CRYPTOSPORIDIUM IN FISH: MORPHOLOGICAL AND MOLECULAR CHARACTERISATION

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Declaration

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

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ABSTRACT

Cryptosporidium is an Apicomplexa protozoan parasite that causes gastrointestinal illness in a wide range of vertebrate hosts, including humans. Little is known of the epidemiology of *Cryptosporidium* in fish. This study investigated the prevalence of *Cryptosporidium* in goldfish (*Carassius auratus)* (n=216) and mullet (*Mugil cephalus*) (n=13). Goldfish can be host to a range of *Cryptosporidium* sp. and research has shown that mullet has been host to genotype 3, therefore sampling from these breeds of fish could provide further characterisation. The fish were acquired from three sources in Metropolitan Perth, Western Australia; Vebas Aquarium (n=16), Water Garden Life fish farm (n=200) and a local bait shop in Fremantle (n=13). Intestines and stomachs were dissected and half kept for histology and the remaining half used for DNA extraction. All samples were initially screened at the 18S locus by quantitative PCR (qPCR) and positives further analysed by nested PCR and sequencing at the 18S and actin loci. Further subtyping was conducted on human-infectious species at the glycoprotein 60 (*gp60*) locus. The overall prevalence by qPCR was 30.1% (69/229) (CI 24.2-36.1). Of these only 34 samples amplified at the 18S locus and 23 clean sequences were obtained, with the remaining 11 sequences exhibiting mixed chromatograms. At the actin locus, 6 samples were successfully amplified and 3 clean chromatograms were obtained. Sequencing and phylogenetic analysis at the 18S locus identified *C. parvum* (n=2), *C. hominis* (n=10) and a novel species (n=11), which was identical to a novel genotype identified in a single isolate from a goldfish from a previous Honours project. Phylogenetic analysis confirmed that this novel genotype was genetically distinct and most closely related to *C. scopthalmi* (10.4% genetic distance). At the actin locus, all three isolates sequenced belonged to the novel genotype, which again grouped with *C. scopthalmi* and exhibited 14.1% genetic distance at this locus. Subtyping of *C. hominis* and *C. parvum* isolates at the *gp*60 locus was successful for 3 *C. hominis* isolates and all were typed as 1bA10G2, which is the main subtype involved in human

outbreaks of cryptosporidiosis. Unfortunately, the parasite could not be identified in histological sections, which may be due to the patchy distribution of *Cryptosporidium* infections and rapid tissue autolysis. This is the first characterisation of the novel genotype at the actin locus and provides further support for its species status. The identification of human-infectious species in these fish is of public health concern as it may enable control of cryptosporidiosis outbreaks. Future research should focus on analysis of a wider range of fish species as well as clinical signs and histology to better understand the host range and pathogenicity of the novel genotype and the prevalence of human-infectious species in fish.

ACKNOWLEDGEMENTS

I would like to sincerely thank my supervisor Professor Una Ryan, and co-supervisors Doctor Andrea Paparini, Doctor Susan Kueh and Associate Professor Alan Lymbery for their guidance, assistance and patience throughout the year. I would also like to thank all the members of the Vector and Waterborne Pathogens Research Group for their help and support throughout the project. Alphabetically ordered they are Alireza Abdi, Doctor Jill Austen, Amanda Barbosa, Annachiara Codello, Alex Gofton, Telleasha Greay, Khalid Al Habsi, Professor Peter Irwin, Doctor Elvina Lee, Doctor Charlotte Oskam, Kimberly Loh, Sylvia Squire and Doctor Rongchang Yang. I would like to also thank the researchers in the SABC who guided me through some of the laboratory work. Finally, I would like to thank my family, friends and colleagues who encouraged me to pursue the research and supported me through the process.

GLOSSARY OF TERMS

Symbol/Abbreviation	Meaning
bp	base pair
°C	Celsius
CD4+	white blood cells
CI	confidence interval
cm	centimetres
DAPI	4', 6-diamidino-2-phenylindole
DNA	deoxyribonucleic acid
et al	and others
g	grams
gp	glycoprotein
H and E	Haematoxylin and Eosin
H_20	Water
ID_{50}	Infective dose at 50%
KDa	Kilodalton
M	molar
mm	millimetres
mM	millimoles
mg	milligrams
$MgCl_2$	magnesium chloride
min	minutes
mL	millilitres
n	total number
PCR	Polymerase Chain Reaction
pH	potential of Hydrogen used to specify the acidity or alkalinity of an aqueous solution
rRNA	ribosomal Ribonucleic Acid
sny	Snyder
sp./spp.	species/species'
TAE	Tri Acetate
WHO	World Health Organisation
w/v	weight of solute per volume of solvent
V	volts
yrs	years
µL	microliters
µm	micrometres
µM	micromoles
-	negative
+	positive
~	approximately

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CHAPTER 1: INTRODUCTION

1.1 INTRODUCTION

Cryptosporidium is a protozoan parasite belonging to the phylum Apicomplexa that infects a wide range of vertebrate hosts including humans, birds, reptiles and fish (Silva *et al*, 2013; Ryan *et al*, 2014). Of particular concern is the zoonotic nature of some *Cryptosporidium* species and its pathogenicity and epidemiology (Koinari *et al*, 2013). In immunocompetent humans the infection is usually self-limiting, however, in compromised immune systems, it can be the cause of chronic diarrhoea, cachexia, lack of appetite and malnutrition (Xiao, 2010). In extreme cases cryptosporidiosis, the associated disease, can be fatal if left untreated (Armadi *et al*, 2002; Valenzuela *et al*, 2014; Iqbal *et al*, 2015). In Human Immunodeficiency Virus (HIV) patients, some studies have found that *Cryptosporidium* can actually accelerate the disease to full blown Acquired Immune Deficiency Syndrome (AIDS) (Karanja *et al*, 1997).

Cryptosporidiosis is one of the leading causes of diarrhoea and vomiting worldwide and is generally more prevalent in countries with little access to clean fresh water (Tian *et al*, 2012; Maity *et al*, 2015; Obateru *et al*, 2016). *Cryptosporidium* is, therefore, highly prevalent in developing countries and more common in paediatric populations (Sarkar *et al*, 2014). The prevalence of *Cryptosporidium*, in humans, has been reported globally to be 6.2% in developing countries but only 2.1% in developed countries (Aggarwal *et al*, 2005). A recent study identified *Cryptosporidium* as the second most common cause of diarrhoea worldwide and death in children, after rotavirus (Kotloff *et al*, 2013; Striepen, 2013). It is also associated with retarded cognitive and functional development in children for up to 7 years' post-infection, during which time they have no immunity against additional *Cryptosporidium* parasitisation (Kirkpatrick *et al*, 2002; Kotloff *et al*, 2013; Valenzuela *et al*, 2014).

Although human cryptosporidiosis is more prevalent in developing countries, sporadic episodes do occur in Westernised societies, costing millions in public health burden. The ongoing cost of waterborne disease is estimated at $21.9 billion (annually), and *Cryptosporidium* forms a substantial percentage of this (Corso *et al*, 2003). The largest outbreak, which occurred in Milwaukee, Wisconsin, was estimated to cost $96.2 million, and HIV sufferers accounted for 74% of infections and there were 69 deaths in the HIV population that related directly to the outbreak. Once an outbreak occurs in the water supplies, it can be extremely difficult to eradicate due to the parasite's resistance to disinfection procedures (Ociai *et al*, 2005).

Cryptosporidium can also result in major economic losses in livestock, particularly newborn calves, causing retardation of growth and death. It is mostly a problem in ruminants and the poultry industry, affecting turkeys and chickens (De Graaf *et al*, 1999, Meireles *et al*, 2006; Ng *et al*, 2006; Zhou *et al*, 2004).

1.2 TAXONOMY

The parasite was first described in the gastric glands of mice by Ernest Edward Tyzzer (Tyzzer 1907), who proposed the name *Cryptosporidium muris* (Tyzzer, 1910). In 1912, Tyzzer described an additional species, *Cryptosporidium parvum*, which differed in its oocyst size and location of infection site (Tyzzer, 1912). However, it was not considered of scientific importance, at the time, and was not followed up by subsequent studies for 48 years (Cited in Fayer and Xiao, 2008). The next significant published report was in 1955, when *Cryptosporidium* was discovered in the gastrointestinal tract of turkeys and associated with morbidity in its host (Slavin, 1955). Today there are at least 31 valid

species (Ryan and Xiao, 2014, Ryan *et al*, 2014, Fayer and Xiao, 2008; Holubová *et al*, 2016; Kváč *et al*, 2016; Zahedi *et al*, 2016) (Table 1). More than 17 species have been identified in humans, but the most common are *C. parvum* and *Cryptosporidium hominis* (Xiao, 2010; Ryan and Xiao, 2014), with *C. hominis* the main cause of human cryptosporidiosis in most countries (Xiao, 2010; Ryan and Power, 2012).

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

Species name	**Author(s)**	**Type host(s)**	**Major host(s)**	**Reports in humans**
C. avium	Holubová *et al*, 2016	Red-crowned parakeets (*Cyanoramphus novaezealandiae*)	Birds	None reported
C. proliferans	Kváč *et al*, 2016	East African mole rat (*Tachyoryctes splendens*)	Rodents	None reported
C. rubeyi	Li *et al*, 2015	California ground squirrel *(Spermophilus beecheyi)*	Squirrels	None reported
C. scophthalmi	Alvarez-Pellitero *et al*, 2004; *Costa et al*, 2016	Turbot *(Scophthalmus maximus)*	Turbot	None reported
C. huwi	Ryan *et al*, 2015	Guppy *(Poecilia reticulata)*, Neon tetra (*Paracheirodon innesi)* and Tiger barb (*Puntius tetrazona)*	Fish	None reported
C. erinacei	Kváč *et al*, 2014b	European hedgehog *(Erinaceus europaeus)*	Hedgehogs, horses	Kváč *et al*, 2014a
C. scrofarum	Kváč *et al*, 2013	Pig *(Sus scrofa)*	Pigs	Kváč *et al*, 2009a; Kváč *et al*, 2009b
C. viatorum	Elwin *et al*, 2012	Human *(Homo sapiens)*	Humans	Elwin *et al*, 2012; Insulander *et al*, 2013
C. tyzzeri	Tyzzer, 1912; Ren *et al*, 2012	Mouse *(Mus musculus)*	Rodents	Rasková *et al*, 2013
C. cuniculus	Robinson *et al*, 2010	European rabbit *(Oryctolagus cuniculus)*	Rabbits	Chalmers *et al*, 2009; Anon, 2010; Molloy *et al*, 2010; Chalmers *et al*, 2011; Anson *et al*, 2014; Koehler *et al*, 2014
C. ubiquitum	Fayer *et al*, 2010	Cattle *(Bos Taurus)*	Ruminants,	Commonly

