas the remaining 4 had soft stools (Table 1). Of the paediatric patients from Al-Mafraq hospital, 57.1% (4/7) had diarrhoea, while the remaining 3 had soft stools (Table 2). Leukemia was the most common type of cancer experienced by the patients that were positive for *Cryptosporidium* (n = 12), followed by lymphoma (n = 2). Data on the type of cancer was unavailable for 6 patients (Table 1).

3.2. *Cryptosporidium* species and *gp60* subtypes

Sequences were obtained for 29/30 positives from the two groups. In addition, screening of the additional 30 faecal samples from paediatric patients from both hospitals, which were negative for *Cryptosporidium* by microscopy, identified another 3 positives. A total of 32 samples typed. All were identified as *C. parvum*. Of the 22 typed *Cryptosporidium* positives from the paediatric oncology patients, from King Hussein Medical Centre for Cancer, 21 were typed as IIaA17G2R1 and one as IIaA16G2R1 (Table 1). The 7 typed positives from the paediatric patients from Al-Mafraq hospital were typed as IIaA17G2R1 (n = 5) and IIaA16G2R1 (n = 2). The 3 additional positives from the 30 microscopy negative control samples were typed as IIaA17G2R1 (Supplementary Table 1). There was no heterogeneity within individual subtypes. Representative *gp60* sequences from *C. parvum* subtypes IIaA17G2R1 and IIaA16G2R1 were submitted to GenBank under accession numbers MF770731 – MF770734.

4. Discussion

The present study compared the prevalence of *Cryptosporidium* species and *gp60* subtypes amongst paediatric oncology and non-oncology patients with diarrhoea. As expected, the prevalence of *Cryptosporidium* was significantly higher amongst paediatric oncology patients with diarrhoea (14.4%), compared to non-oncology paediatric patients with diarrhoea (5.1%), with 2.8 times more cryptosporidiosis cases amongst the former. This result is similar to a previous study in Egypt, which detected *Cryptosporidium* in 24% of children with acute lymphoblastic leukemia (ALL) on maintenance chemotherapy, compared to 3% in control patients with diarrhoea (Hassanein et al., 2012). In addition to being a cause of diarrhoea in oncology patients, *Cryptosporidium* has been associated with colon cancer (Sulżyc-Bielicka et al., 2012) and has been shown to induce low-to-high grade intestinal dysplasia in immunocompromised mice (Abdou et al., 2013).

Previous studies in Jordan (for which the immune status is unknown) have reported prevalences ranging from 1.5–37.7% in humans and 3.9–18.7% in production animals using both microscopy and molecular tools (Youssef et al., 2000; Nimri, 2003; Mahgoub et al., 2004; Hijjawi et al., 2010; Hijjawi et al., 2016(. These differences likely reflect differences in detection methods, sample size, area of collection and microscopist skill etc.

During the present study, only one species, *C. parvum* and two *gp60* subtypes (IIaA16G2R1, IIaA16G2R1), were detected in all the typed isolates. This was an unexpected finding as in two previous genotyping studies in Jordan, up to 4 *Cryptosporidium* species (*C. parvum*, *C. hominis*, *C. meleagridis*, and *C. canis*) and six subtype families (IIa, IIc, IId, 1b, 1d and IIIa) were detected in human isolates (Hijjawi et al., 2010; Hijjawi et al., 2016). In the previous two studies, *C. parvum* and *C. hominis* were the dominant species and were detected in almost equal frequencies (Hijjawi et al., 2010; Hijjawi et al., 2016). The lack of identification of the *C. parvum* IId subtype and also *C. hominis* is surprising, as previous studies have shown that both are common in humans in Middle Eastern countries including Jordan, although most studies have reported that *C. parvum* is the dominant *Cryptosporidium* species (Sulaiman et al., 2005; Meamar et al., 2007; Al-Brikan et al., 2008; Pirestani et al., 2008; Hijjawi et al., 2010; Iqbal et al., 2011; Nazemalhosseini-Mojarad et al., 2011; Taghipour et al., 2011; Alyousefi et al., 2013; Sharbatkhori et al., 2015), which may explain the lack of detection of *C. hominis*. The IIa subtype family has been previously detected in Jordan children, however, this is the first report of the IIaA17G2R1 and IIaA16G2R1 subtypes in humans in Jordan. The IIaA16G2R1 subtype has also been previously reported from one cattle isolate from Jordan (Hijjawi et al., 2016).

The dominant subtype, IIaA17G2R1, was detected in 91.3% (95% CI, 72–98.9) of paediatric oncology patients with diarrhoea and 71.4% (95% CI, 29–96.3) of paediatric patients with diarrhoea only. The high prevalence of this subtype, particularly amongst oncology patients, suggests that an outbreak may have been occurring in oncology patients at the King Hussein Medical Centre for Cancer and Al-Mafraq hospital during the collection period (summer 2016). This may also explain the lack of detection of the *C. parvum* IId subtype and *C. hominis*. However, Multi-Locus Sequence Typing (MLST) is required to confirm this. Alternatively, the low heterogeneity observed at the subtype level, may reflect intensive and stable transmission of *C. parvum* in this region. For example, a study of several human populations in Tunisia reported that the *C. hominis* subtype IaA26G1R1, was the most dominant subtype (50%), suggesting stable anthroponotic cryptosporidiosis transmission (Essid et al., 2017). IIaA17G2R1 is a relatively common subtype that has been reported in livestock (Alves et al., 2006; Xiao et al., 2007; Mi et al., 2014; Kaupke and Rzeżutka, 2015) and has also been responsible for an outbreak of cryptosporidiosis in a youth summer camp in North Carolina in 2009 (CDC, 2011). In the latter study, IIaA17G2R1 was identified in faecal samples from livestock and humans at the camp, indicating that zoonotic transmission may have occurred. In the present study, the source of the *Cryptosporidium* infection is unknown but may have been due to the consumption of contaminated food or water. In order to identify the risk factors involved in the acquisition of *Cryptosporidium* infections in children and immunocompromised individuals in Jordan, a well-designed case control study, with detailed collection of data on water and food sources, animal and human contact and immune status is required. As only one molecular study has been conducted on species and subtypes of *Cryptosporidium* in livestock in Jordan (Hijjawi et al., 2016), further studies on larger numbers of animal and human samples, as well as water samples are essential to determine the transmission dynamics of cryptosporidiosis in Jordanian children.

A limitation of the present study is that while a compromised immune system can be assumed for the paediatric oncology patients, the immune status of non-oncology paediatric patients with diarrhoea is unknown, as this is not part of routine monitoring for children in

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Jordan hospitalised for diarrhoea. In addition, in the present study, the prevalence of *Cryptosporidium* species and subtypes in these children was accessed using microscopy only and therefore the true prevalence is likely underestimated. For example, one previous which compared microscopy and molecular analysis on Jordanian human patients identified a prevalence of 1.8% by microscopy and > 19% by quantitative PCR (qPCR) confirming the superior sensitivity of PCR (Hijjawi et al., 2010). The finding of an additional 3 positives in the present study in the 30 microscopy negative samples, further supports this.

In conclusion, a high prevalence of cryptosporidiosis was detected amongst paediatric oncology patients. Routine screening for *Cryptosporidium* should be conducted for all oncology patients undergoing chemotherapy, preferably by PCR. The detection of cryptosporidiosis in these patients however presents specific challenges for the treatment, as the only FDA approved drug, nitazoxanide, is ineffective in immunocompromised individuals (Amadi et al., 2009). A previous study reported the eradication of *Cryptosporidium* in four children with acute lymphoblastic leukemia using paromomycin or azithromycin (Trad et al., 2003), and therefore these therapies should be considered for paediatric oncology patients. There is no vaccine for cryptosporidiosis and given the parasite's high infectivity, robustness, and resistance to disinfection (Ryan et al., 2016), improved therapeutics particularly for immunocompromised individuals are urgently required. The advent of whole genome sequencing has identified several promising drug targets including inosine-5′-monophosphate dehydrogenase (IMPDH) (essential for purine salvage) and acyl-coenzyme A synthetases (LC-ACS) which are essential in fatty acid metabolism (Ryan and Hijjawi, 2015), which holds promise for the future.

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Appendix 9

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Research paper

Cryptosporidium homai n. sp. (Apicomplexa: Cryptosporidiiae) from the guinea pig (*Cavia porcellus*)

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hsp70

ABSTRACT

The morphological, biological, and molecular characterisation of a new *Cryptosporidium* species from the guinea pig (*Cavia porcellus*) are described, and the species name *Cryptosporidium homai* n. sp. is proposed. Histological analysis conducted on a *post-mortem* sample from a guinea pig euthanised due to respiratory distress, identified developmental stages of *C. homai* n. sp. (trophozoites and meronts) along the intestinal epithelium. Molecular analysis at 18S rRNA (18S), actin and *hsp70* loci was then conducted on faeces from an additional 7 guinea pigs positive for *C. homai* n. sp. At the 18S, actin and *hsp70* loci, *C. homai* n. sp. exhibited genetic distances ranging from 3.1% to 14.3%, 14.4% to 24.5%, and 6.6% to 20.9% from other *Cryptosporidium* spp., respectively. At the 18S locus, *C. homai* n. sp. shared 99.1% similarity with a previously described *Cryptosporidium* genotype in guinea pigs from Brazil and it is likely that they are the same species, however this cannot be confirmed as actin and *hsp70* sequences from the Brazilian guinea pig genotype are not available. Phylogenetic analysis of concatenated 18S, actin and *hsp70* sequences showed that *C. homai* n. sp. exhibited 9.1% to 17.3% genetic distance from all other *Cryptosporidium* spp. This clearly supports the validity of *C. homai* n. sp. as a separate species.

