Toxoplasma gondii infection in

Australian felines

Anthea Brennan

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Declaration

I declare that this thesis is a result of my own work and has not been submitted for another degree or qualification. All information derived from published or unpublished work of others has been acknowledged in the text. A reference list is provided at the end of the thesis.

Anthea M. Brennan

Date: 29/11/15

Author contributions

Included in this thesis is a manuscript that is under revision. I am the primary author of this publication. According to the University of Sydney policy on submission of a thesis by publication, a signed statement regarding the contribution of co-authors is submitted for each published work. Below is the manuscript, followed by the co-authorship confirmation statements for each of these.

Comparison of genotypes of *Toxoplasma gondii* in domestic cats from Australia with latent infection or fatal toxoplasmosis- Anthea Brennan, Shannon L. Donahoe, Julia A. Beatty, Katherine Belov, Scott Lindsay, Katherine A. Briscoe, Jan Šlapeta, Vanessa R. Barrs

Abbreviations

+ve	Positive
3'/5'SAG 2	3'/5' Surface Antigen 2
ACT	Australian Capital Territory
AIDS	Aquired Immunodeficiency Syndrome
alt.SAG 2	Alternate Surface Antigen 2
Apico	Apicoplast
BTUB	β-tubulin
CNS	Central Nervous System
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphate
DSH	Domestic Short Hair
ELISA	Enzyme Linked Immunosorbent Assay
FeLV	Feline Leukaemia Virus
FIV	Feline Immunodeficiency Virus
FS	Female Speyed
GLMM	General Linear Mixed Model
GRA6	Dense Granule 6
H&E	Haematoxylin and Eosin
IFN	Interferon
IgG	Immunoglobulin G
IHC	Immunohistochemistry
LDM	Long Distance Migration
M	Molar
MHC	Major Histocompatibility Complex
mM	Millimolar
MN	Male Neutered
NF-κβ	Nuclear Factor Kapa Beta
NSW	New South Wales
PBS	Phosphate Buffered Solution
PBS-Tw	Phosphate Buffered Solution with added Tween 20
PCR	Polymerase Chain Reaction
QLD	Queensland
RFLP	Restriction Fragment Polymorphism
SAG 1	Surface Antigen 1
SAG 3	Surface Antigen 3
SNP	Single Nucleotide Polymorphism
SPF	Specific Pathogen Free
ToxoDB	Toxo Database
UVTHS	University Veterinary Teaching Hospital
-ve	Negative
VIC	Victoria
VPDS	Veterinary Pathology and Diagnostic Services
WA	Western Australia

Abstract

Toxoplasma gondii is a significant human and animal parasite with worldwide occurrence. The population structure and prevalence in definitive hosts and intermediate hosts has been well studied in a number of regions including North and South America and Europe. While *Toxoplasma gondii* has been previously reported in a number of mammalian species in Australia, there is a surprising lack of information regarding the population structure of *T.gondii* in Australia and current rates of exposure in domestic companion animal hosts.

Members of the felidae family are the only known definitive host of *T. gondii* in which sexual reproduction of the parasite occurs.Sexual reproduction results in shedding of environmentally resillient oocysts in faeces. All strains of *T. gondii* can be traced back to a feline host. As such, an understanding of *T. gondii* infection among feline hosts is an important first step to understanding general prevalence and population structure of the parasite in a particular area. The seroprevalence of *T. gondii* in cats worldwide is estimated to be 30-40%.

In addition to being a definitive host of *T. gondii,* domestic cats are susceptible to clinical disease due to *T.gondii*. Although uncommon, feline toxoplasmosis occurs in immunosuppressed cats, congenitally infected kittens and occasionally in otherwise clinically healthy individuals. Little is known about whether parasite genotype is associated with severity of disease in naturally occurring toxoplasmosis of domestic cats.

The first aim of this research was to expand knowledge of feline *T. gondii* infections in Australia by determining the seroprevalence of *T. gondii* in owned Australian cats and identifying risk-factors for infection. The second aim was to determine whether the genotype of *T. gondii* is a significant determinant of whether cats develop clinical toxoplasmosis.

To estimate seroprevalence of *T. gondii* in Australian owned cats, *Toxoplasma* specific IgG ELISAs were performed on sera from 425 owned domestic Australian cats. A multivariate

analysis of the results from a questionnaire given to the owners was used to evaluate lifestyle factors which could contribute to increased likelihood of infection.