			rodents, primates	reported (cf. Fayer *et al*, 2010; Elwin *et al*, 2012)
C. xiaoi	Fayer *et al*, 2010	Sheep *(Ovis aries)*	Sheep and goats	Adamu *et al*, 2014
C. ryanae	Fayer *et al*, 2008	Cattle *(Bos Taurus)*	Cattle	None reported
C. macropodum	Power and Ryan, 2008	Kangaroo *(Macropus giganteus*)	Marsupials	None reported
C. fragile	Jirků *et al*, 2008	Toad *(Duttaphrynus melanostictus)*	Toads	None reported
C. fayeri	Ryan et al, 2008	Kangaroo *(Macropus rufus)*	Marsupials	Waldron *et al*, 2010
C. bovis	Fayer *et al*, 2005	Cattle *(Bos taurus)*	Cattle	Khan *et al*, 2010; Ng *et al*, 2012; Helmy *et al*, 2013
C. suis	Ryan *et al*, 2004	Pig *(Sus scrofa)*	Pigs	Xiao *et al*, 2002; Leoni *et al*, 2006; Cama *et al*, 2007; Wang *et al*, 2013
C. galli	Pavalasek, 1999; Ryan *et al*, 2003	Birds *(Spermestidae, Frangillidae, Gallus gallus, Tetrao urogallus, Pinicola enucleator)*	Birds	None reported
C. hominis	Morgan-Ryan *et al*, 2002	Human *(Homo sapiens)*	Humans	Most common species in humans
C. molnari	Alvez-Pellitero and Sitja-Bobadilla, 2002	Gilthead sea bream *(Sparus aurata)* and European seabass) *(Dicentrarchus labrax)*	Fish	None reported
C. canis	Fayer *et al*, 2001	Dog *(Canis familiaris)*	Dogs	Many reports (Lucio-Forster *et al*, 2010)
C. andersoni	Lindsay *et al*, 2000	Cattle (*Bos Taurus*)	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	Pavlasek *et al*, 1995	Emerald monitor *(Varanus prasinus)*	Lizards	None reported
C. baileyi	Current *et al*, 1986	Chicken *(Gallus gallus*)	Birds	None reported
C. parvum	Tyzzer, 1912	Cattle *(Bos Taurus)*	Ruminants	Commonly reported in humans
C. meleagridis	Slavin, 1955	Turkey *(Meleagris gallopavo)*	Birds and humans	Commonly reported in humans
C. serpentis	Levine, 1980	Snakes *(Elaphe guttata, E. subocularis, Sanzinia madagascarensus*)	Snakes and lizards	None reported
C. felis	Iseki, 1979	Cat *(Felis catis)*	Cats	Many reports (cf. Lucio-Forster *et al*,

				2010)
C. wrairi	Vetterling *et al*, 1971	Guinea Pig *(Cavia porcellus*)	Guinea pigs	None reported
C. muris	Tyzzer, 1907; and 1910	house mouse *(Mus musculus*)	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

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. The similarities between *Cryptosporidium* and gregarines have been supported by extensive microscopic, molecular, genomic and biochemical data (Bull *et al*, 1998; Carreno *et al*, 1999; Hijjawi *et al*, 2002; Leander *et al*, 2003a; Hijjawi *et al*, 2004; Rosales *et al*, 2005; Barta and Thompson, 2006; Valigurová *et al*, 2007; Boxell *et al*, 2008; Karanis *et al*, 2008; Zhang *et al*, 2009; Borowski *et al*, 2008; 2010; Hijjawi, 2010; Hijjawi *et al*, 2010; Templeton *et al*, 2010; Karanis and Aldeyarbi, 2011; Koh *et al*, 2013, 2014; Huang *et al*, 2014; Aldeyarbi and Karanis, 2015; 2016a; 2016b; Valigurová *et al*, 2015), which have served as the basis for the formal transfer of *Cryptosporidium* from subclass Coccidia, class Coccidiomorphea to a new subclass, Cryptogregaria, within the class Gregarinomorphea (Cavalier-Smith, 2014). *Cryptosporidium* is currently the sole Cryptogregaria and is described as an epicellular parasite of vertebrates possessing a gregarine-like feeder organelle but lacking an apicoplast (Cavalier-Smith, 2014).

1.2.1 Delimiting species in *Cryptosporidium*

Initial research on *Cryptosporidium* delimited species based on the oocyst morphology and host occurrence of the parasite (Ryan and Xiao, 2014). Unfortunately, *Cryptosporidium* oocysts are among the smallest of any apicomplexan stage (4-6µm) and lack distinct morphological features that could differentiate the species and therefore molecular characterisation is essential (Fall *et al*, 2003; Xiao, 2010).

To allow researchers to delimit *Cryptosporidium*, a set of guidelines are now routinely followed: (1) morphometric studies of the oocysts; (2) genetic characterisation at two loci and submission of GenBank accession numbers; (3) some evidence of host range and specificity and (4) compliance with International Code of Zoological Nomenclature (ICZN) (Xiao *et al*, 2004).

1.3 LIFE CYCLE OF *CRYPTOSPORIDIUM*

Cryptosporidium have a complex life cycle which involves both asexual and sexual reproduction. Environmentally-resistant oocysts representing the infective life cycle stage of the parasite are excreted in the faeces (Fayer and Xiao, 2008). These oocysts are extremely resilient, due to their thick trilaminar walls, and can survive many months in a watery environment and are resistant to disinfectants including chlorine in drinking water (Chen *et al*, 2002, 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 and this causes release of four infective sporozoites, through a suture opening, which then attach to the apical membrane of the epithelial host cell and undergo successive rounds of asexual and sexual reproduction (Fayer and Xiao, 2008) (Fig 1). The motile sporozoites approach a potential host cell anterior end first and actively invade the

cell (Fayer *et al*, 1997; Tzipori and Widmer, 2000; Wetzel *et al*, 2005). 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 (Fayer *et al*, 1997; Hijjawi *et al*, 2010). Two types of meronts have been described in the *Cryptosporidium* life cycle; type I and type II meronts (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 (Current, 1990; Current and Garcia, 1991, Tzipori and Widmer, 2000; 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 fertilize the macrogamont forming a zygote (Current, 1990; Current and Garcia, 1991; 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.

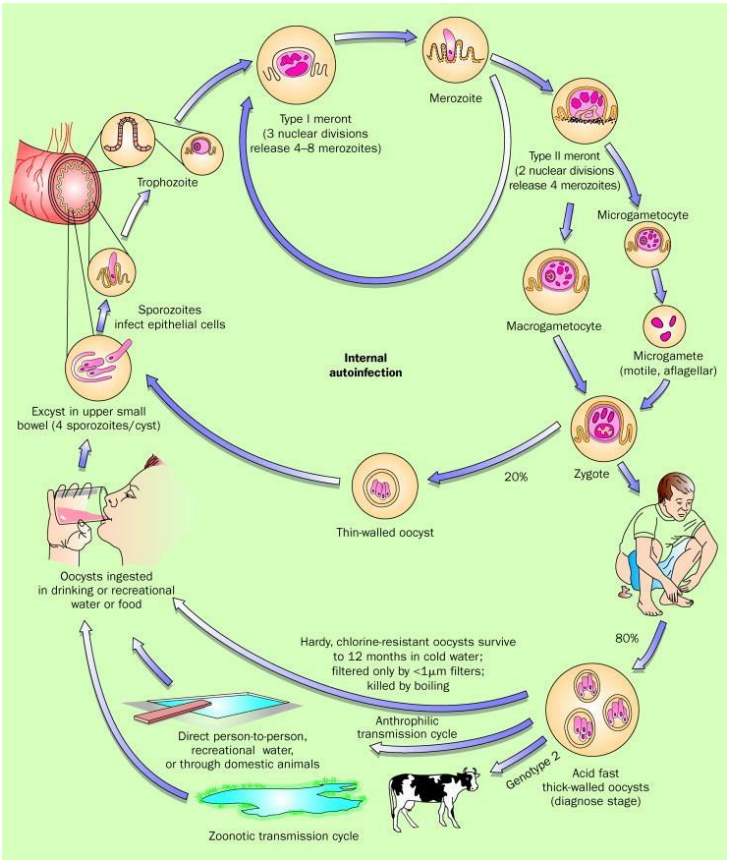

Figure 1: The life cycle of *Cryptosporidium parvum* (Kosek *et al*, 2001).

1.4 EPIDEMIOLOGY AND TRANSMISSION STUDIES

1.4.1 Routes and mechanisms of transmission

Cryptosporidium outbreaks can be generated through contaminated water, person-person, animal-person and contaminated food, via the faecal-oral route (Johnson *et al*, 1995; Rose *et al*, 1997; Fayer, 2004; Koinari *et al*, 2013). Transmission can also occur by inhalation resulting in respiratory cryptosporidiosis (Sponseller *et al*, 2014). Very little is known about the prevalence and genetic diversity of species of *Cryptosporidium* in marine environments and the role that marine animals play in transmission of these parasites to humans. However, both non-zoonotic and zoonotic *Cryptosporidium species* and genotypes have been identified in marine mammals. For example, Roberedo-Fernandez *et al*, (2014) identified the zoonotic *C. parvum* in beached dolphins (*Delphinus delphus*) on the Galician Coast, Northwest Spain, suggesting that marine mammals may become

infected from human activities in the water, and in turn transmit the parasite to other marine animals such as fish, cetaceans or humans (Reboredo-Fernandez *et al,* 2014).

Cryptosporidium is particularly suited to waterborne transmission as the environmental stage, the oocyst, is highly resistant to disinfection (Korich *et al,* 1990; Chauret *et al,* 2001; Painter *et al,* 2015) and is excreted in large quantities (10^8-10^9 oocysts in a single bowel movement) (Cordell, 2001; Yoder and Beach, 2007; Yoder *et al,* 2012) for up to 60 days after cessation of gastrointestinal symptoms (Jokipii and Jokipii, 1986; Stehr-Green *et al,* 1987). In addition, the low infectious dose (10-100 oocysts) (DuPont *et al,* 1995; Chappell *et al,* 2006), means that ingestion of a relatively small amount of contaminated water is sufficient to initiate infection in a susceptible individual. The long incubation period of cryptosporidiosis (averaging 7 days) (Chalmers and Davies, 2010), delays the identification of the source and implementation of interventions to de-contaminate the source thus perpetuating transmission.

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). Given that the 50% infective dose (ID_{50}) for *C. hominis* is less than one tenth that of the *C. parvum* IOWA isolate (DuPont *et al,* 1995), ingestion of only one oocyst of a more infectious isolate may lead to a higher incidence of infection in the general immunocompetent population.

1.5 CLINICAL SIGNS AND TREATMENT

The symptoms of cryptosporidiosis are usually self-limiting in immunocompetent people but can be chronic in compromised immune systems, such as in children under 5 years and HIV and cancer patients (Carr *et al,* 1998). The general symptoms associated with

cryptosporidiosis include diarrhoea, vomiting, nausea, lack of appetite and cramps (Chalmers and Davies, 2010) Immunocompetent patients frequently experience chronic diarrhoea, weight loss and abdominal pain, with a wasting syndrome that can lead to death (Armadi *et al,* 2002). *Cryptosporidium* is often found in other organs in HIV patients such as those in biliary tract (Sponseller *et al,* 2014).

Although hundreds of drugs have been tested for prophylaxis and treatment of cryptosporidiosis in animals and humans, only one, Nitazoxanide (Alinia®), has been approved for use in humans by the 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).