1. Introduction

Cryptosporidium spp. are protozoan parasites responsible for gastroenteritis in a wide range of vertebrates including humans, domestic and wild animals and are a common cause of waterborne outbreaks worldwide (Zahedi et al., 2016; Ryan et al., 2016; Efstratiou et al., 2017). The parasite is transmitted via the faecal-oral route with both zoonotic and anthroponotic transmission cycles (Ryan et al., 2014). Currently relatively little is known about the molecular characteristics, host specificity, pathogenicity and zoonotic importance of *Cryptosporidium* spp. in wild and domestic rodents (Appelbee et al., 2005; Ziegler et al., 2007a,b; Ryan et al., 2014; Kváč et al., 2016; Li et al., 2016). To date, of the 33 recognised *Cryptosporidium* spp. (cf. Ryan et al., 2016; Jezkova et al., 2016), 11 species including *C. proliferans*, *C. meleagridids*, *C. tyzerri*, *C. andersoni*, *C. ubiquitum*, *C. wrairi*, *C. parvum*, *C. suis*, *C. meleagridis*, *C. muris* and *C. rubeyi* and over 20 genotypes of unknown species status have been reported in rodents with a prevalence ranging from 1% to 63% (Table 1) (Qi et al., 2015; Song et al., 2015; Stenger et al., 2015; Zahedi et al., 2016; Li et al., 2016).

The guinea pig (*Cavia porcellus*) is one of eight species in the genus Cavia (Rodentia: Caviidae), and is endemic to South America. Based on available archaeological and molecular data, it has been living in the region since the Miocene-Pliocene boundary, and it has been suggested that *C. porcellus* was initially derived from *Cavia tschudii*, when the Amerindia peoples of Peru started to domesticate guinea pigs 4500–7000 years ago. Eventually, the utility of the domesticated form of guinea pig as a food source or pet and laboratory animal, has resulted in its worldwide distribution including Australia (Dunnum and Salazar-Bravo, 2009).

Currently, *C. wrairi* is the only valid *Cryptosporidium* spp. described in guinea pigs (*Cavia porcellus*), with strong host specificity and no reports of human infection (Vetterling et al., 1971; Chrisp et al., 1990; Spano et al., 1997; Lv et al., 2009; Gressler et al., 2010; Smith et al., 2010). Previous experimental infections indicated that *C. wrairi* was infective to mice, lambs and calves, causing a sparse infection in ruminants, however as genotyping was not conducted, this cannot be confirmed (Angus et al., 1985; Chrisp et al., 1992).

The present study examined the morphological, biological and

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Table 1
Cryptosporidium species and genotypes reported in rodents.

Species/genotype	Major host	Report in rodents	References	Reports in humans
C. proliferans	Rodents	Spiny mouse (*Acomys cahirinus*), Tristram's jird(*Meriones tristrami*), Lesser gerbil (*Gerbilus gerbilus)*, Mongolian gerbil (*Meriones unguiculatus*), Bushy-tailed jird (*Sekeetamys calurus*), Southern multimammate mouse (Mastomys coucha), Natal multimammate mouse (*Mastomys natalensi*), Brandt's voles (*Microtus brandti)*, House mouse (*Mus spp.*), Eastern gray squirrel (*Sciurus carolinensis*), East African mole-rat(*Tachyoryctes splendens*)	Kváč et al., 2016	No reports in humans to date
C. rubeyi	Rodents	Golden-mantled ground squirrels (*Callospermophilus lateralis*), Belding's ground squirrels (*Urocitellus beldingi*), California ground squirrels (*Otospermophilus beecheyi*), Black-tailed prairie dogs (*Cynomys ludovicianus*)	Atwill et al., 2004; Pereira et al., 2010; Li et al., 2015; Stenger et al., 2015	No reports in humans to date
C. scrofarum	Pigs	Asian house rat (*Rattus tanezumi),* Brown rat (*Rattus norvegicus*)	Ng-Hublin et al., 2013	Occasionally reported in humans. Kváč et al., 2009a; Kváč et al., 2009b
C. tyzzeri	Rodents	Mice (*Mus musculus*), Brown rats (*Rattus norvegicus*), Large-footed bat (*Myotus adversus*), Yellow-necked mouse (*Apodemus flavicollis*), Bank vole (*Myodes glareolus*), Common vole (*Microtus arvalis*)	Morgan et al., 1999; Bajer et al., 2003; Karanis et al., 2007; Lv et al., 2009; Ren et al., 2012; Kváč et al., 2013	Occasionally reported in humans. Rasková et al., 2013
C. ubiquitum	Ruminants, rodents, primates	Deer mouse (*Peromyscus*), Eastern grey squirrels (*Sciurus carolinensis*), Red squirrel (*Sciurus vulgaris*), Eastern chipmunk (Tamias striatus), Large Japanese field mouse (*Apodemus speciosus),* Prehensile-tailed porcupines (*Coendou prehensilis*), Wood chuck (*Marmota monax*)	Perez and Le Blancq, 2001; Feng et al., 2007; Ziegler et al., 2007a,b; Fayer et al., 2010; Murakoshi et al., 2013; Li et al., 2014; Song et al., 2015; Stenger et al., 2015; Qi et al., 2015; Li et al., 2016	Commonly reported. Gatei et al., 2006; Tiangtip and Jongwutiwes, 2002; Gatei et al., 2003; Palmer et al., 2003; Gatei et al., 2006; Leoni et al., 2006; Muthusamy et al., 2006; Azami et al., 2007; Al Brikan et al., 2008; Neira et al., 2012; Hasajová et al., 2014; Li et al., 2014; Chappell et al., 2015; Petrincová et al., 2015; Spanakos et al., 2015.
C. suis	Pigs	Rodents	Bodager et al., 2015	Occasionally reported in humans. Xiao et al., 2002; Leoni et al., 2006; Cama et al., 2007; Wang et al., 2013; Bodager et al., 2015
C. suis-like	Pigs	Asian house rat (*Rattus tanezumi)*	Ng-Hublin et al., 2013	No reports in humans to date.
C. andersoni	Cattle	Marmots *(Marmota monax),* Campbell's dwarf hamster (*Phodopus campbelli*), Golden hamster (*Mesocricetus auratus*)	Lv et al., 2009; Wang et al., 2012	Numerous reports. Leoni et al., 2006; Morse et al., 2007; Waldron et al., 2011; Agholi et al., 2013; Jiang et al., 2014; Liu et al., 2014; Hussain et al., 2017
C. parvum	Ruminants	Eastern grey squirrel (*Sciurus carolinensis*), Ground Squirrels (*Spermophilus beecheyi*), Siberian chipmunk (*Tamias sibiricus*), Wood mice (*Apodemus sylvaticus*), White-footed mouse (*Peromyscus leucopus*), Capybara (*Hydrochoerus hydrochaeris*), Yellow-bellied marmot (*Marmota flaviventris*), Bamboo rats (*Rhizomys sinensis*), Campbell's dwarf hamster (*Phodopus campbelli*), Golden hamster (*Mesocricetus auratus*)	Chalmers et al., 1997; Matsui et al., 2000; Torres et al., 2000; Perez and Le Blancq, 2001; Bajer et al., 2003; Lv et al., 2009; Ng-Hublin et al., 2013; Zhao et al., 2015; Li et al., 2016	Commonly reported in humans
C. meleagridis	Birds and humans	Deer mouse (*Peromyscus*)	Feng et al., 2007; Bodager et al., 2015	Commonly reported in humans. Morgan et al., 2000; Cama et al., 2003; Gatei et al., 2006; Muthusamy et al., 2006; Leoni et al., 2006; Berrilli et al., 2012; Elwin et al., 2012; Neira et al., 2012; Silverlås et al., 2012; Kurniawan et al., 2013; Sharma et al., 2013; Wang et al., 2014; Adamu et al., 2014; Ghaffari and Kalantari, 2014; Ryan and Xiao, 2014; Rahmouni et al., 2014; Stensvold et al., 2014; Stensvold et al., 2015
C. wrairi	Guinea pigs	Guinea pig (*Cavia porcellus*), California ground squirrels (*Spermophilus beecheyi*)	Vetterling et al., 1971; Chrisp et al., 1990; Spano et al., 1997; Lv et al., 2009	No reports in humans to date
C. muris	Rodents	Wild rats (*Rattus* sp.), Mice (*Mus* sp.), Girrafes house mice (*Mus musculus*), Eastern grey squirrel (*Sciurus carolinensis*), Golden hamster (*Mesocricetus auratus*), Japanese field mouse (*Apodemus argenteus*), Bank voles (*Clethrionomys glareolus*), Campbell hamster (*Phodopus campbelli*), Siberian hamster (*Phodopus sungorus*), Golden hamster (*Mesocricetus auratus*), East African mole rat (*Tachyoryctes splendens*), Large Japanese field mouse (*Apodemus speciosus*)	Chalmers et al., 1997; Torres et al., 2000; Hurkova et al., 2003; Hikosaka and Nakai, 2005; Ziegler et al., 2007a,b; Kváč et al., 2008; Lv et al., 2009; Wang et al., 2012; Murakoshi et al., 2013; Ng-Hublin et al., 2013; Song et al., 2015; Zhao et al., 2015	Many reports – Gatei et al., 2006; Leoni et al., 2006; Muthusamy et al., 2006; Azami et al., 2007; Al Brikan et al., 2008; Neira et al., 2012; Hasajová et al., 2014; Petrincová et al., 2015; Spanakos et al., 2015
Beaver genotype	Rodents	North American beaver (*Castor canadensis*)	Feng et al., 2007	No reports in humans to date