Of the 425 cats tested in this study 38% (n=162) were seropositive. The prevalence in different geographic regions ranged from 16-71%. Cats fed raw beef or raw kangaroo in their diet or cats that hunted rodents were significantly more likely to be seropositive

To identify the genotypes associated with latent and active infections in Australian cats, tissue samples were collected from cats undergoing routine post mortem examination at the University Veterinary Teaching Hospital (n=28). Serology to detect *T. gondii* specific IgG was performed after collection of heart blood from cats of unknown *T. gondii* serostatus. Cases were chosen based on the likelihood of a cat being exposed to the parasite. Results of a PCR targeting the *B1* gene to detect *T. gondii* DNA were positive in tissue samples from 11 of 17 (65%) seropositive cats tested including four with clinical toxoplasmosis and seven with latent infections, as determined by serology, histologic findings and immunohistochemistry. Three of the four cats with clinical toxoplasmosis were immunosuppressed. *T. gondii* type II (ToxoDB genotype #3) was determined in four cats with clinical toxoplasmosis and three cats with latent toxoplasmosis using PCR-RFLP at 12 loci (*SAG1, 5'SAG2* and *3'SAG2*, *altSAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1* and *Apico*) and direct sequencing of the multi-copy *B1* gene. Novel *T. gondii B1* gene polymorphisms were detected in two strains (at nucleotide posititions 233, 366 and 595) and a *B1* gene polymorphism unique to Australia was identified in another (guanine/adenine at nucleotide position 378). One cat was co-infected with two or more type-II like strains at *3'SAG2*. The results of this study suggest that the infecting *T. gondii* genotype is not a determinant of clinical disease in cats naturally infected with *T. gondii* and type II strains are the most prevalent in Australia.

The ToxoDB#3 genotype has been previously identified in Australian hosts. Given the high prevalence in the present study, it appears that this genotype may be endemic to Australian hosts and this discovery warrants further investigation of a larger sample set using high resolution techniques such as those presented in this thesis to confirm this.

Naturally infected cats with active and latent infection were both infected with the same strain, indicating the infecting strain is not the major determinant of disease progression and that other host factors may be of greater importance.

Throughout this program of research, epidemiological aspects of feline *T. gondii* infections in Australia were elucidated further. Key findings include a seroprevalence of 38% among owned Australian cats, the identification of a cat which was infected with more than one strain of *T. gondii,* the discovery that infections with the same ToxoDB#3 genotype can cause both clinical and latent infection in cats and the confirmation of diversity at the B1 locus in felines, which has been previously found in Australian wildlife species. This is the first survey of feline infections in owned Australian cats to evaluate lifestyle factors which contribute to the likelihood of exposure and it is also the first study to genetically characterise infecting strains in cats with clinical toxoplasmosis. Further studies are needed to fully understand the population structure and also the prevalence of *T. gondii* in Australia as current data is lacking in other domestic and wildlife species.

Chapter 1. Introduction

Chapter one of this thesis introduces the parasite, describes the life cycle of *T. gondii* and reviews the literature on the worldwide population structure of the parasite and current knowledge about the relationship between strain and virulence in *T. gondii* infections. The second half of this chapter focuses on *T. gondii* infections in cats and reviews data on *T. gondii* in Australia. This chapter concludes with the aims of the studies that make up this thesis.

1.1 *Toxoplasma gondii*

T. gondii is an obligate intracellular coccidian parasite that causes toxoplasmosis. It is one of the most prevalent parasites and can infect almost any nucleated cell in all species of warm-blooded animals (Beazley and Egerman, 1998). Members of the felidae family are the definitive hosts as sexual reproduction of the parasite can only occur in the intestinal epithelium of felids. All other hosts are intermediate hosts, in which *T. gondii* can undergo asexual division (Beazley and Egerman, 1998).

T. gondii is responsible for the disease toxoplasmosis. The course of disease and clinical presentation of toxoplasmosis in each species, and even in each individual, vary greatly depending on genetic background, immunological status, inoculum size, and virulence of the strain (Beazley and Egerman, 1998; Robert-Gangneux and Darde, 2012).

1.2 Discovery of the parasite and history clinical observations

Toxoplasma gondii was discovered accidently in 1908 by Charles Nicolle who was studying *Leishmania* in the native rodent *Ctenodactylus gundi* in North Africa (Innes, 2010). The parasite was named for the arc-like appearance of the tachyzoites and bradyzoites that Nicolle found.