The use of ART (antiretroviral therapy) to maintain a high CD4+ immune cell titre, resulting in a strengthened immune system has helped combat opportunistic *Cryptosporidium* in HIV+ and AIDS patients (Miao *et al,* 2000; Carr *et al,* 1998). In the livestock industry, paramomycin has shown to reduce oocyst shedding and diarrhoea in lambs as well as improve their growth rate (Quilez *et al,* 2000; Johnson *et al,* 2000). Nasir *et al,* (2013) reported that azithromycin was also effective in reducing *C. parvum* oocyst shedding in calves.

There are currently no vaccines available for cryptosporidiosis and due to the infective and widespread nature of *Cryptosporidium,* the most effective control of transmission is appropriate sanitation procedures (Ryan *et al,* 2014) and guidelines are available for control strategies, following the recommendations of the WHO Neglected Disease Initiative, in 2002 (Savioli *et al,* 2006).

1.6 DETECTION AND CHARACTERISTION

1.6.1 Microscopic methods

In earlier research, prior to the advent of molecular techniques, the only way to identify *Cryptosporidium* species was microscopic examination of purified or un-purified oocysts (Fayer *et al,* 2000). This technique however lacks sensitivity, is labour intensive and prone to human error (Fayer *et al,* 2000).

The most common staining techniques include differential staining methods (eg. Methylene Blue and Ziehl-Neelson), flourochrome staining and negative staining techniques (eg. Malachite green and merbromide) (Kawamoto *et al,* 1987; Cambell *et al,* 1992; Chichino *et al,* 1991; Elliot *et al,* 1999).

Due to lack of distinguishing oocyst features however, morphology cannot be used to identify species and therefore molecular characterisation is required to identify to species level (Morgan *et al,* 1998; Fayer *et al,* 2000).

1.6.2 Immunological methods

Immunological based methods include polyclonal fluorescent antibody tests, latex agglutination reactions, immunofluorescence (IF) with monoclonal antibodies (mAbs), enzyme-linked immunosorbent assays (ELISA), reverse passive haemagglutination (RPH), immunoserology using immunofluorescence detection and ELISA, and solid-phase qualitative immunochromatographic assays (Fayer *et al,* 2000). Cross-reactivity with other microorganisms can however occur due to the non-specific nature of antibody-based methods and this can limit their use (Fayer *et al,* 2000).

1.6.3 Molecular detection and characterisation methods

Molecular tools have become the most reliable technique for identifying *Cryptosporidium* species, genotypes and subtypes. This has led to an increased understanding of the epidemiology, taxonomy, and zoonotic potential of *Cryptosporidium* (Xiao, 2010).

1.6.3.1 18S ribosomal RNA (18s rRNA)

The 18S rRNA locus is considered the most reliable locus for detection and identification of all *Cryptosporidium* species and genotypes. It is multicopy and has both hypervariable and semi-conserved regions which facilitate specific and sensitive detection (Xiao, 2010).

1.6.3.2 Actin locus

The actin gene is a widely distributed and highly conserved single copy microfilament protein that is thought to play a role in sporozoites gliding motility and penetration into host cells (Kim *et al*, 1991). Sequencing of this gene is another commonly employed characterisation technique as polymorphisms occur along this whole gene, caused by its ability to evolve rapidly (Xiao *et al*, 2004).

1.6.3.3 Subtyping tools for Cryptosporidium

Subtyping (fingerprinting) tools are often used to better understand the transmission dynamics of *Cryptosporidium* species, particularly *C. hominis* and *C. parvum* in humans, animals and wildlife (Xiao, 2010). The 60kDa glycoprotein (*gp60/40/15*) gene is the most heterogeneous locus in the genome and as this gene encodes a protein that helps in the invasion of gastro-intestinal cells and zoite attachment, it is therefore biologically relevant (Strong *et al*, 2000; Xiao, 2010). Multilocus fragment typing (MLFT) and multilocus sequence typing (MLST) are used to enhance the resolution of the subtyping produced by *gp60* sequence analysis.

1.6.3.4 Next Generation Sequencing

The majority of molecular characterisation techniques, to date, have been based on Sanger Sequencing (chain termination) (Sanger *et al*, 1977). A limitation of this approach is that *Cryptosporidium* species and genotypes of low relative abundance or mixed infections may not be detected. Furthermore, Sanger Sequencing is a labour intensive, costly and time consuming technique. The advent of second generation, high-throughput sequencing (HTS) platforms that have allowed fast and efficient sequencing of genes, genomes and metagenomes (Metzker *et al*, 2010). By applying this technique to the host samples of mixed *Cryptosporidium*, it may be possible to identify the specific infections responsible for the parasitisation at a reduced cost (Taniuchi *et al*, 2011).

1.7 *CRYPTOSPORIDIUM* IN FISH

Fish make up a large percentage of Global dietary intake. In developing countries, fish are considered an inexpensive source of protein, important to both fish farmers and consumers. It has been estimated that, in the year 2000, the global consumption of fish, annually, consisted of up to 16% of the protein dietary intake per annum, and consumption continues to increase. In Asia, the intake is 26%, which is considerably higher than in other countries which were >10%. It is estimated that the trade raises US $57 billion per year, internationally (Tidwell and Allan, 2000).

Cryptosporidium has been described in both fresh and marine water piscine species with parasitic stages located either on the stomach or intestinal surface, or at both sites (Table 2). 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). Hoover and colleagues also noted a similar infection in an unnamed species of marine fish (Hoover *et*

al, 1981). Three years later, Levine (1984) named it *Cryptosporidium nasorum* based on its presumed host specificity (Levine, 1984). However no oocyst measurements or helpful taxonomic features were recorded and the fact that only developmental stages on the intestinal microvillous surface were described, has resulted in *C. nasorum* being considered a *nomen nudem* (i.e, a name that is invalid because an insufficient description was published) (Xiao *et al,* 2004; Ryan *et al,* 2004a; Xiao *et al,* 2004).

Currently three species are recognised as piscine host specific; *Cryptosporidium molnari,* which was originally described in gilthead sea bream (*Sparus aurata*) and European sea bass (*Dicentrarchus labarx*) (Alvarez-Pellitero and Sitja-Bobadilla, 2002) and was characterised genetically in 2010 (Palenzuela *et al*, 2010), (2) *Cryptosporidium scophthalmi* was described in turbot in 2004 (*Psetta maxima*. sny. *Scopthalmus maximus*) (Alvarez-Pellitero *et al,* 2004) and characterized genetically in 2016 (Costa *et al,* 2016), and (3) *Cryptosporidium huwi* (previously piscine genotype 1) (Ryan *et al,* 2015). Molecular characterisation has identified additional piscine genotypes (piscine genotypes 2-8, *C. molnari*-like genotype and 7 un-named novel genotypes) as well as *C. parvum*, *C. xiaoi, C. scrofarum, C. hominis,* rat genotype III and 1 potential novel species in fish (Murphy *et al,* 2009; Reid *et al,* 2010; Zanguee *et al,* 2010; Barugahare *et al,* 2011; Morine *et al,* 2012; Koinari *et al,* 2013; Ryan and Xiao, 2014; Certad *et al,* 2015; Yang *et al,* 2015; Morgan, 2015; Ryan *et al,* 2015; Yang *et al,* 2016).

Table 2*: Cryptosporidium* species and genotypes reported in fish.

Species	Host	Site of Infection	Size (µm) L x W	Reference	GenBank accession number (18SrRNA)
C. nasorum	Naso Tang (*Naso literatus*)	Intestine	3.6 x 3.6	Hoover *et al.* 1981	-
Cryptosporidium sp.	Carp (*Cyprinus carpio*)	Intestine	-	Pavlasek, 1983	-
Cryptosporidium sp.	Cichlid (*Oreochromis* sp.)	Stomach	-	Landsberg and Paperna, 1986	-
Cryptosporidium sp.	Brown trout (*Salmo trutta*)	Intestine	-	Rush *et al*, 1987	-
Cryptosporidium sp.	Barramundi (*Lates calcarifer*)	Intestine	-	Glazebrook and Campbell, 1987	-
Cryptosporidium sp.	Rainbow trout (*Oncorhynchus mykiss*)	Stomach	5-7	Freire-Santos *et al.* 1998	-
Cryptosporidium sp.	Red drum (*Sciaenops ocellatus*)	Stomach	7 x 4	Camus and Lopez, 1996	-
Cryptosporidium sp.	Pleco (*Plecostomus* sp.)	Intestine and Stomach	-	Muench and White, 1997	-
Piscicryptosporidium reinchenbachklinkei	Gourami (*Trichogaster leeri*)	Stomach	2.4-3.18 x 2.4-3.0	Paperna and Vilenkin, 1996	-
Piscicryptosporidium cichlidis (previously *Cryptocystidium villithecum)*	Cichlid (*Oreochromis* sp.)	Stomach	4.0-4.7 x 2.5-3.5	Paperna and Vilenkin, 1996	-
Piscicryptosporidium sp. (previously Chloromyxum-like)	Gilthead sea bream (*Sparus aurata*)	Stomach	-	Paperna and Vilenkin, 1996	-
C. molnari	Gilthead sea bream (*Sparus aurata*), European sea bass (*Dicentrarchus labrax*), Murray cod (*Maccullochella peelii peelii*)	Stomach (and intestine)	4.72 (3.23–5.45) × 4.47 (3.02–5.04)	Alvarez-Pellitero and Sitja-Bobadilla, 2002, Palenzuela *et al*, 2010, Barugahare *et al*, 2011	HM243548, HM243550, HQ585890
C. molnari-like (LC12)	Peach anthias (*Pseudanthias dispar*)	-		Yang *et al*, 2015	KR610356
C. scophthalmi	Turbot (*Scophthalmus maximus*)	Intestine	4.44 (3.7–5.03) × 3.91 (3.03–4.69)	Alvarez-Pellitero *et al*, 2004; Costa *et al*, 2016	KR340589, KR34058
C. huwi (previously piscine genotype 1)	Guppy (*Poecilia reticulata*)	Stomach	4.6 x 4.4	Ryan *et al*, 2004a; 2015	AY524773, KC46997, KC469778
Cryptosporidium sp.	Alewife (*Alosa pseudoharengus*)	-	-	Ziegler *et al*, 2007	-
Piscine genotype 2	Angelfish (*Pterophyllum scalare*), Oscar fish (*Astronatus ocellatis*)	Stomach	3.4 x 4.1	Murphy *et al*, 2009; Morgan, 2015; Yang *et al*, 2015	FJ769050, KR610347
Piscine genotype 3	Mullet (*Mugil cephalus*)	Intestine	-	Reid *et al*, 2010; Yang *et al*, 2015	HM989833, KR610348
Piscine genotype 4	Golden algae eater (*Crossocheilus aymonieri*), Kupang damsel (*Chrysiptera hemicyanes)* Oscar	Intestine	-	Reid *et al*, 2010; Morine *et al*, 2012	HM989834, KR610346