(continued on next page)

Table 1 *(continued)*

Species/genotype	Major host	Report in rodents	References	Reports in humans
Chipmunk genotype I	Rodents	Chipmunk sp. (*Tamias sp.*), Eastern grey squirrel (*Sciurus carolinensis*), Deer mice (*Peromyscus maniculatus*)	Jiang et al., 2005; Feltus et al., 2006; Feng et al., 2007; Kváč et al., 2008; Insulander et al., 2013; Lebbad et al., 2013; Guo et al., 2015; Song et al., 2015	Emerging human pathogen Feltus et al., 2006; ANOFEL, 2010; Lebbad et al., 2013; Guo et al., 2015
Chipmunk genotype II	Rodents	Eastern chipmunk (*Tamias striatus*)	Feng et al., 2007; Stenger et al., 2015	No reports in humans to date
Chipmunk genotype III	Rodents	Siberian chipmunk (*Tamias sibiricus*)	Lv et al., 2009	No reports in humans to date
Deer mouse genotype I	Rodents	Deer mouse (*Peromyscus*)	Feng et al., 2007	No reports in humans to date
Deer mouse genotype II	Rodents	Deer mouse (*Peromyscus*)	Feng et al., 2007	No reports in humans to date
Deer mouse genotype III	Rodents	Deer mouse (*Peromyscus*)	Feng et al., 2007	No reports in humans to date
Deer mouse genotype IV	Rodents	Deer mouse (*Peromyscus*)	Feng et al., 2007	No reports in humans to date
Ferret genotype	Rodents	Siberian chipmunk (*Tamias sibiricus*), River otters (Lontra canadensis), Red squirrel (*Sciurus vulgaris*), Guinea pig (*Cavia porcellus*), Hamster (*Phodopus sungorus*)	Kváč et al., 2008; Lv et al., 2009; Feng et al., 2011; Li et al., 2016	No reports in humans to date
Ground squirrel genotype I	Rodents	Thirteen-lined ground squirrel (*Ictidomys tridecemlineatus*)	Stenger et al., 2015	No reports in humans to date
Ground squirrel genotype II	Rodents	Black-tailed prairie dog (*Cynomys ludovicianus*)	Stenger et al., 2015	No reports in humans to date
Ground squirrel genotype III	Rodents	Thirteen-lined ground squirrel (*Ictidomys tridecemlineatus*)	Stenger et al., 2015	No reports in humans to date
Hamster genotype	Rodents	Siberian hamster (*Phodopus sungorus*)	Lv et al., 2009	No reports in humans to date
Mouse genotype II	Rodents	House mouse (*Mus musculus)*	Foo et al., 2007; Silva et al., 2013	No reports in humans to date
Mouse genotype III	Rodents	House mouse (*Mus musculus)*	Silva et al., 2013	No reports in humans to date
Muskrat genotype I	Rodents	Muskrat (*Ondatra zibethicus*), Boreal red-backed vole (*Myodes rutilus*).	Zhou et al., 2004; Feng et al., 2007	No reports in humans to date
Muskrat genotype II	Rodents	Muskrat (*Ondatra zibethicus*), Deer mouse (*Peromyscus maniculatus*), Meadow vole (*Microtus pennsylvanicus*)	Ziegler et al., 2007a,b; Robinson et al., 2011	No reports in humans to date
Naruko genotype	Rodents	Large Japanese field mouse (*Apodemus speciosus*)	Murakoshi et al., 2013	No reports in humans to date
Rat genotype I	Rodents	Brown rat (*Rattus norvegicus*)	Kimura et al., 2007; Chalmers et al., 2010; Ng-Hublin et al., 2013	No reports in humans to date
Rat genotype II	Rodents	Brown rat (*Rattus tanezum*),Wild black rat (*Rattus rattus*), Brown rat (*Rattus norvegicus*)	Lv et al., 2009; Ng-Hublin et al., 2013; Silva et al., 2013	No reports in humans to date
Rat genotype III	Rodents	Asian house rat (*Rattus tanezumi)*, Wild black rat *(Rattus rattus)*	Lv et al., 2009; Ng-Hublin et al., 2013; Silva et al., 2013	No reports in humans to date
Rat genotype IV	Rodents	Tanezumi rat (*Rattus tanezumi*), Asian house rat (*Rattus tanezumi)*, Brown rat (*Rattus norvegicus*)	Ng-Hublin et al., 2013	No reports in humans to date
Skunk/skunk-like genotype	Skunk	Eastern grey squirrel (*Sciurus carolinensis*), American red squirrels (*Tamiasciurus hudsonicus*)	Feng et al., 2007; Ziegler et al., 2007a,b	Several reports. Zhou et al., 2004; Feng et al., 2007; Ziegler et al., 2007a,b; Robinson et al., 2008; Feng et al., 2011; Elwin et al., 2012
Vole genotype	Rodents	Meadow vole (*Microtus pennynsylvanicus)*)	Feng et al., 2007	No reports in humans to date
Novel genotype	Guinea pigs	Guinea pig (*Cavia porcellus*)	Huber et al., 2007	No reports in humans to date

Appendix 9

Table 2
List of faecal samples collected for this study, from guinea pigs, held at the University of Western Australia Animal Care Services, Perth, Australia.

Sample ID	Date	Type of sample
Z1	12/12/2016	Faeces – Pooled
Z2	12/12/2016	Faeces – Individual
Z3	12/12/2016	Faeces – Individual
Z4	12/12/2016	Faeces – Pooled
Z5	12/12/2016	Faeces – Pooled
Z6	12/12/2016	Faeces – Pooled
Z7	12/12/2016	Faeces – Pooled
Z8	12/12/2016	Faeces – Pooled
Z9	12/12/2016	Faeces – Pooled
Z10	12/12/2016	Faeces – Pooled
Z11	12/12/2016	Faeces – Pooled
Z12	12/12/2016	Faeces – Pooled
Z13	12/12/2016	Faeces – Pooled
Z14	12/12/2016	Faeces – Individual
Z15	12/12/2016	Faeces – Pooled
Z16	12/12/2016	Faeces – Pooled
Z17	12/12/2016	Faeces – Pooled
Z18	12/12/2016	Faeces – Individual
Z19	12/12/2016	Faeces – Pooled
Z20	12/12/2016	Faeces – Pooled
Z21	12/12/2016	Faeces – Pooled
Z22	12/12/2016	Faeces – Pooled
Z23	12/12/2016	Faeces – Pooled
Z24	12/12/2016	Faeces – Pooled
Z25	12/12/2016	Faeces – Pooled
Z26	12/12/2016	Faeces – Pooled
Z27	12/12/2016	Faeces – Pooled
Z28	12/12/2016	Faeces – Individual
E89	01/11/2016	Faeces – Individual

molecular characteristics of a *Cryptosporidium* sp. detected in the gastrointestinal tract and faeces of guinea pigs. Based on the collective data from the present study, the *Cryptosporidium* spp. detected in these guinea pigs is genetically and biologically distinct from all species of *Cryptosporidium* described previously, and we propose the species name *Cryptosporidium homai* n. sp. For clarity, we herein refer to this novel species by its proposed name.

2. Materials and methods

2.1. Source of sample and sample processing

A guinea pig, which was part of a group of experimental animals held at the University of Western Australia Animal Care Services, Perth, Australia, presented with audible respiratory distress (rattled breathing sounds, difficulty in breathing and chin coated in saliva) and as a result was euthanised. *Post-mortem* examination was performed and intestinal and lung sections were sent to a specialist veterinary laboratory for further histopathological examination, and during routine handling, individual faecal sample were collected and stored at 4 °C until required. Further to the initial histopathology and molecular analysis, additional faecal samples (n = 28) were collected either from individual animals or pooled from animals kept in the same enclosure for further molecular analysis (Table 2).