The first conclusive case of *T. gondii* causing human disease was identified by Wolf and colleagues (1939), in an infant girl who had convulsive seizures three days after birth. She died at one month old and a post mortem examination found *T. gondii* in lesions of encephalomyelitis and retinitis. Tissue samples fed to rabbits and mice caused encephalitis in both species.

The veterinary importance of *T. gondii* was first indicated when the first canine toxoplasmosis case was diagnosed in 1910 (Dubey, 2008; Mello, 1910). Subsequently the parasite was found to be associated with disease in several other species, most notably being associated with abortions in sheep in New Zealand (Hartley and Marshall, 1957).

1.3 Life cycle of the parasite
The life cycle of *Toxoplasma gondii* is summarised in figure 1.1 and discussed below.

Tissue cyst in intermediate host ingested by definitive host (cat)

Unsporulated oocysts passed in faeces contaminate the environment and sporulate after one to five days

Oocysts in water or plant matter are ingested by intermediate hosts

Tissue cysts in intermediate hosts consumed by carnivores

Tachyzoites in infected mother travel across the placenta and infect fetus

Fig1.1 The life cycle of *T. gondii*

1.3.1 Sexual phase

The sexual stage (also referred to as the enteroepithelial stage) of the parasite's life cycle occurs within the intestines and can be completed within 3-10 days in 97% of naïve cats (Dubey, 1996). Ingested tissue cysts are the most common infectious stage of the *T. gondii* life cycle that will cause cats to excrete oocysts (Dubey, 1996; Dubey and Frenkel, 1976). After ingestion, enzymes break down cyst walls and bradyzoites are released into the stomach and small intestine (Jacobs et al., 1960). Following digestion of the cyst wall, bradyzoites penetrate the intestinal wall and begin development of several generations of *T. gondii* and five pre-determined asexual stages (A-E) develop (Dubey and Frenkel, 1972). There are several generations within each asexual stage; after a number of generations male and female gamonts are formed and fertilisation occurs to form an oocyst (Dubey and Frenkel, 1972).

Oocysts are one of three infectious stages in the life cycle of *T. gondii*. Once they have been formed, they are passed (unsporulated and, therefore, non-infectious) in faeces (Fig 1.1). Exposure to air and moisture for one to five days results in sporulation. Once sporulated, the oocyst contains two sporocysts, which contain four sporozoites. The sporozoites are the infectious agent. They are extremely resistant to environmental pressure (Hutchison, 1965).

1.3.2 Asexual phase

The stage of the life cycle, which occurs in both definitive and intermediate hosts, referred to as the extraintestinal cycle, is asexual (Fig 1.1). After the digestion of oocysts

or tissue cysts, sporozoites or bradyzoites (in the case of tissue cyst ingestion) excyst and penetrate intestinal epithelial cells. The sporozoites and bradyzoites then divide asexually in two via a process called endodyogeny to become tachyzoites. Tachyzoites are a rapidly multiplying, invasive stage of *T. gondii,* which can multiply in almost any cell and are carried around in the blood stream to infect other tissues. Tachyzoites enter cells via active penetration and are protected from host immune action by a self-made parasitophosphorous vacuole (Dobrowolski and Sibley, 1996). Following penetration of the intestinal epithelium, tachyzoites continue to multiply until the cell ruptures, after which tachyzoites will travel through the blood stream to infect new cells. The presence of tachyzoites evokes a very strong host interferon gamma (IFN) cell mediated immune response which eventually controls the tachyzoite stage, but not without causing significant damage to the host (Montoya and Liesenfeld, 2004). The amount of inflammation required to control the tachyzoite stage causes cell death and damage, which can manifest as visible clinical signs (Montoya and Liesenfeld, 2004). This strong immune response forces tachyzoites to encyst.

Once encysted, tachyzoites become bradyzoites, which are morphologically similar, but functionally different. Tissue cysts grow intracellularly and the bradyzoites within multiply at a much slower rate than tachyzoites. Cysts are most commonly formed in the central nervous system, muscles and visceral organs. It is these cysts that facilitate *T. gondii*'s ability to reinfect an individual as cysts persist for the life of the host and are not affected by the host immune action against tachyzoites (Saeij et al., 2005).