	fish (*Astronatus ocellatis*), Neon tetra *(Paracheirodon innesi*)				
Piscine genotype 5	Angelfish (*Pterophyllum scalare*), Butter bream (*Monodactylidae)*, Golden algae eater (*Crossocheilus aymonieri)*	-	-	Zanguee *et al*, 2010; Yang *et al*, 2015	KR610344
Piscine genotype 5-like (KS05)	Oscar fish (*Astronatus ocellatis*),	-	-	Yang *et al*, 2015	KR610345
Piscine genotype 6, piscine genotype 6-like	Guppy (*Poecilia reticulata*), Gourami (*Trichogaster trichopterus)*	-	-	Zanguee *et al*, 2010, Morine *et al*, 2012	HM991857, JQ995776
C. parvum, *C. parvum*-like *C. xiaoi* and *C. scrofarum*	Whiting (*Sillago vittata*), Barramundi (*Lates calcarifer*)			Reid *et al*, 2010, Gibson-Kueh *et al*, 2011	-
Cryptosporidium sp.	Barramundi (*Lates calcarifer*)	Distal stomach and proximal small intestine		Gabor *et al*, 2011	-
Piscine genotype 7	Red eye tetra (*Moenkhausia sanctaefilomenae*)	-	-	Morine *et al*, 2012; Yang *et al*, 2015	JQ 995773, KR610354
Piscine genotype 8	Oblong silver biddy (*Gerres oblongus)*	-	-	Koinari *et al*, 2013; Yang *et al*, 2015	KC807985, KR610349
Cryptosporidium sp. (LC51)	Azure damsel (*Chrysiptera hemicyanea)*	-	-	Yang *et al*, 2015	KR610351
Cryptosporidium sp. (CA68)	Platyfish (*Xiphophorus maculatus)*	-	--	Yang *et al*, 2015	KR610353
Cryptosporidium sp.(LC38)	Goldfish (*Carassius auratus)*	-	-	Yang *et al*, 2015	KR610357
Cryptosporidium sp. (LC01)	Orange clownfish *(Amphiprion percula*)	-	-	Yang *et al*, 2015	KR610350
Cryptosporidium sp. (KS02)	Oscar fish (*Astronatus ocellatis*)	-	-	Yang *et al*, 2015	KR610352
Cryptosporidium sp.	Koi carp (*Cyprinus carpio*)	Disseminated infection in multiple tissues		Yang *et al*, 2016	KX033348
Cryptosporidium sp. (JM29)	Goldfish (*Carassius auratus)*	-	-	Morgan, 2015	-

In 1996, Paperna and Vilenkin (1996) proposed a new genus, designated *Piscicryptosporidium,* for *Cryptosporidium*-like species infecting a number of piscine hosts. The genus included two species, *P. reichenbachklinkei* and *P. cichlidaris* previously described as *Cryptosporidium* sp., in cichlid fishes, of the genus *Oreochromis*. Several unique features were cited to support the genus including the covering of the surface of the parasitophorous sac by rudimentary microvilli and the localisation of the oocysts deep within the gastric mucosa (Paperna and Vilenkin, 1996). However, these apparently differential features have also been described in some mammalian *Cryptosporidium* spp. For example, *C. parvum* has been occasionally found within some cells (Beyer *et al*, 2000; Marcial and Madara, 1986) and microvilli are usually retained in different mammalian species (Alvarez-Pellitero and Sitja-Bobadilla, 2002).

No molecular data was provided by Paperna and Vilenkin, (1996) to support the genus or species, however more recent characterisation of *C. molnari, C. scolpthalmi, C. huwi* and piscine genotypes indicate that piscine-derived species and genotypes of *Cryptosporidium* are genetically very distinct and primitive to all other species (Ryan *et al*, 2004a; 2015; Palenzuela *et al*, 2010; Reid *et al*, 2010; Zanguee *et al*, 2010; Barugahare *et al*, 2011; Morine *et al*, 2012; Yang *et al*, 2015; 2016). However, further studies at additional loci are required to confirm whether or not *Piscicryptosporidium* is valid.

The prevalence of *Cryptosporidium* in fish is highly variable, ranging from 0.8% to 100% mostly among juvenile fish (Landsberg and Paperna, 1986; Sitjà-Bobadilla *et al*, 2005; Alvarez-Pellitero *et al*, 2004; Murphy *et al*, 2009; Reid *et al*, 2010; Zanguee *et al*, 2010; Morine *et al*, 2012; Koinari *et al*, 2013; Certad *et al*, 2015; Morgan, 2015; Yang *et al*, 2015). *Cryptosporidium molnari* has been reported with maximum prevalence and

intensity in gilthead sea bream occurring in spring, followed by summer (Sitja-Bobadilla *et al,* 2005). *Cryptosporidium scopthalmi* has been reported in up to 100% of juvenile fish, from some farms, with larger fish generally showing less infections less frequently (Alvarez-Pellitero *et al,* 2004). Baragahare *et al*. (2011) looked at freshwater Australian Murray cod, acquired from a breeding facility. 3 cohorts of fish were collected: 9 month old, 6 month old and one group showing signs of morbidity. Fish displaying signs of deterioration and stunted growth were selected. Sequencing revealed that as many as 21 out of 22 fish were infected with *C. molnari*-like species in the 6-month old cohort (Baragahare *et al,* 2011).

The pathogenesis of *Cryptosporidium* in fish has not been extensively studied, but research has indicated that infected fish exhibit several clinical signs, with morbidity and mortality of parasitized fish dependent on several variables, which include (1) the species of *Cryptosporidium*; (2) the age of the fish; (3) coinfection factors; (4) the size of the fish; and (5) type of fish. For example, *C. molnari* has been associated with clinical signs in gilthead sea bream, consisting of whitish faeces, abdominal swelling and ascites which resulted in mortalities in fingerlings (Alvarez-Pellitero and Sitja-Bobadilla, 2002). The accumulation of *C. molnari* oocysts within the mucosal tissue resulted in cellular injury including consecutive necrosis, vacuolation, and sloughing of epithelial cells, but no inflammation reaction was observed (Alvarez-Pellitero and Sitja-Bobadilla, 2002). However, leucocyte inflammatory infiltration has been reported for *C. scolpthalmi* infections (Alvarez-Pellitero *et al,* 2004) and in gastric cryptosporidiosis in juvenile red drum (*Sciaenops ocellatus*) (Camus and López, 1996).

Other studies of *Cryptosporidium*-infected fish have reported anorexia, regurgitation of food, emaciation, poor growth rates, swollen coelomic cavities, atrophy of skeletal muscle,

tucked abdomen, listlessness and increased mortality, particularly in larval and juvenile fish (Hoover *et al*, 1981, Gratzek, 1993; Camus and López, 1996; Ryan *et al*, 2004b; Murphy *et al*, 2009; Gabor *et al*, 2011), and that *Cryptosporidium* distorted the normal mucosal architecture (Landsberg and Paperna, 1986; Paperna, 1987; Camus and López, 1996; Alvarez-Pellitero *et al*, 2004; Ryan *et al*, 2004a; Gabor *et al*, 2011). Another study reported that co-infections with bacteria (*Vibrio harveyi*) and *C. molnari* resulted in greater histopathologic gastric damage relative to infections with *C. molnari* alone (Sitjà-Bobadilla *et al*, 2006). In that study, 410 gilthead sea bream were monitored, from the Spanish coast. The most common bacterial genus detected was *Vibrionatriegens*, a group of commensal bacteria, and the authors reported that *Cryptosporidium* was more prevalent in fish with a high bacterial burden (Sitja-Bobadilla *et al*, 2006).

Cryptosporidium huwi was associated with high mortalities amongst guppies and was detected in the stomach, with oogonial and sporogonial stages observed deep within the epithelium, similar to *C. molnari* (Ryan *et al*, 2004a). Previous studies on piscine genotype 2, which was identified in a hatchery, revealed that infected fish exhibited variable levels of emaciation, poor growth rates, swollen coelomic cavities, anorexia, listlessness and increased mortality (Murphy *et al*, 2009). In affected fish, large numbers of protozoa were identified both histologically and ultrastructurally associated with the gastric mucosa. A high prevalence of *Cryptosporidium* was associated with high mortalities in immature barramundi (*Lates calcarifer*) in Australia (Gabor *et al*, 2011). The parasitized fish were in grow-out a facility, which is a potentially stressful environment, which could have contributed to the high rate of infections found in these juvenile fish (Gabor *et al*, 2011).

1.8 AIMS AND HYPOTHESES

In 2012, a novel genotype (JM29 - see Table 2) was identified in goldfish, during an Honours project, (*Carassius auratus*) from a local fish farm, Water Garden Life (Morgan, 2015). The main aim of this study is to characterise further this novel genotype in goldfish and to attempt to further characterise piscine genotype 3 previously described from sea mullet (*Mugil cephalus*) obtained from a local Western Australian bait shop (Reid *et al*, 2010; Yang *et al*, 2015). Further exploring the *Cryptosporidium* in these fish will increase our understanding of the phylogenetic relationships that may exist between species and genotypes.

Specifically, this project aimed to:

1. Identify previously described genotypes, as well as any novel genotypes, from goldfish and sea mullet in Western Australia.
2. Use histological analysis to better understand the life cycle of piscine-derived *Cryptosporidium* spp.
3. Conduct phylogenetic analyses to determine the validity and evolutionary relationships of piscine-derived *Cryptosporidium* species.

The hypotheses of this project are:

1. Both previously described and novel genotypes will be identified in goldfish and sea mullet which will expand our knowledge and understanding of *Cryptosporidium* species.
2. Life cycle stages of *Cryptosporidium* will be observed in histological analysis.
3. Phylogenetic analysis will provide insights into the validity and evolutionary relationships of piscine-derived *Cryptosporidium* species.

CHAPTER 2: MATERIALS AND METHODS

2.1 Overview

A flow diagram of the methodology used is depicted in Figure 2. Intestines and stomachs were dissected and removed. Half was kept for histology and immunofluorescent antibody staining. The remaining half was used for DNA extraction, followed by quantitative PCR (qPCR). Positives from qPCR were amplified by nested PCR at the 18S and actin loci and phylogenetic analysis conducted on the sequences. If *C. hominis* or *C. parvum* was detected, then subtyping at the glycoprotein 60 (*gp60*) locus was conducted.

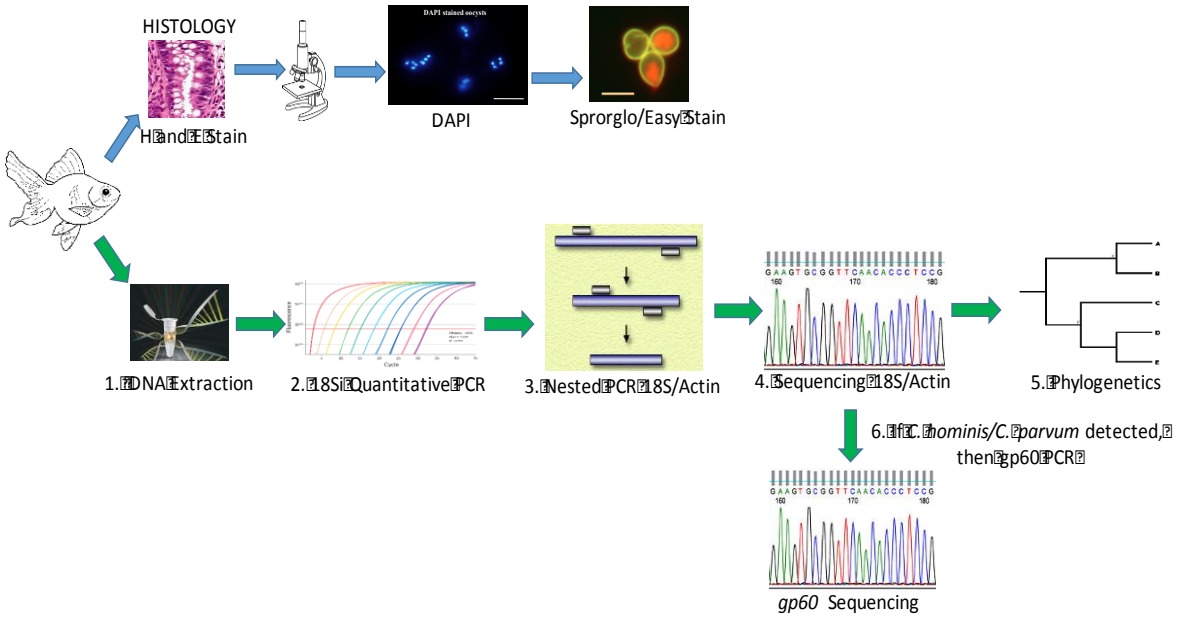

Figure 2. Sampling overview. Stomach and intestines were dissected out, 50% was kept for several histological methods and 50 % was kept for several PCR methods.