2.2. Histopathology

Sections of intestinal tissue were fixed in 10 mg/100 ml phosphate buffered formalin for at least 24 h, dehydrated in an ethanol-xylene series and embedded in paraffin wax. Two micrometer tissue sections

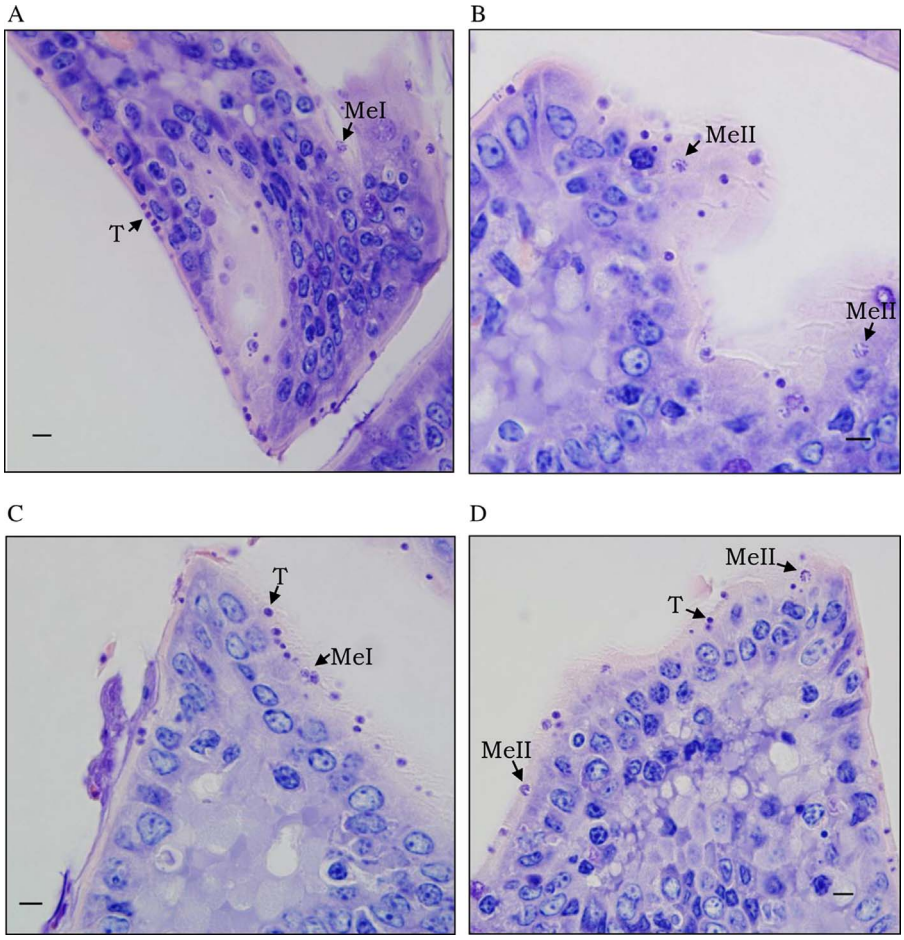

Fig. 1. (A–D) Giemsa-stained section of intestinal epithelium showing moderate to heavy epicellular infection by *Cryptosporidium,* associated with minimal host inflammatory response (isolate E89). Trophozoites (T) and meronts (Me) generally measured less than five micrometres as is typical for *Cryptosporidium*. There was a predominance of merogony with both type I (MeI) and type II meronts (MeII) present. Scale bar: 5 µm.

Appendix 9

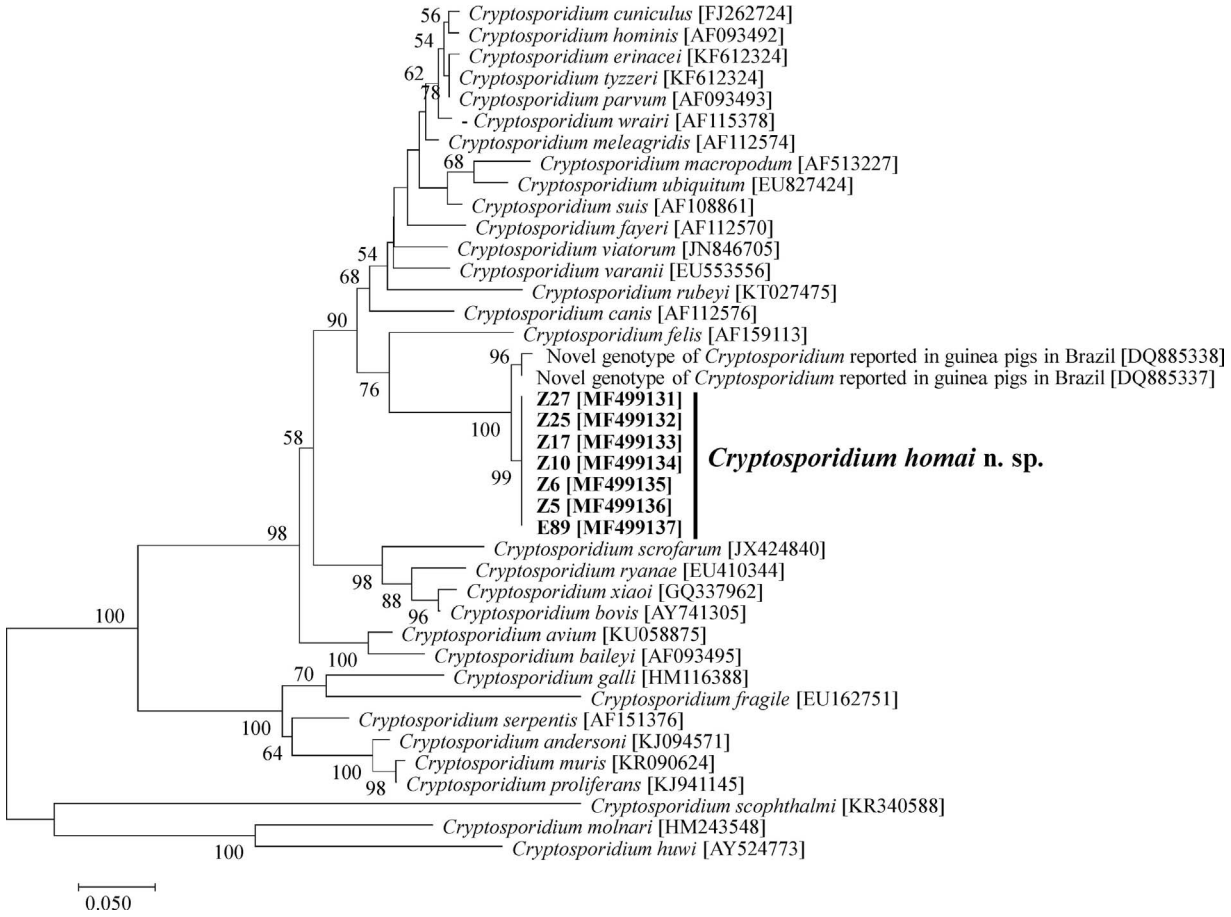

Fig. 2. Evolutionary phylogenetic relationship between *C. homai* n. sp. and *Cryptosporidium* species described to date as inferred by maximum likelihood (ML) analysis of 18S rRNA locus. Percentage support (> 50%) from 1000 pseudoreplicates from ML analyses is indicated at the left of the supported node. Scale bars indicate the number of substitutions per nucleotide position.

were dewaxed in xylene, rehydrated in an ethanol series and stained by haematoxylin & eosin (H & E) or Giemsa. Giemsa stock solutions were made up with 0.75 g Giemsa powder, 65 ml methanol and 65 ml glycerol, and diluted 1:10 with tap water immediately prior to use. Giemsa stained tissue sections were acidified with 10% acetic acid for 15 s, and placed in freshly diluted Giemsa stain, preheated in a microwave (Kambrook model 686LE, 1150W) on medium for 30 s, followed by microwaving on low for 30 s. Slides were rinsed in tap water followed by absolute ethanol, before permanent mounting in DPX (Dako).

2.3. DNA isolation

Following five cycles of freeze-thaw, genomic DNA was extracted from 250 mg of each faecal sample (n = 29), using a Power Soil Kit (MO BIO, Carlsbad, California, USA) in accordance to the manufacturer's instructions. An extraction blank (no faecal sample) was used in each extraction group. Purified DNA was stored in −20 °C prior to molecular analyses. DNA extraction and post-DNA extraction procedures were performed in separate dedicated laboratories.

2.4. PCR amplification

A nested PCR approach was used to amplify an approximately 825 bp 18S rRNA fragment using the primers SSU-F2 (5′-TTCTAGAGCTAATACATGCG-3′) and SSU-R2 (5′-CCCATT TCCTTCGAAACAGGA-3′) for the primary PCR and SSU-F3 (5′-GGAAGGGTTGTATTTATTAGATAAAG-3′) and SSU-R4 (5′-AAGGAGTAAGGAACAACCTCCA-3′) for the nested PCR (Xiao et al., 1999). Each 25 µl PCR mixture contained 1 µl of genomic DNA, 1 × Go Taq PCR buffer (KAPA Biosystems, South Africa), 3.75 mM $MgCl_2$, 400 µM of each dNTPs, 0.4 µM of forward and reverse primers and 1 U Kapa DNA polymerase (Kapa Biosystems, South Africa). The PCR cycling conditions consisted of an initial denaturation step at 94 °C for 3 min followed by 40 cycles of 94 °C for 45 s, 58 °C for 90 s, and 72 °C for 1 min, followed by a final extension step at 72 °C for 7 min. An approx. ~818 bp fragment of the actin gene was amplified as previously described (Ng et al., 2006), with the following modifications; denaturation time was increased from 30 s to 45 s, annealing time from 20 s to 30 s and extension time from 40 s to 1 min. PCR amplification of an approximately 325 bp fragment of the *hsp70* gene was performed using a nested PCR as previously described (Hong et al., 2014).