1.4 Transmission

It was not until the invention of the Sabin-Feldman dye test that the life cycle and transmission routes of *T. gondii* were able to be explored (Weiss and Dubey, 2009). The invention of this sensitive and specific test allowed researchers to investigate the epidemiological characteristics of the parasite in humans and several other animals. Subsequently, more *T. gondii* infections were diagnosed than could possibly be caused by congenital transmission alone, which was assumed to be the main route of infection, specifically in sheep (Hartley and Marshall, 1957; Innes, 2010). This prompted further investigations into the possible transmission routes of *T. gondii.*

It is now known that the wide spread distribution of *T. gondii* can be attributed to the many mechanisms of transmission and that *T. gondii* can be transmitted both horizontally and vertically. Horizontal transmission can occur via ingestion of tissue cysts from intermediate hosts, ingestion of oocysts in water, soil or vegetation contaminated by infected feline faeces and via organ transplants and blood transfusions (Fig1.1) (Beazley and Egerman, 1998; Montoya and Liesenfeld, 2004). Vertical transmission occurs when tachyzoites are transmitted across the placenta after a naïve female is infected during the gestational period. Susceptibility to both horizontal and vertical transmission routes have been reported in a variety of species including dogs, cats, grey kangaroos and humans (Al-Qassab et al., 2009; Parameswaran et al., 2009b; Powell and Lappin, 2001; Wilson et al., 1980).

1.4.1 Horizontal transmission and infectious agents

Carnivorism and bradyzoites

In 1960, Jacobs and colleagues discovered that the parasitophosphorous vacuole of *T. gondii* could be broken down via proteolytic enzymes in the gut and that bradyzoites could survive within the acidic gut environment. This discovery revealed that bradyzoites could survive long enough in the gut to infect a new host once a tissue cyst has been ingested (Jacobs et al., 1960). These findings were supported by epidemiological research, which suggested that areas where the consumption of undercooked or rare meat was common, had a higher prevalence of toxoplasmosis (Innes, 2010).

Infection after the consumption of tissue cysts from immediate hosts occurs when proteolytic enzymes breakdown the parasitophosphorous vacuole surrounding the tissue cyst. This releases bradyzoites, which quickly differentiate into tachyzoites and penetrate the intestinal epithelium and begin asexually reproducing (Fig 1.1).

Infection due to carnivorism is considered to make up a significant portion of cases and it is this transmission route, which allows the parasite diversity to be perpetuated sexually in feline hosts (Herrmann et al., 2012). Bradyzoites can be found within tissue cysts in several species, which are used for human and pet consumption including sheep, pigs, chickens and beef (Dubey et al., 2005b; Opsteegh et al., 2011; Wang et al., 2013). Bradyzoites are also found in game meat (Dubey et al., 2013; Mancianti et al., 2013; Pan et al., 2012; Parameswaran et al., 2010).

Several human toxoplasmosis outbreaks have been traced back to the consumption of contaminated meat. In Korea, two outbreaks of acute toxoplasmosis were attributed to the consumption of undercooked pork, and in America and French Guiana outbreaks of acute toxoplasmosis were attributed to consumption of undercooked game meat (Carme et al., 2002; Choi et al., 1997; Ross et al., 2001).

Oocysts

The discovery of *T. gondii* transmission through undercooked meat accounted for several cases of toxoplasmosis, which could not be explained by congenital transmission. However, it did not account for similar exposure rates in vegetarians and people whose diet included meat. This disparity called for further investigation into a potential third route of transmission (Rawal,1959). The third route was discovered by William Hutchison who found a new environmentally resistant form of the parasite in the faeces of cats, which he associated with the nematode parasite *Toxocara cati* (Hutchison, 1965). The Isospora-like oocysts were found to be extremely environmentally resistant, and remained infectious after 12 months in water (Hutchison, 1965). Further experiments established that *T. gondii* could be transmitted independently of *T. cati* ova as the *T. gondii* oocysts could be found in worm-free cat faeces (Dubey, 1968).

Once the oocyst was discovered as an independent infectious agent it became clear that cats had a crucial role in the transmission of *T. gondii.* The sexual life cycle of *T. gondii* within feline intestinal tracts was discovered some five years later and led to *T. gondii* being classified as a coccidian parasite (Hutchison, 1969; Hutchison et al., 1970).