2.2 SAMPLING

A total of 229 commercial fish were purchased between April and July 2016. All fish were obtained from sources located in Perth, Western Australia; a local aquarium (Vebas)

(n=16), a fish farm (Water Garden Life) (n=200) and a local bait retailer (n=13) (Table 3). The mullet was purchased frozen.

The farmed fish were maintained in an outdoor pond environment. The water supply to the ponds on the fish farm was from the underground water table (bore water and was pumped into outdoor and indoor ponds without any filtration system). These fish were collected from the farm in four batches approximately three weeks apart, during the Australian wet season, between April – August, 2016.

Table 3. Sources of the 229 fish purchased in this study.

Fish species	**Family**	**Aquarium**	**Farmed fish**	**Local bait retailer**	**Total fish/species**
Goldfish (*Carassius auratus*)	Cyprinidae	16	200	0	216
Sea Mullet* (*Mugil cephalus*)	Mugildae	0	0	13	13

*These samples were obtained frozen.

Upon arrival to the laboratory, live fish were maintained in an aquarium environment with filtered air. Fish were euthanized in an ice slurry under Animal Ethics Permit RW 2734-16, in subsets of 10 at a time. The Mullet were purchased frozen. Fish were then weighed and measured and dissections were conducted using scissors and forceps, which were sterilised in 100% ethanol, between each fish. Stomach and intestines were excised from each fish. 5mm sections of tissue were fixed in a 10% formalin solution, for histological examination, while another 5mm section was minced on clean glass slides with sterile blades. These samples were stored in 1.5 ml Eppendorf tubes and frozen at -20°C, for DNA extraction.

DNA was always extracted within 24 hours of sample collection. Faecal samples were obtained from fish where possible and were stored in 1.5 ml Eppendorf tubes at -4°C until further processing. Physical separation was maintained between dissection of samples and DNA extraction of samples, in two separate laboratories, to ensure minimal contamination.

2.3 GENOMIC DNA EXTRACTION

A Qiagen DNeasy Blood and Tissue Kit (Duesseldorf, Germany) was used to extract DNA from ~25mg of stomach and intestinal tissue. The manufacturer's instructions were followed in the first batch of goldfish, which advised 200µL of elution buffer. However, the DNA yield was too dilute, therefore the elution buffer was reduced to 50µL, to increase the final DNA concentration. To further optimise DNA elution, the elution buffer was maintained on the DNA membrane for a minimum of 30 minutes at 57°C and eluted by centrifugation at full speed in a microfuge for 15 min. Once eluted, the elution buffer was re-applied to the membrane, and the 15 min spin was repeated; DNA was then stored at -70°C.

2.4 PCR (POLYMERASE CHAIN REACTION)

All primers used in the present study are listed in Table 4. Samples were initially screened at the 18SrRNA locus using a quantitative PCR (qPCR) and qPCR positives were subsequently amplified at the 18SrRNA and actin loci using a nested PCR protocol. Subtyping was conducted at the glycoprotein 60 (*gp60*) locus (Figure 2).

Table 4. Primer sequences used to amplify products at each locus.

Locus	Primer	Direction	Sequence	Length	Reference
qPCR					
18Si	18S i/F	Forward	5'-AGT GAC AAG AAA TAA CAA	298	Morgan *et al*, 1997

			TAC AGG-3'		
	18S i/R	Reverse	5'-CCT GCT TTA AGC ACT CTA ATT TTC-3'		
Nested PCR					
18S rRNA					
Primary reaction	SHP1	Forward	5'-ACC TAT CAG CTT TAG ACG GTA GGG TAT-3'	~ 763	Silva *et al*, 2013
	SHP2	Reverse	5'-TTC TCA TAA GGT GCT GAA GGA GTA AGG-3'		
Secondary reaction	SHP3	Forward	5'-ACA GGG AGG TAG TGA CAA GAA ATA ACA-3'	~ 588	Silva *et al*, 2013
	SSU-R3	Reverse	5'-AAG GAG TAA GGA ACA ACC TCC A-3'		Xiao *et al*, 1999
Actin					
Primary reaction	AIIF1	Forward	5'-GGD GCA ACR ACY TTR ATC TTC-3'	~830	Koinari *et al*, 2013
	Act6R	Reverse	5'-GGD GCA ACR ACY TTR ATC TTC-3'		
Secondary reaction	AIIF2	Forward	5'-GAY GAR GCH CAR TCV AAR AGR GGT AT-3'	~818	Ng *et al*, 2006
	AIIR1	Reverse	5'-TTD ATY TTC ATD GTH GAH GGW GC-3'		
gp60					
Primary reaction	AL3531	Forward	5′-ATA GTC TCG CTG TAT TC-3′	300	Zhou *et al*, 2003
	AL3534	Reverse	5′-GCA GAG GAA CCA GCA TC-3′		
Secondary reaction	AL3532	Forward	5′-TCC GCT GTA TTC TCA GCC-3′		
	AL3533	Reverse	5′-GAG ATA TAT CTT GGT GCG-3′	420-450	Zhou *et al*, 2003

2.4.1 Quantitative PCR (qPCR)

qPCR reactions (25 µl) contained: 1X PCR Buffer, 2mM $MgCl_2$, 0.4 mM of each dNTP, 0.5 µM of each primer (18Si F and 18Si R), 0.2mM TaqMan Probe, 1U Taq Polymerase. A positive and negative control was used for each qPCR. One µL of *C. xiao* obtained during a previous studywas used as a positive control. The negative control contained no template DNA. Reactions were carried out in a Rotor-Gene-Q 3000 (FisherBiotech, Australia) and were subjected to the following PCR cycles: an initial hold of 95°C for 6 minutes, followed by 50 cycles of 94°C for 20 seconds and 60°C for 90 seconds.

2.4.2 18S Nested PCR

A two-step nested PCR approach was used to amplify qPCR positives at the 18S rRNA locus (Figure 2 and Table 4). PCR reactions (25 µL) for both external and internal reactions consisted of the following: 2 µL (2.5mM) $MgCl_2$, 2.5µL of 10x Buffer (10x

*Taq*man with green dye and 1.5 mM $MgCl_2$) (KAPA Biosystems, Massachusetts, USA), 200 µM each of deoxynucleotide triphosphate (dNTP) (Promega, Wisconsin, USA), 0.8µL (0.4µM) each forward primer (external SHP1 and internal SHP3), 0.8µL (0.4µM) reverse primer (external SHP2 and internal SSUR3), 0.1 unit of Taq Polymerase (KAPA Biosystems), 1µL DNA and ultrapure PCR Grade H_2O, to make each reaction to 25µL in volume.

The conditions used to amplify the 18S locus were as follows: a preliminary cycle of 94°C for 3 minutes, followed by repetitive cycles (35 cycles for the external and 45 cycles for the internal reactions) of 94° for 30 seconds, 55°C for 30 seconds and 72°C for 1 minute, a final extension time of 72°C for 5 minutes and a hold temperature of 12°C. The secondary PCR used 1µL of the primary PCR product as a template for the DNA. In all PCR's a positive and a negative control were used. The positive control was *C. xiaoi* and the negative control included no template DNA.

2.3.3 ActinPCR

The actin PCR was conducted in a volume of 25 µl consisting of 0.25 µM, forward primer, 0.25 µM reverse primer, 200µM of each deoxynucleotide triphosphate (dNTP), 2.5µL 10x Buffer, 0.625 U Platinum Taq, 3mM $MgCl_2$ and 1µL DNA. The PCR conditions were: a preliminary step of 94°C for 2 minutes, 58°C for 1 minute and 72°C for 2 minutes. This was followed by 50 cycles of 94°C for 30 seconds, 58°C for 20 seconds and 72°C for 40 seconds and a final extension time of 72°C for 7 minutes. The same conditions were used in both primary and secondary rounds of PCR, and 1µL of primary product was used for the secondary PCR (Ng *et al*, 2006).

2.4.4 *gp60* PCR

If *C. hominis* or *C. parvum* were identified, further subtyping was conducted at the *gp60* locus. The 25 µl PCR reactions consisted of 1x Buffer, 3mM $MgCl_2$, 200µM of each dNTPs, 0.2 µM forward and reverse primers, 0.1 U of Taq Polymerase and 1µL DNA for the primary PCR or 1µL of primary product for the secondary PCR and water 25 µl. The PCR reactions were subjected to the following conditions: 95°C for 3 minutes, followed by 35 cycles of 90°C for 45 seconds, 50°C for 45 seconds and 72°C for 10 minutes (Glaberman *et al*, 2002).

2.5 GEL ELECTROPHORESIS

Products from the secondary PCRs at the 18S rRNA, actin and *gp60* loci were separated by gel electrophoresis using a 1% *(w/v)* agarose gel (Fisher Biotec, Australia) in Tris-Acetate (TAE) buffer (consisting of 40 mM Tris-HCl, 20 mM EDTA at pH 7.0). The DNA was stained with SYBR® safe DNA gel stain (Invitrogen, Oregon, USA). 5µL of 100 bp ladder (Promega, Madison, USA), was used as a reference marker in the first well. Each gel was run at 85V for 30-40 minutes in a gel tank (BioRAD, USA). Gels were then visualised under a transilluminator (FisherBiotech, Australia).

2.6 SEQUENCING

Positive bands of the correct size (Table 4), were excised from the agarose gel with sterile blades and purified using an in-house filter tip-based method (Yang *et al*, 2013). Briefly, positive bands were cut from the gel and the gel fragment transferred to a 100 µl filter tip (with the tip cut off) (Axygen, FisherBiotech, WA), and then placed in a 1.5 ml Eppendorf

tube and spun at full speed in a microfuge for 15 seconds. The filter tip was then discarded and the eluent was retained and used for sequencing without any further purification.

The secondary PCR products were sequenced in both forward and reverse directions, according to the SABC in-house sequencing manual using a Big Dye version 3.1 Terminator Cycle Sequencing Kit (Applied Biosystems, Massachusetts, USA).