No-template and extraction reagent blank controls were included in every PCR run. Positive control DNA (*C. macropodum*) was also added to every run to validate the PCRs. PCR setup and DNA handling procedure were performed in separate physically contained PCR-hoods, and post-PCR procedures were performed in a separate laboratory.

2.5. Sequence and phylogenetic analysis

Nested PCR products were electrophoresed through 1% agarose gels, and DNA fragments of the expected size (bp) for the 18S, actin and *hsp70* assays were excised from the gels and purified for Sanger sequencing using an in-house filter tip method (Yang et al., 2013). Purified PCR products from all three assays, were sequenced independently in both directions using an ABI Prism™ Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, California, USA) according to the manufacturer's instructions at 58 °C, 58 °C and 56 °C annealing temperature for the 18S rRNA, actin and *hsp70* loci, respectively.

Appendix 9

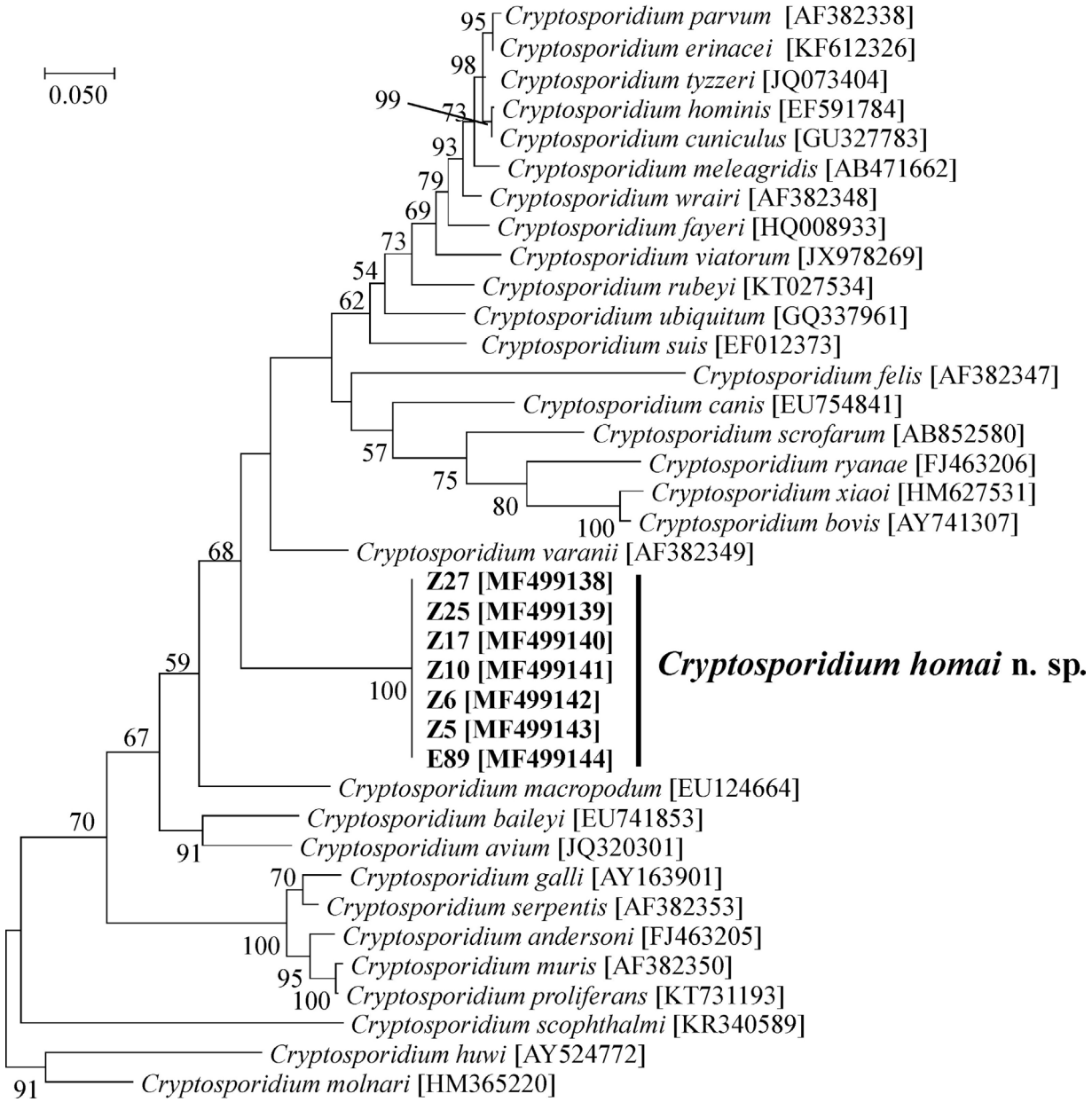

Fig. 3. Phylogenetic relationships between *C. homai* n. sp. and other *Cryptosporidium* species inferred by ML analysis of actin gene. Percentage support (> 50%) from 1000 pseudoreplicates from ML analyses is indicated at the left of the supported node. Scale bars indicate the number of substitutions per nucleotide position.

Sanger sequencing chromatogram files were imported into Geneious Pro 8.1.6 (Kearse et al., 2012), and the nucleotide sequences of each gene was curated, analysed and aligned with reference sequences from GenBank using Clustal W (http://www.clustalw.genome.jp). The most suitable nucleotide substitution model was assessed in MEGA7 (Kumar et al., 2015). Distance, Parsimony and Maximum Likelihood (ML) trees were constructed using MEGA version 7 (Kumar et al., 2015). Bootstrap support for branching was based on 1000 replications. Sequences have been deposited in GenBank under the accession numbers MF499131–MF499151.

3. Results

3.1. Prevalence and histological analysis

In the present study, *C. homai* n. sp. was detected in 24.1% (7/29–95% CI: 10.3%–43.5%) of faecal samples collected from guinea pig enclosures by PCR and sequencing at 3 loci. Histological analysis of the small intestine of one guinea pig (isolate E89), indicated moderate to heavy epicellular infection of the intestinal epithelium by *Cryptosporidium,* associated with a minimal host inflammatory response. Trophozoites and meronts generally measured < 5 µm, as is typical for *Cryptosporidium*. There was a predominance of merogony with both type I and type II meronts present (Fig. 1). The lamina propria was expanded by mild to moderate predominantly lymphocytic-plasmocytic inflammatory infiltrate with the occasional neutrophils, eosinophils and necrotic cells. An average of 1–2 and up to 3 mitotic figures were observed per intestinal crypt in 40–50% of crypts per high power field (40× objective). Mildly tortuous intestinal glands or crypts and the prominence of mitotic figures are suggestive of intestinal epithelial hyperplasia.