Sequencing reactions (10 µl) consisted of 3.2µM primer (forward or reverse), 1µL Big Dye version 3.1 (Applied Biosystems Massachusetts, USA), 1.5µL of 5 x reaction buffer (Applied Biosystems Massachusetts, USA) with 6.5µL purified DNA. PCR conditions included a preliminary step of 95°C for 2 minutes, 27 cycles of 95°C for 10 seconds, 55°C for 5 seconds and 60°C for 4 minutes, with a final hold temperature of 12°C.

All 10µl of this reaction was transferred into separate 0.5µL Eppendorf tube and DNA precipitated using 1µL (125mM) Ethylenediaminetetraacetic acid (EDTA), 1µL (5.3mM) Sodium Acetate (NaOAc) and 25µL 100% Ethanol (ETOH). The tube was mixed by and left to precipitate for 20 minutes, followed by 30 minutes in the microfuge at maximum speed. The supernatant was gently aspirated by vacuum and discarded, leaving the pellet of DNA at the bottom of the tube. Pellets were washed with 125µL of 70% ETOH, followed by 15 minutes in the microfuge at maximum speed at room temperature. The wash step was repeated and then tubes were opened and exposed to a MAXI Speed Vac (WIKA, Czech Republic) for 2-3 minutes, to dry the pellet and then submitted for sequencing at the WA State Agricultural Biotechnology Centre (SABC).

2.7 STATISTICAL ANALYSES

Prevalences were expressed as proportion (%) of samples positive by qPCR and 95% confidence intervals calculated assuming a binomial distribution using the software Quantitative Parasitology
3.0 (Rózsa *et al*, 2000).

A Student's t-test was performed using PAST (Hammer Ø, Harper DAT, Ryan PD. PAST: Paleontological Statistics Software Package for Education and Data Analysis. Palaeontol Electronica. 2001;4(1):9) to determine if there were pairwise differences in weight or length, between goldfish parasitized with *Cryptosporidium* and those that were not.

2.8 PHYLOGENETIC ANALYSIS

Sequencing chromatograms were preliminarily assessed using Chromas Lite version 2.5.1, to verify quality, peak intensity, ambiguities and automated base calling. (http://www.technelysium.com.au). Sequences providing mixed chromatograms or low signal to noise ratios were discarded. Quality-checked sequences were then aligned with reference sequences from GenBank (http://www.ncbi.nlm.nih.gov) using Clustal W (http://www.clustalw.genome.jp). Distance, parsimony and maximum likelihood trees were constructed using MEGA version 7 (http://www.megasoftware.net) (Tamura *et al*, 2011). Genetic distances were calculated in MEGA using the Kimura 2 parameter model.

2.9 MICROSCOPY

2.9.1 H & E Staining

Samples that tested positive at the 18S locus were suspended in paraffin wax blocks and cut into slides, to provide morphological evidence and location of the parasite (stomach or

intestines). To increase the surface area, longitudinal sections of tissue were cut. This increased the likelihood of finding *Cryptosporidium*. The slides were initially stained with hematoxylin and eosin (H & E).

2.9.2 DAPI/Easy Stain™/Sporoglo™ staining

Silanized Slides, of positive DNA samples, were stained with an immunofluorescent antibody, Sporoglo™ (Waterborne Inc, USA) which binds to *Cryptosporidium* life cycle stages and Easy Stain™ (BTF, Sydney) which binds to *Cryptosporidium* oocyst walls using an adapted method from Boxell *et al.* (2008). Paraffin wax was removed by washing in xylene and ethanol and then rehydrated with Phosphate-buffered Saline (PBS), pH 7.4. Slides were first counter-stained with DAPI (4',6-diamidino-2-phenylindole), a fluorescent stain that binds strongly to A-T rich regions in DNA, for 2-5 minutes. The slides were rinsed with PBS and stained with Sporoglo™ for 45 minutes at room temperature. Slides were rinsed again with PBS and then stained with Easy Stain™ for 30 minutes at room temperature. They were rinsed once more with PBS and mounted with anti-fade mounting media. Visualisation was conducted using an Olympus D21 digital camera magnified by an Olympus BX51 fluorescence microscope.

CHAPTER 3: RESULTS

3.1 *CRYPTOSPORIDIUM* PREVALENCE

Of the 229 fish screened at the 18S locus by qPCR, 69 were positive for *Cryptosporidium* - an overall prevalence of 30.1% (CI 24.2-36.1). The prevalence of *Cryptosporidium* in goldfish at the fish farm was 24.4%, 25% at Vebas aquarium and mullet, the prevalence was 30.8% (Table 5). The overall prevalence in goldfish was 30.1% (CI 24.0-36.2). The prevalence appeared to be higher during the June and July sampling which coincides with peak winter rainfall, however more extensive research is required to confirm this.

Table 5. Prevalence of *Cryptosporidium* in fish from the three locations sampled in the present study.

Location	**Fish**	**# Samples**	**qPCR (+)**	**% Prevalence by qPCR (95 CI)**
Vebas Aquarium	Goldfish (*Carassius auratus*)	16	4	25.0 (3.8-46.2)
Water Garden Life Fish Farm	Goldfish (*Carassius auratus*)	200	61	20.4 (15.8-25.0)
Bait Shop	Mullet (*Mugil cephalus*)	13	4	30.8 (5.7-55.9)

Of the 69 qPCR positives, only 34 could be amplified by nested PCR at the 18S locus, an overall prevalence of 14.8% (CI 10.2-19.5) at this locus. None of the fish from Vebas aquarium could be amplified by nested PCR and only one of the 4 qPCR positives from Mullet were amplified by nested PCR at the 18S locus.

Samples that screened positive and negative by qPCR were statistically compared using a t-test to determine whether there was a correlation between the fish size (weight and length) and likelihood of infection. A p-value > 0.05 was deemed significant. Analysis revealed that weight and length were not correlated with the presence of *Cryptosporidium* in the fish ($p > 0.05$) as the weight and length of the fish were similar in both positive and negative parasitisation samples (Figure 3 and Table 6). The weight of the goldfish from

Vebas aquarium varied between 3.19 – 5.98 g and the length varied between 6.2 – 5.98 cm. The weight of the goldfish from Water Garden Life varied between 0.01 – 15.05 g and the length varied between 4.4 – 16.2 cm. The weight of the Mullet varied between 180.1 - 501.56g and the length varied between 25.7 – 34.4 cm

Table 6. Statistical analysis of the correlation between the presence of *Cryptosporidium* and the length and weight of the fish. Results of the t-test were not significant with *p*-values >0.05.

Location	**Fish**	***p*-value (weight)**	***t*-value (weight)**	***p*-value (length)**	***t*-value (length)**
Vebas Aquarium	Goldfish (*Carassius auratus*)	0.76	0.311	0.811	-0.244
Water Garden Life Fish Farm	Goldfish (*Carassius auratus*)	0.082	-1.751	0.490	-0.692
Bait Shop	Mullet (*Mugil cephalus*)	0.907	0.119	0.987	0.017

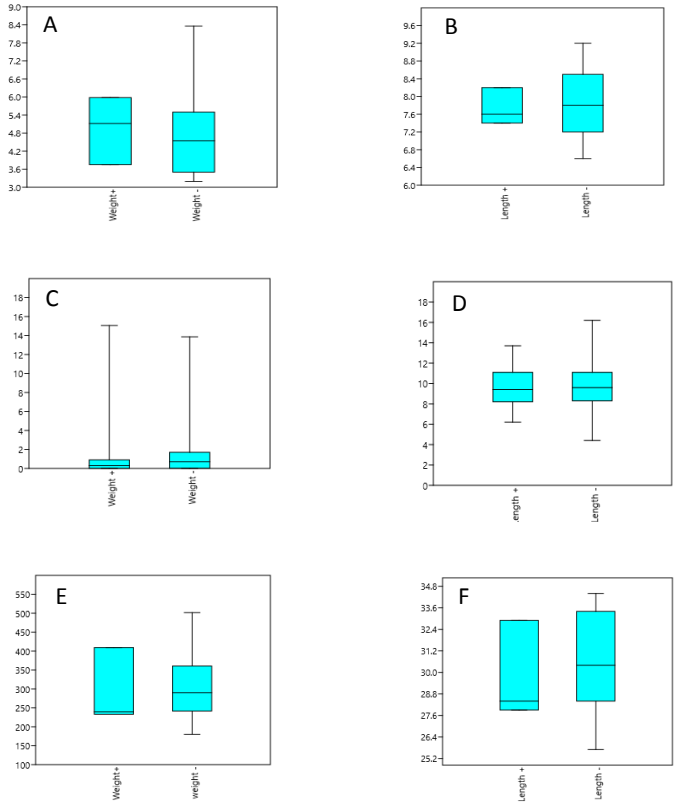

Figure 3. The weight in grams (A, C and E) and length in cm (B, D and F) of fish that tested negative and positive by qPCR, for *Cryptosporidium*. Vebas aquarium (A and B), Water Garden Life fish farm (C and D) and the bait shop (E and F) are displayed as

Boxplots with the median value represented by the line through the middle of each plot. Positive isolates are shown on the left and negative are shown on the right of each box.

3.2 *CRYPTOSPORIDIUM* SPECIES IN FISH

3.2.1 18S rRNA Locus

Of the 34 samples that amplified at the 18S rRNA locus, 23 clean sequences were obtained. Of these, 10 (43.5%, 23.2-63.7 95 CI) (isolates 137, 138, 139, 143, 144, 145, 152, 159, 160 and 209) were identified as *C. hominis*, 2 (8.7%, 0-20.2 95 CI) (isolates 214 and 204) were identified as *C. parvum* and 11 (47.8%, 27.4- 68.2 95 CI) (isolates 27, 50, 119, 146, 128, 156, 157, 175, 196, 198, 202) were a novel genotype and 100% identical to a single sequence (isolate 29) obtained in 2015, by a previous Honours student (Jacqui Morgan), in a goldfish from the same fish farm (Table 6). The remaining 11 amplicons produced mixed chromatograms when sequenced, presumably due to mixed infections. All the successfully sequenced isolates were from goldfish were from the Water Garden Life fish farm.

Table 7. *Cryptosporidium* species and subtypes and identified at the 18S, actin and *gp60* loci.

Isolate code	18S Locus	Actin locus	*gp60* locus
27	Novel	-	-
50	Novel	Novel	-
119	Novel	Novel	-
128	Novel	-	-
137	*C. hominis*	Novel	-
138	*C. hominis*	-	-
139	*C. hominis*	-	-
143	*C. hominis*	-	*C. hominis*
144	*C. hominis*	-	-
145	*C. hominis*	-	-
146	Novel	-	-
152	*C. hominis*	-	*C. hominis*
156	Novel	-	-
157	Novel	-	-
159	*C. hominis*	-	-
160	*C. hominis*	-	-
175	Novel	-	-
196	Novel	-	-
198	Novel	-	-

202	Novel	-	-
204	*C. parvum*	-	*C. hominis*
209	*C. hominis*	-	-
214	*C. parvum*	-	-

Phylogenetic analysis was conducted using the Kimura 2-parameter model using *Eimeria faurei* (AF345998) as the outgroup. Analysis using both Distance and Maximum Likelihood (ML) produced trees with similar topologies (data not shown). The 10 isolates there were identified as *C. hominis* by sequence analysis, produced only short sequences and were not included in the phylogenetic tree (Figure 4), but based on phylogenetic analysis of these shorter sequences (~110 bp), grouped with *C. hominis* reference isolate AB830587. The two *C. parvum* sequences grouped with *C. parvum* reference isolate AF108864. The 11 samples identified as a novel genotype grouped in a separate clade with isolate 29 (Jacqui Morgan) and were most closely related to *C. scopthalmi* (KR340588) (Figure 4). The genetic distance between the novel genotype and *C. scopthalmi* was 10.4% (Table 8). The genetic distance between the novel genotype and all other species of *Cryptosporidium* was 15.5-24.0%.