3.2. Sequence and phylogenetic analysis C. homai n. sp. at the 18S, actin and hsp70 loci

Phylogenetic relationships were inferred by Distance, Parsimony

Appendix 9

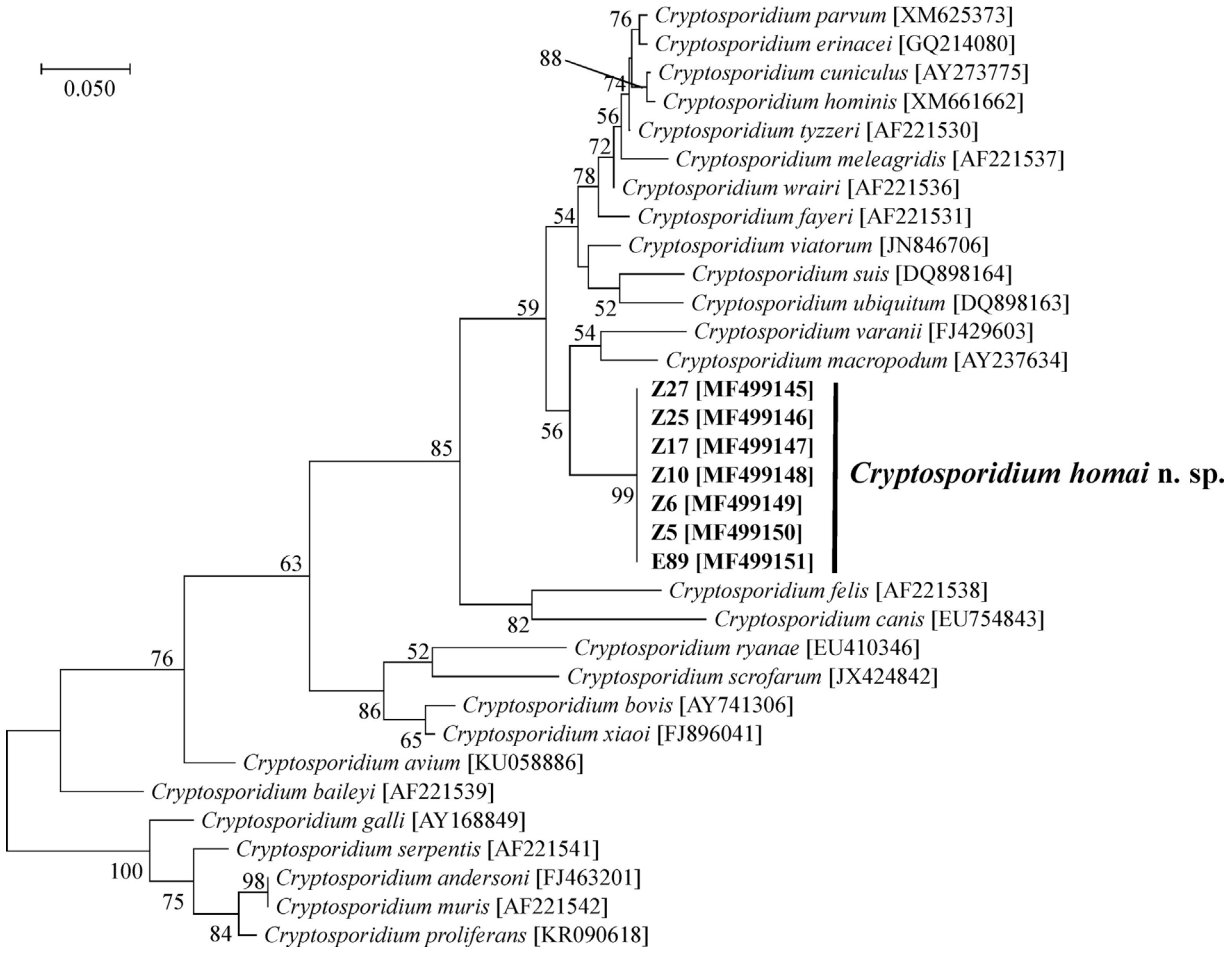

Fig. 4. Phylogenetic relationships between *C. homai* n. sp. and other *Cryptosporidium* species inferred by ML analysis of partial *hsp70* gene sequences. Percentage support (> 50%) from 1000 pseudoreplicates from ML analyses is indicated at the left of the supported node. Scale bars indicate the number of substitutions per nucleotide position.

and Maximum Likelihood (ML) analyses at 18S, actin and *hsp70* loci, based on 825, 818 and 325 bp of nucleotide sequences, respectively, and produced trees with mostly similar topologies with some exceptions (Figs. 2–4). An ML tree was also inferred from concatenated 18S, actin and *hsp70* sequences (Fig. 5).

At the 18S locus, all *C. homai* n. sp. (n = 7, which included 6 faecal samples and the intestinal sample, E89) were identical and grouped in a separate clade, sharing 99.1% identity with a novel genotype of *Cryptosporidium* spp. reported in guinea pigs (*C. procellus*) from Brazil (7 and 6 SNPs difference over 676 bp of submissions DQ885337 and DQ885338, respectively) (Huber et al., 2007). *C. homai* n. sp exhibited 3.1% genetic distance from the closest species, *C. felis*, 3.6% genetic distance from *C. wrairi*, and 3.2% (*C. suis*) to 15.4% (*C. scophthalmi*) genetic distance from all other *Cryptosporidium* spp.

At the actin locus, *C. homai* n. sp. again grouped separately and exhibited 14.4% genetic distance from the closest species, *C. varanii*, 18.4% genetic distance from *C. wrairi* and genetic distances ranging from 15.7% (*C. suis*) to 24.5% (*C. scophthalmi*) from all other *Cryptosporidium* spp.

Phylogenetic analysis of the *hsp70* gene, also confirmed the genetic distinctness of *C. homai* n. sp., where it exhibited 6.6% genetic distance from the closest species, *C. suis*, 7% genetic distance from *C. wrairi*, and 7.1% (*C. varanii*) to 20.9% (*C. serpentis*) genetic distance from all other *Cryptosporidium* spp.

An ML tree inferred from concatenated 18S, actin and *hsp70* sequences grouped *C. homai* n. sp. with *C. varanii* and *C. macropodum,* with 9.1% and 10% genetic distance respectively. Based on phylogenetic analysis using concatenated sequences, *C. homai* n. sp. exhibited 10.8% genetic distance from *C. wrairi*, the only valid *Cryptosporidium* sp. described in guinea pigs, and exhibited between 17.3% (*C. proliferans*) and 9.7% (*C. suis*) genetic distance from all other *Cryptosporidium* spp.

3.3. Taxonomic summary and species description

Order: Cryptogregarida (Cavalier-Smith, 2014).
Family: Cryptosporidiidae
Species name: *C. homai* n. sp.
Type host: Guinea pigs (*Cavia porcellus*)
Other natural hosts: Unknown
Type locality: Perth, Western Australia
Site of infection: Intestine
Prepatent period: Unknown
Patent period: Unknown
Material deposited: partial sequences of 18S, actin and *hsp70* genes were submitted to GenBank under accession numbers MF499131–MF499151.
Etymology: This species is named *C. homai* n. sp. in honor of my late aunt, Ms. Homa Hoorfar.

4. Discussion

In the present study, post-mortem analysis of a guinea pig euthanised due to respiratory distress, identified an intestinal infection with a *Cryptosporidium* species, which on the basis of molecular analysis is a new species, named *C. homai* n. sp. The new species was detected in 24.1% of faecal samples from guinea pigs held at an experimental

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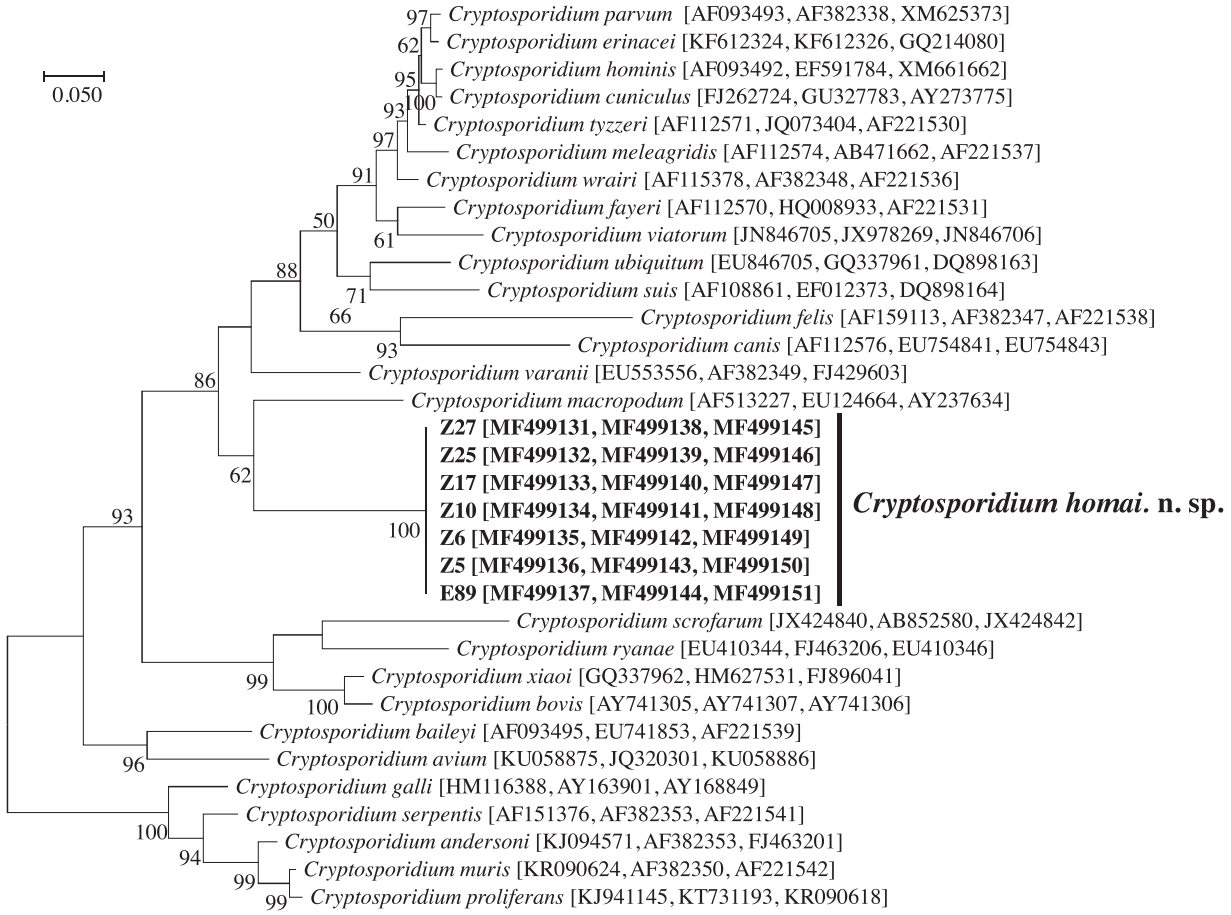

Fig. 5. Phylogenetic relationships between *C. homai* n. sp. and other *Cryptosporidium* species inferred ML analysis of concatenated sequences constructed from partial DNA sequences of 18S, actin and *hsp70* loci. Percentage support (> 50%) from 1000 pseudoreplicates from ML analyses is indicated at the left of the supported node. Scale bars indicate the number of substitutions per nucleotide position.

animal facility. The exact prevalence is difficult to determine as pooled faecal samples were obtained from enclosures, however the high prevalence is likely due to the close proximity of animals to each other, which would facilitate transmission. Very little is known about *Cryptosporidum* in guinea pigs. Surveys of pet guinea pigs in Italy (n = 80) and Ecuador (n = 40) failed to detect *Cryptosporidium* (d'Ovidio et al., 2015; Vasco et al., 2016). Another study in Brazillian guinea pigs (*Cavia aperea aperea*), detected *Cryptosporidium* in 3 of 5 faecal samples by microscopy, and *Cryptosporidium* was also detected in one guinea pig on a farm in the UK, but no genotyping was conducted in either study (Gressler et al., 2010; Smith et al., 2010). Other studies have identified *C. wrairi* in guinea pigs (Lv et al., 2009; Feng et al., 2011), and until recently this was the only *Cryptosporidium* spp. identified in guinea pigs.