Figure 4: Phylogenetic relationship of *Cryptosporidium* species and genotypes as inferred by Distance analysis of ~590 bp of sequence at the 18S rRNA locus. Percentage support (>50%) from 500 pseudoreplicates from Distance analysis at the left of the supported node using the Kimura 2-parameter model.

Table 8. Pairwise genetic distances (%) between the novel genotype and piscine *Cryptosporidium* species and genotypes at the 18S locus based on the Kimura-2 parameter model.

	Novel genotype	***C. scopthalmi***	***C. huwi***	***C. molnari***	**Piscine genotype 2**	**Piscine genotype 3**	**Piscine genotype 4**	**Piscine genotype 5**	**Piscine genotype 7**	**Piscine genotype 8**
Novel genotype	0									
C. scopthalmi	10.4	0								
C. huwi	20.3	20.6	0							
C. molnari	22.3	20.9	10.8	0						
Piscine genotype 2	22.8	22.6	13.0	12.0	0					
Piscine genotype 3	24.1	22.6	11.6	11.6	5.0	0				
Piscine genotype 4	24.1	22.2	12.6	13.1	5.4	4.7	0			
Piscine genotype 5	22.2	21.2	13.3	11.2	7.0	8.8	8.8	0		
Piscine genotype 7	20.3	21.4	4.1	10.5	12.7	11.6	13.4	13.0	0	
Piscine genotype 8	21.4	20.1	11.6	11.6	6.4	6.0	10.5	6.7	11.6	0

Note piscine genotype 6 was not included in this analysis as only a short sequence (<300 bp) is available but separate analysis revealed that the genetic distance between it and the novel genotype is ~23.9%.

3.2.2 Actin Locus

Only 6 samples were successfully amplified at the actin locus, and of these, three produced mixed chromatograms, while the remaining three amplicons (50, 119, 137) produced clean chromatograms and 642 bp of readable sequence. Phylogenetic analysis was conducted using *Monocystis agilis* (AY391264) as an outgroup. Analysis using Distance and ML produced trees with similar topologies (data not shown). Phylogenetic analysis revealed that the three isolates (50, 119, 137), were a novel genotype that grouped most closely with *C. scopthalmi* with a genetic distance of 14.1 % (Figure 5 and Table 9). The genetic distance between the novel genotype and all other *Cryptosporidium* species ranged from 16.9-28.8%.

Table 9. Pairwise genetic distances (%) between the novel genotype and all other species of *Cryptosporidium* at the actin locus based on the Kimura-2 parameter model.

	Novel genotype	***C. scopthalmi***	***C. huwi***	***C. molnari***
Novel genotype	0			
C. scopthalmi	14.1	0		
C. huwi	26.0	23.6	0	
C. molnari	18.6	16.9	16.8	0

Note actin sequences for the other piscine genotype are not available.

3.2.3 *gp*60 Locus

Isolates that were typed as *C. hominis* (n=10) and *C. parvum* (n=12) at the 18S locus were subtyped at the *gp60* locus. Of the 12 isolates, five were successfully amplified and of these, two produced mixed chromatograms, while three isolates (143, 152, 204) were typed as *C. hominis* subtype 1bA10G2.

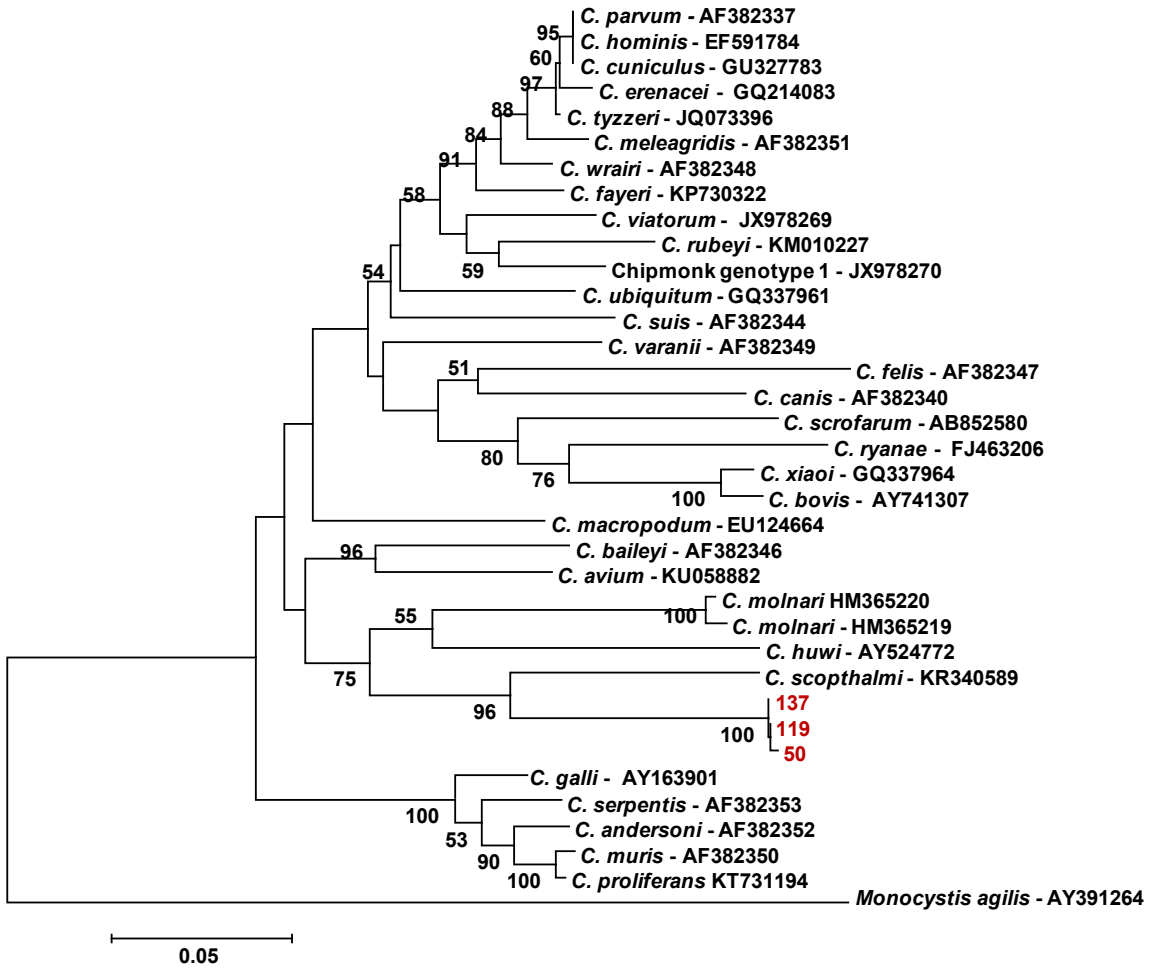

Figure 5: Phylogenetic relationship of *Cryptosporidium* species and genotypes as inferred by Distance analysis of ~642 bp of sequence at the actin locus. Percentage support (>50%) from 500 pseudoreplicates from Distance analysis at the left of the supported node using the Kimura 2-parameter model.

3.3 MICROSCOPY

Unfortunately, no oocysts or life-cycle stages were observed in H & E stained sections. Several unsuccessful attempts were made to stain the silanized slides with Easy Stain™ and Sporoglo ™, however high background staining was observed, which obscured the sections and due to time constraints, it was not possible to troubleshoot these issues.

CHAPTER 4: DISCUSSION

4.1 PREVALENCE OF *CRYPTOSPORIDIUM*

In the present study, the overall prevalence of *Cryptosporidium* in fish by qPCR was 30.1%, with 25% prevalence in goldfish from a local aquarium (Vebas), 24.4% prevalence in goldfish from a local fish farm (Water Life Gardens) and 30.8% prevalence in mullet from a local bait shop. Previous studies have reported a large variation in prevalences ranging from 0.8 to 100% (Sitjà-Bobadilla *et al*, 2005; Murphy *et al*, 2009; Reid *et al*, 2010; Zanguee *et al*, 2010; Morine *et al*, 2012; Koinari *et al*, 2013; Certad *et al*, 2015; Ryan *et al*, 2015; Yang *et al*, 2015a; Yang *et al*, 2016). The prevalence in ornamental fish is generally higher than wild-caught fish, presumably due the high stocking densities and low water exchange rates practiced in the rearing of ornamental fish, and hence, higher chance of exposure and infection. For example, Reid *et al.* (2010) observed a prevalence of only 2.4% (6/255) in wild marine fish, whereas in 2010, Zanguee *et al.* detected a prevalence of 10.5% (21/200) in freshwater aquarium fish (Zanguee *et al*, 2010). However, in 2011, a prevalence of only 3.5% was reported in freshwater ornamental fish, collected from 8 retail outlets across Perth (Morine *et al*, 2012). More recently, prevalences of 5.3 and 7.1% respectively have been reported in ornamental fish (Yang *et al,* 2015; Ryan *et al*, 2015). Another study of *Cryptosporidium* in freshwater fish from Lake Geneva (LacLéman) from six species of fish, identified a high overall prevalence (36.6%-15/41) (Certad *et al*, 2015).

In the present study, the overall prevalence by nested PCR (14.8%) was much lower than qPCR (30.1%) and qPCR positives from Vebas and the bait shop could not be amplified and typed by nested PCR. The lower prevalence observed by nested PCR compared to qPCR in the present study, is most likely due to the fact that (1) qPCR is much more

sensitive than conventional PCR (Hadfield *et al*, 2011) and (2) the size of the amplicons generated, as the qPCR amplicon size was 298 bp, whereas the secondary amplicon size for the 18S nested PCR was 588 bp. It is well known that shorter amplicons amplify much more efficiently than longer amplicons (Shagin *et al*, 1999). It is also possible that some of the qPCR positives were due to non-specific amplification, however the qPCR assay has been extensively validated (Yang *et al*, 2015b).

4.2 SEQUENCING AND PHYLOGENETIC ANALYSIS AT THE 18S AND ACTIN LOCI

Sequencing and phylogenetic analysis identified three species of *Cryptosporidium* in goldfish (1) *C. parvum* (n=2), (2) *C. hominis* (n=10) and (3) a novel genotype (n=11). *Cryptosporidium hominis* and *C. parvum* are the main species infecting humans (Xiao, 2010) and have been previously reported in fish (Reid *et al*, 2010; Koinari *et al*, 2013; Certad *et al*, 2015). In the study by Reid *et al.* (2009), *C. parvum* was identified in a marine fish, School whiting (*Sillago vittata*) at the 18S rRNA locus, and the *C. parvum* subtype identified at the *gp60* locus was IIaA18G3R1, which is one of the most commonly reported *C. parvum* subtypes in humans and cattle in Australia (Ryan and Power, 2012). In the study by Koinari *et al.* (2013), *C. parvum* was identified in cultured Nile tilapia (*Oreochromis niloticus*) (n=2), wild caught marine mackerel scad (*Decapterus macarellus*) (n=2) and wild freshwater silver barb (*Barbonymus gonionotus*) (n=1) at the 18S locus. Subtyping at the *gp60* locus identified *C. parvum* subtypes IIaA19G4R1 and IIaA14G2R1 in Nile tilapia, *C. parvum* subtype IIaA15G2R1 in one mackerel scad isolate and interestingly *C. hominis* subtype IdA15G1 in the second isolate indicating that this fish was infected with both *C. parvum* and *C. hominis*. In the single barb positive, *C. parvum* subtype IIaA19G4R1 was identified. In both the latter two studies, no oocysts or life cycle

stages were observed in the infected fish hosts due to substantial autolysis of tissues, which has been reported as an issue for *Cryptosporidium* detection in piscine hosts (Zanguee *et al*, 2010). Fish are known to have a very rapid rate of tissue autolysis compared to homeotherms (Roberts, 2012) and many of the fish were dead for up to 4 hours prior to being processed which contributed to the problem. In the most recent study, in freshwater fish from Lake Geneva (LacLéman), *C. parvum* was detected at a prevalence of 86.7% (13/15), mixed *C. parvum* and *C. molnari* infections were identified in 6.7% (1/15) of infections and *C. molnari* in 6.7% -1/15) (Certad *et al*, 2015). In that study, developmental stages of *C. parvum* were found in the stomach and intestine, indicating that *C. parvum* was infecting fish, rather than being passively, carried which has important public health implications.

In the present study, subtyping of the 10 isolates that were identified as *C. hominis* at the 18S locus was successful for three isolates and all three were identified as *C. hominis* subtype 1bA10G2. This is a dominant subtype responsible for *C. hominis*-associated outbreaks of cryptosporidiosis in the United States, Europe and Australia (Zahedi *et al*, 2016) and is therefore of public health significance.

A novel genotype was identified in 11 samples which was genetically distinct but most closely related to *C. scophthalmi* at the 18S locus and this was confirmed in a subset of samples at the actin locus. *Cryptosporidium scophthalmi* was first described in turbot (*Scophthalmus maximus*) from different farms on the coast of North Western Spain (Alvarez-Pellitero *et al*, 2004). The parasite was found mainly in the intestinal epithelium and very seldom in the stomach but at the time was not characterised genetically (Alvarez-Pellitero *et al*, 2004). As discussed in the introduction, delimiting species in *Cryptosporidium* requires genetic characterisation at two loci and submission of the

genetic data to GenBank (Xiao *et al,* 2004). Due to the lack of genetic data for *C. scophthalmi*, it was therefore considered a nodem nudem (Ryan and Xiao, 2014; Ryan *et al,* 2015). However, in 2016, genetic data at two loci (18S and actin) and histological analysis of a *C. scophthalmi*-like isolate found in the intestinal mucosa of farmed turbot was provided, which confirmed the validity of *C. scophthalmi* as a species (Costa *et al*, 2016). In that study, depending on the stage of the infection, *C. scophthalmi* organisms were present either as extracytoplasmatic stages lining the intestinal epithelium, and/or as sporogonial clusters found deeply embedded in the intestinal mucosa of turbot. This simultaneous presence of different *Cryptosporidium* stages has been described for other piscine *Cryptosporidium* species, including *C. scophthalmi, C. molnari* and *C. huwi* (Alvarez-Pellitero and Sitjà-Bobadilla, 2002; Alvarez-Pellitero *et al,*2004; Ryan *et al*, 2004; Palenzuela *et al*, 2010; Ryan *et al*, 2015). The feature of sporulation taking place deep within the intestinal epithelium, contrasts with the epicellular location of *Cryptosporidium* species from other vertebrates (Ryan *et al*, 2014).

In the most recent study on *C. scophthalmi*, histopathological damage to the intestinal mucosa of turbot was extensive in heavily infected specimens, often causing epithelium necrosis and loss of tissue integrity, due to the presence of large vacuoles, containing *Cryptosporidium* clusters, occupying wide zones of the epithelium (Costa *et al*, 2016), similar to that previously reported for *C. scophthalmi* (Alvarez-Pellitero *et al*, 2004). Both *C. molnari* and *C. huwi* have been associated with necrosis and sloughing of gastric mucosa epithelial cells (Alvarez-Pellitero and Sitjà-Bobadilla, 2002; Ryan *et al*, 2004; Palenzuela *et al*, 2010).

In the present study, the histological and clinical impact of the novel genotype in goldfish is unknown but as it is closely related to *C. scophthalmi,* infection with the novel genotype

may result in similar histological damage. However, further research is required to confirm this.

Genetic analysis of the novel genotype in the present study provides support that the novel genotype may be a valid species. The genetic distance between *C. scophthalmi* and the novel genotype was 10.4% and 14.1% at the 18S and actin loci respectively, which is greater than many currently accepted species. For example, the genetic distance at both the 18S and actin loci between *C. parvum* and *C. erinacei* is 0.5% (Kvác *et al*, 2014) and the genetic distance between *C. muris* and *C. andersoni* at the 18S and actin loci is 0.9% and 3.5% respectively. However, histological analysis is required to provide a morphological basis for the novel genotype as new *Cryptosporidium* species. An important criterion for species delimitation for *Cryptosporidium* is the provision of morphological data, therefore research is required before the species of the novel genotype can be confirmed.

4.2 FUTURE CONSIDERATIONS

It is unclear as to whether the fish were parasitized with *Cryptosporidium* or if detection of *Cryptosporidium* DNA in these fish was due to mechanical ingestion of the oocysts. Histologically there was no evidence of infection, therefore future studies should focus on extensive histological characterisation of the novel genotype using both H & E staining and fluorescent antibody staining. *Cryptosporidium* infections in fish frequently have a patchy distribution (Ryan *et al*, 2015) and it is therefore possible that infections were missed in the histological analysis. To increase the likelihood of identifying oocysts, many sections from each sample should be cut.

A more comprehensive study should be conducted sampling all year round, to determine if the prevalence is higher during high rainfall periods. Previous research has suggested that seasons may play a role in prevalence of *Cryptosporidium.* For example, Sitja- Bobadilla *et al.* (2005) reported a seasonality distribution of *C. molnari* in the gilthead sea bream. Unlike the current study however, maximum prevalence and intensity of *C. molnari* occurred in spring followed by summer (Sitja-Bobadilla *et al*, 2005). It is also important to examine different ages groups, as previous studies have shown that the age could be a contributing factor to increased prevalence of *Cryptosporidium*. Alvarez-Pellitero *et al.* (2004) reported that up to 100% of juvenile turbot were parasitised by *Cryptosporidium* (Alvarez-Pellitero *et al*, 2004). Sitja-Bobadilla *et al* (2005) also reported that infection levels were higher in pre-growing and early on-growing juvenile fish (Sitja-Bobadilla *et al*, 2005). A case report that looked at intensively reared barramundi (*Lates calcarifer*) reported that cryptosporidiosis outbreaks tended to occur in the juvenile population and caused severe stock losses (Gabor *et al*, 2011). In other hosts, such as cattle and humans, age has also been found to be a determining factor, with neonates and the young being mostly affected (Kotloff *et al*, 2013; Sarkar *et al*, 2014). Juvenile and adult fish should be collected over several seasons, to determine whether the prevalence of *Cryptosporidium* is higher in the juvenile population and if the prevalence changes over the time period.

In the current study, the only samples infected with the novel genotype were the goldfish from Water Garden Life fish farm. Further studies should focus on determining the host range of the novel genotype, by screening a wide range of fish hosts and a much larger number of fish from each host group. The novel genotype has never been reported in humans and therefore its zoonotic potential is uncertain, however the finding of *C. hominis* and *C. parvum* in these fish is of public health importance and the source of the *C. hominis* and *C. parvum* infection in these fish needs to be investigated further. The water used at

Water Garden Life Fish farm was recirculated bore water (underground bore water), with no filters and future studies should screen the water for the presence of *C. hominis* and *C. parvum* oocysts.

Previous attempts to reconstruct the evolutionary relationships between *Cryptosporidium* species in fish, using nested PCR and conventional Sanger sequencing-based approaches, have been hampered by a lack of concordance between the commonly utilised markers, 18S rRNA and actin (Yang *et al*, 2015). This inconsistency has resulted in conflicting phylogenetic trees at the actin and 18S loci, with the main clades identified by the 18S not reproduced at the actin locus. It is likely that the discrepancy is due to frequent mixed infections and in the current study there was also evidence of mixed infections as evidence by mixed chromatograms. Elucidating this diversity is important to advance our understanding of parasite-host interactions, host specificity, epidemiology, phylogeny and public health and veterinary implications of piscine-derived *Cryptosporidium* sp.

Unless cloning is performed however, the traditional Sanger method cannot be used to sequence a mixture of amplicons obtained by the co-amplification of multiple genetic variants. Unlike the Sanger method, at adequate sequencing depths, massive parallelization of the sequencing reactions allowed by next generation sequencing (NGS), can allow resolving mixtures of amplicons. NGS has already been successfully exploited to characterize the genotypes present in mixed human infections of influenza virus (H1N1) (Ghendin *et al*, 2011) and cytomegalovirus (HCMV) strains (Gorzer *et al*, 2010).

Recently, deep sequencing strategies have been employed to uncover the extent of mixed infections in fish using the NGS platform, the Ion Torrent (Paparini *et al*, 2016). In that study however, not all mixed infections could be resolved, as the samples could not be

amplified using single-round PCR, due to low parasite DNA concentrations in the samples. It is generally recognised that nested PCR approaches have an inherent risk of contamination and have previously been shown to exhibit strong amplification biases and/or stochastic variation (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 between the first and second round. In molecular parasitology studies, however, nested PCR is often critical to obtain enough DNA copies to sequence by the Sanger method. This is an inherent problem with *Cryptosporidium* epidemiology, as environmental water samples, gastric/intestinal tissues from fish or faeces from wildlife, frequently contain very low numbers of oocysts and high levels of PCR inhibitors, and therefore nested PCR is often necessary to amplify the parasite DNA. The benefits of NGS, and quantitative comparisons between alternative DNA sequencing methods are therefore hampered by the need to use nested PCR to produce sufficient DNA products for analyses. Previous studies have also reported a strong bias when using a nested PCR approach for Ion Torrent sequencing (Whiteley *et al*, 2012).

The pathogenicity of the novel genotype in goldfish is currently unknown but future studies should focus on determining clinical signs of cryptosporidiosis in fish, as outbreaks of cryptosporidiosis can cause economic losses in fish stock as well as pose a risk to human health. Further research into the epidemiology, host specificity and zoonotic nature of *Cryptosporidium*, particularly the novel species, is important both for our understanding of the evolutionary history of piscine-derived *Cryptosporidium* species and for disease management practices.

As previously suggested, goldfish are often released into native waterways and as a result have dominated some of the ecosystems (for example Vasse River Busselton, WA). Future

studies, should also look at the impact of *Cryptopsporidium* on wild Goldfish and, subsequently, any impact on native species. Wild Goldfish could act as vectors of *Cryptosporidium* into WA ecosystems posing a considerable threat to native species.

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