A previous study identified a novel *Cryptosporidium* genotype in guinea pigs (*C. procellus*) obtained from an indoor public market, in Rio de Janeiro, Brazil (Huber et al., 2007), which shared 99.1% similarity with *C. homai* n. sp. at the 18S locus (7 and 6 SNPs difference over 676 bp of submissions DQ885337 and DQ885338, respectively). Phylogenetic analysis grouped them in a clade together with high bootstrap support, suggesting that they are likely the same species. Unfortunately, sequences at the actin and *hsp70* loci were unavailable for this genotype to confirm this.

Trophozoites and meronts of *C. homai* n. sp. measured < 5 µm, but oocysts were not observed. However, it is widely accepted that morphology is not a useful criterion for delimiting *Cryptosporidium* spp. (Fall et al., 2003). Phylogenetic analysis at the 18S, actin and *hsp70* loci confirmed the genetic distinctness of *C. homai* n. sp. which exhibited genetic distances ranging from 3.1% to 15.4%, 14.4% to 24.5%, and 6.6% to 20.9% from all other *Cryptosporidium* spp., respectively. Phylogenetic analysis of concatenated 18S, actin and *hsp70* sequences also exhibited 9.1–17.3% genetic distances between *C. homai* n. sp. and other *Cryptosporidium* spp. This clearly supports the species status of *C. homai* n. sp., as these differences are greater than between many currently accepted species. For example, the genetic distance at the 18S and actin loci between *C. hominis* and *C. cuniculus* is 0.4% and 1.6%, respectively (Kvác et al., 2014), and the genetic distance between *C. muris* and *C. andersoni* at the 18S, actin, *hsp70* loci is 0.7%, 3.5% and 2.2%, respectively (Holubová et al., 2016).

In the present study, *C. homai* n. sp. did not group with *C. wrairi* (the only currently valid species in guinea pigs) and exhibited 3.6%, 18.4% and 7.0% genetic distance from this species at 18S, actin and *hsp70* loci, respectively and is clearly a separate species from *C. wrairi*. The phylogenetic relationship of *C. homai* n. sp. to other *Cryptosporidium* spp. is however still ambiguous; at the 18S locus, it was most closely related to *C. felis*, while at the actin locus, it was closest to *C. varanii* (14.4% genetic distance), at the *hsp70* locus, it grouped most closely with *C. suis* (6.6%), and a concatenated analysis of all 3 loci, grouped it most closely with *C. varanii* (9.1%). Analysis at additional loci or whole genome analysis will shed more light on the evolutionary relationships between *C. homai* n. sp. and other *Cryptosporidium* spp.

The pathogenic potential of *C. homai* n. sp. is unknown. Histopathological analysis indicated minimal host inflammatory responses, with the lamina propria expanded by mild predominantly lymphocytic-plasmocytic inflammatory infiltrate with the occasional neutrophils, eosinophils and necrotic cells. Intestinal mucosal cells are usually replaced from germinal cells in the crypts, as the older epithelial cells are sloughed at the tips of villi. The occasional mitotic figure is

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expected in the normal healthy animal as renewal of cells. But large numbers indicate a response to the infection. Histopathological analysis of the infected guinea pig in the present study revealed large numbers of mitotic figures which suggest increased replacement of intestinal cells from hyperplasia. More structured studies are required to clearly define the clinical signs (if any) caused by *C. homai* n. sp.

The host range of *C. homai* n. sp. and its zoonotic potential are also currently unknown, but it has not been previously reported in any other host, suggesting that it may be host specific, however, further analysis is required to determine this.

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SHORT COMMUNICATION

First report of *Cryptosporidium* species in farmed and wild buffalo from the Northern Territory, Australia

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Abstract A molecular epidemiological survey of *Cryptosporidium* from water buffalo (*Bubalus bubalis*) in the Northern Territory in Australia was conducted. Fecal samples were collected from adult farmed ($n=50$) and wild buffalo ($n=50$) and screened using an 18S quantitative PCR (qPCR). Positives were typed by sequence analysis of 18S nested PCR products. The qPCR prevalence of *Cryptosporidium* species in farmed and wild buffalo was 30 and 12 %, respectively. Sequence analysis identified two species: *C. parvum* and *C. bovis,* with *C. parvum* accounting for ~80 % of positives typed from the farmed buffalo fecal samples compared to 50 % for wild buffalo. Subtyping at the 60 kDa glycoprotein (gp60) locus identified *C. parvum* subtypes IIdA19G1 ($n=4$) and IIdA15G1 ($n=1$) in the farmed buffalo and IIaA18G3R1 ($n=2$) in the wild buffalo. The presence of *C. parvum*, which commonly infects humans, suggests that water buffaloes may contribute to contamination of rivers and waterways with human infectious *Cryptosporidium* oocysts, and further research on the epidemiology of *Cryptosporidium* in buffalo populations in Australia is required.

Keywords *Cryptosporidium* · Buffalo · 18S · *C. parvum* · *C. bovis* · gp60

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Introduction

Cryptosporidium is an important protozoan parasite that infects a wide range of animals including humans (Xiao 2010). The parasite is fecal orally transmitted via water, food, or direct contact (Burnet et al. 2014). Clinical symptoms in immunocompetent individuals can include self-limiting watery diarrhea, abdominal pain, nausea, and vomiting but in immunocompromised individuals, infections can result in chronic or protracted diarrhea (Chalmers and Davies 2010). The environmental stage of the parasite (oocyst) is resistant to inactivation by commonly used drinking water disinfectants (Baldursson and Karanis 2011; Burnet et al. 2014). As a result of this, contamination of water supplies is a major mode of transmission, and *Cryptosporidium* was the etiological agent in 60.3 % (120) of the waterborne protozoan parasitic outbreaks reported worldwide between 2004 and 2010 (Baldursson and Karanis 2011). Of the twenty-nine recognized *Cryptosporidium* species (Ryan and Hijjawi 2015; Zahedi et al. 2015), *C. parvum* and *C. hominis* have been responsible for the majority of infections in humans (Xiao 2010).

Livestock animals have been implicated as a source of human cryptosporidiosis based on molecular epidemiological studies conducted in various countries (Xiao 2010; Santín 2013; Abeywardena et al. 2014; Abeywardena et al. 2015). However, relatively little is known about the range of species and genotypes of *Cryptosporidium* in other members of the family Bovidae, including water buffalo (*Bubalus bubalis*), but to date, *C. parvum*, *C. ryanae*, *C. bovis*, *C. ubiquitum*, a "*C. suis*-like" genotype, and *C. ryanae* variants have been reported (Gómez-Couso et al. 2005; Cacciò et al. 2007; Feng et al. 2012; Venu et al. 2012; Abeywardena et al. 2013a; Abu Samra et al. 2013; Amer et al. 2013; Helmy et al. 2013; Inpankaew et al. 2014; Mahfouz et al. 2014; Ma et al. 2015; Abeywardena et al. 2015; Aquino et al. 2015; Helmy et al. 2015).

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Water buffalo are one of the important livestock animals in many regions of the developing world. The world's water buffalo population was estimated as 195 million in 2011, being mostly distributed in Asia, some Mediterranean regions and Latin American countries (Robertson et al. 2014). Water buffalo were brought to Australia between 1824 and 1886 from South-East Asia to provide working animals and meat for the remote northern settlements (Letts 1964). When the early settlements were abandoned, the buffaloes were released, where they became feral populations. Currently, both farmed and feral buffalo herds exist in Australia, with the majority of feral buffaloes in the Northern Territory (in Arnhem Land) with a minimum estimated population of wild buffalo of approximately 98,000 buffalo across 92,000 km^2 of country (Saalfeld 2014).

To date, only one study of *Cryptosporidium* in farmed water buffalo in Victoria, Australia, has been conducted (Abeywardena et al. 2013a), and nothing is known about the prevalence and species of *Cryptosporidium* infecting water buffalo in Northern Australia. Therefore, the aim of the present study was to use molecular tools to better understand the public health risks of *Cryptosporidium* sp. in both wild and farmed buffalo herds to drinking water supplies in the Northern Territory (NT) of Australia.

Materials and methods

Sample collection and processing

Buffalo fecal samples were collected in August 2015 from (1) wild buffalo ($n = 50$) located near the Maude Creek cattle station, 30-km south of Katherine, NT (the herd was originally sourced from the Phelp River, South-Eastern Arnhem Land) and (2) farmed buffalo ($n = 50$) from the Northern Territory Government, Department of Primary Industry and Fisheries (DPIF) buffalo farm (Beatrice Hill Farm), near the Adelaide River, NT by Tropical Water Solution staff. Samples were collected from freshly deposited fecal samples into individual 75-ml fecal collection pots using a scrapper to expose and scoop from the center of the scat pile. All samples were stored at 4 °C, shipped to Murdoch University, and stored at 4 °C until analyzed. All fecal samples were collected from adult animals; the wild buffalo were between 2–5 years of age, and the farmed buffalo were between 3 and 4 years of age. Approval for faecal collection was obtained from the Northern Territory Buffalo Industry Council and individual property owners directly by phone. As the samples were collected directly from the ground and not per rectum, specific animal ethics approval was not required.

DNA isolation

Genomic DNA was extracted from 250 mg of each fecal sample using a Power Soil DNA Kit (MolBio, Carlsbad, California). A negative control (no fecal sample) was used in each extraction group.

PCR amplification of the 18S rRNA gene

All samples were screened for the presence of *Cryptosporidium* at the 18S rRNA locus using a quantitative PCR (qPCR) previously described (King et al. 2005; Yang et al. 2014). Each 10-μl PCR mixture contained 1× Go Taq PCR buffer (KAPA Biosystems), 3.75 mM $MgCl_2$, 400 μM of each dNTPs, 0.5-μM 18SiF primer, 0.5-μM 18SiR primer, 0.2-μM probe, and 1U/reaction Kapa DNA polymerase (MolBio, Carlsbad, California). The PCR cycling conditions consisted of one pre-melt cycle at 95 °C for 6 min and then 50 cycles of 94 °C for 20 s and 60 °C for 90 s.

Samples that were positive by qPCR were amplified at the 18S locus using primers which produced a 611-bp product as previously described (Silva et al. 2013) with minor modifications; the annealing temperature used in the present study was 57 °C for 30 s, and the number of cycles was increased from 39 to 47 cycles for both primary and secondary reactions. PCR contamination controls were used including negative controls and separation of preparation and amplification areas.

PCR amplification of the gp60 gene

Samples that were typed as *C. parvum* at the 18S locus were subtyped at the 60 kDa glycoprotein (gp60) locus using a nested PCR as previously described (Zhou et al. 2003).

Sequence analysis

The amplified DNA from secondary PCR products was separated by gel electrophoresis and purified for sequencing using an in-house filter tip method (Yang et al. 2013). Purified PCR products from both loci were sequenced independently using an ABI Prism™ Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, California) according to the manufacturer's instructions at 57 and 54 °C annealing temperature for the 18S rRNA and gp60 loci, respectively. Sanger sequencing chromatogram files were imported in Geneious Pro 8.1.6 (Kearse et al. 2012), edited, analyzed, and aligned with reference sequences from GenBank using Clustal W (http://www.clustalw.genome.jp).

Statistical analysis

Statistical analyses (Fisher's exact test) were performed using SPSS 22 for Windows (SPSS Inc. Chicago, USA), to

determine if there was a statistical difference in the prevalence of *Cryptosporidium* in farmed versus wild buffalo.

Results

Prevalence of *Cryptosporidium* in farmed and wild buffalo

The qPCR prevalence of *Cryptosporidium* species in the farmed buffalo was 30 % (15/50) (95 % CI, 17.9–44.6) and was 12 % (6/50) (95 % CI, 4.5–24.3) in the wild buffalo. This difference was significant ($P = 0.048$, $P < 0.05$).

Cryptosporidium species detected in farmed and wild buffalo

Sequence analysis at the 18S locus was only successful for six of the fifteen farmed buffalo positives and four of the six wild buffalo samples. Two *Cryptosporidium* species, *C. parvum* and *C. bovis*, were identified in both types of the samples. In the farmed buffalo samples, five were *C. parvum* and one was *C. bovis*, whereas for the wild buffalo, two were identified as *C. parvum* and two were *C. bovis.*

Sequences at the gp60 locus were obtained for five of the farmed buffalo *C. parvum* positives, and *C. parvum* subtypes IIdA19G1 ($n = 4$) and IIdA15G1 ($n = 1$) were identified. In the wild buffalo, gp60 sequences were obtained for both *C. parvum* positives, and both were identified as IIaA18G3R1.

Discussion

The present study described the prevalence and molecular characterization of *Cryptosporidium* species in the farmed and wild buffalo from the Northern Territory in Australia. The prevalence of *Cryptosporidium* species in the farmed and wild buffalo fecal samples was 30.0 and 12.0 % respectively. A previous study of *Cryptosporidium* in farmed water buffalo in Victoria reported a prevalence of 13.0 % (62/476) (Abeywardena et al. 2013a). Other studies have reported prevalences in buffalo ranging from 5.7 to 62.1 % (Amer et al. 2013; Inpankaew et al. 2014; Abeywardena et al. 2014; Aquino et al. 2015; Ma et al. 2015).

Cryptosporidium parvum is the most commonly reported zoonotic species of *Cryptosporidium* infecting humans and was the most common species detected in farmed buffalo (~80 % of positives typed) and in wild buffalo accounted for 50 % of the positives typed. This species has been detected previously in buffaloes in many countries including Spain, Italy, Australia, Egypt, India, and Thailand (Amer et al. 2013; Cacciò et al. 2007; Gómez-Couso et al. 2005; Maurya et al. 2013; Abeywardena et al. 2013a; Inpankaew et al. 2014; Mahfouz et al. 2014; Aquino et al. 2015). Previous studies have reported that *C. parvum* was the predominant species in young buffaloes (Cacciò et al. 2007; Maurya et al. 2013; Inpankaew et al. 2014), and in the previous study in Australia, *C. parvum* was only detected in buffalo <6 months of age (Abeywardena et al. 2013a). In the present study, however, all buffalo samples were adults (2–5 years), indicating that this species can also commonly infect adult buffaloes.

Cryptosporidium bovis was detected in ~20 and 50 % of the farmed and wild buffalo isolates-typed, respectively. This species has previously been reported in buffalo in Egypt (Helmy et al. 2013), South Africa (Abu Samra et al. 2013), Australia (Abeywardena et al. 2013a), and China (Ma et al. 2015). In the previous study in Australia, *C. ryanae* variants (reported as genotypes 1 and 2) and a *C. suis*-like genotype (reported as genotype 3) were also reported in buffaloes (Abeywardena et al. 2013a). *Cryptosporidium bovis* is predominantly a parasite of livestock and has only been reported in humans on a few occasions (Khan et al. 2010; Ng et al. 2012).

The *C. parvum* subtype IIaA18G3R1 was identified in the two wild buffalo samples. IIaA18G3R1 is a common subtype and has been reported widely in both cattle and humans worldwide including Australia (Plutzer and Karanis 2009; Ryan and Power 2012). The *C. parvum* subtypes IIdA19G1 and IIdA15G1 were identified in the farmed buffalo in the present study. Both are considered zonootic subtypes (Wang et al. 2014). The *C. parvum* IId subtype family has been reported mainly from sheep and goats but has also been reported in humans and cattle (Plutzer and Karanis 2009; Xiao 2010; Wang et al. 2014). The *C. parvum* IIdA15G1 subtype has been detected in livestock and humans (Plutzer and Karanis 2009; Xiao 2010; Wang et al. 2014), including a human in Australia (Ng et al. 2010). The IIdA19G1 subtype is less common but has been reported in humans and animals (Xiao 2010; Wang et al. 2014). In China, IId is the predominant *C. parvum* subtype (Wang et al. 2014), and both of these IId subtypes are commonly found in bovine animals in China including yaks (Wang et al. 2014; Qi et al. 2015). However, this subtype is not common in dairy cattle in Australia (Ng et al. 2012; Abeywardena et al. 2013b). Previous analysis has indicated that *C. parvum* IId subtypes were probably dispersed from Western Asia to other geographical regions (Wang et al. 2014). As water buffalo in Australia came from Asia (Letts 1964), it is likely that the IId subtype family was introduced into Australia with the introduction of buffalo. In the previous study in Australia (Abeywardena et al. 2013a), gp60 subtyping was not conducted.

Conclusion

The preliminary data from the present study indicates that water buffaloes have the potential to contribute to the zoonotic

transmission of *C. parvum* via contamination of water, as buffaloes usually wallow in rivers, streams, and other water sources (Abeywardena et al. 2014). These findings indicate the need (1) for further characterization of the prevalence, intensity of infection, and species of *Cryptosporidium* in buffalo populations across Australia and (2) to determine the levels of oocysts in rivers and waterways flowing from buffalo farms and wild buffalo locations, particularly those that flow into water reservoirs used for drinking water.

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Digital Appendices

Digital appendix 1 - Supplementary Table 3-S1

Supplementary data associated with this table can be found at:

https://figshare.com/s/07288fd4814287029139.

http://dx.doi.org/10.1016/j.ijpara.2017.03.003.

Digital appendix 2 - Supplementary Table 4-S1

Supplementary data associated with this table can be found at:

https://figshare.com/s/bbbde6d24d6a8c13371e.

https://doi.org/10.1016/j.watres.2018.02.005.

Digital appendix 3 - Supplementary Table 5-S1

Supplementary data associated with this table can be found at:

https://figshare.com/s/c006e84b8babf4178e23.

https://doi.org/10.1016/j.scitotenv.2018.07.024.