Transmission by oocysts occurs when a host ingests vegetation or water, which has been contaminated with feline faeces containing oocysts. It is one of the most effective routes of transmission for *T. gondii* due to extreme environmental resistance of oocysts (Fig 1). Depending on environmental conditions, sporulated oocysts can remain infectious for up to one year after shedding. Oocysts can survive water submersion, soil submersion for extended periods of time, and are resistant to detergents (Frenkel et al., 1975; Wainwright et al., 2007).

The development of an assay to differentiate oocyst infections from other infection types by detecting antibodies for sporozoites revealed the relative importance of environmental contamination in the transmission of *T. gondii.* In some instances, 78% of mothers who gave birth to congenitally infected children had acquired infections from oocyst ingestion (Yan et al., 2012).

Contamination of vegetation and water by oocysts is the main transmission route for terrestrial herbivores, which can subsequently infect carnivores through bradyzoite consumption. This is significant because it can perpetuate the cycle between cats and wildlife and subsequently perpetuate the sexual phase of *T. gondii* life cycle. Marine mammals can also be infected via oocyst contamination of seawater and molluscs (Miller et al., 2004; Roe et al., 2013).

1.4.2 Vertical transmission

Congenital transmission

Congenital transmission of *T. gondii* is a result of transplacental transmission of tachyzoites (Abbasi et al., 2003) (Fig 1.1). This route of transmission occurs when a naïve woman is infected during the first two months of gestation. Women infected before this are considered immune and, therefore, unlikely to transmit infection. However, there have been reports of immunocompromised patients transmitting infections which were acquired earlier than one to two months prior to gestation (Garcia, 1968; Gavinet et al., 1997; Vogel et al., 1996). Infection severity and possibility of foetal infection are inversely related; infections in the first trimester are the most severe but least likely to happen (Desmonts and Couvreur, 1974; Sever et al., 1988).

1.5 Population structure of *T. gondii*

The first studies to use methods other than virulence in mouse bioassays to analyse the population structure of *T. gondii* involved isoenzymes and a few RFLP (restriction fragment length polymorphism) markers (Darde et al., 1992; Darde et al., 1988; Sibley and Boothroyd, 1992). Initially, these techniques grouped strains that were virulent to mice together, while non-virulent strains seemed to be more diverse (Sibley and Boothroyd, 1992). These results were confirmed by Howe and Sibley (1995) using RFLP at other loci and the techniques were expanded to test more isolates from different continents and a number of different host species. Of the isolates tested 84% could be categorised into three clonal types which suggested an epidemic population where

sexual recombination of *T. gondii* was a rare event (Howe and Sibley, 1995). However, the isolates tested were mostly from humans or domestic animals in only a few regions. As further studies expanded on the markers used and the locations sampled, it was revealed that the *T. gondii* population is not entirely clonal. In South America, higher resolution techniques with better discriminatory markers and microsatellites found genotypes in French Guiana that were distinct from those found previously and these populations had more diversity between strains as well (Ajzenberg et al., 2004; Lehmann et al., 2004). Later, Su and colleagues (2006) confirmed that clonality was not the rule by using standardised RFLP across nine unlinked genomic markers to genotype Brazilian samples and to genotype previously typed strains with more loci as most previous techniques only typed at a single SAG2 locus.

As more genetic data was generated from a broader geographic range and different hosts, distinct subgroups were found in South America, Europe, Africa, Asia, and Central America (Lehmann et al., 2006). Su and colleagues (2012) later used a combination of RFLP, microsatellite and sequence based markers (based on introns in housekeeping genes) to cluster genotypes into major groups of related strains. The 956 isolates from diverse geographic locations could be grouped into six clades, which were likely formed because of the interbreeding of a small number of founding populations. Some of the clades showed related yet divergent genotypes suggesting a mutational drift and/or recombination while others were made up of one genotype. This study confirmed that in Northern America and Europe, *T. gondii* exhibits a highly clonal population with three predominant genetic lineages: Type I, Type II, Type III and a less common haplogroup 12. South America exhibited a high level of diversity with a mixture of diverse genotypes and clonal genotypes. (Su et al., 2003; Su et al., 2012).

More recently, Shwab and colleagues (2014) gathered information on all of the isolates that had been typed using the 10-loci RFLP technique designed by Su and colleagues (2006; 2010) and generated a report on the distribution and abundance of *T. gondii* genotypes throughout the world. The 1457 samples in this report were typed into 189 genotypes and again confirmed the findings of previous authors that *T. gondii* exhibits a clonal population structure in Northern America and Europe, and that Southern America exhibits an epidemic population comprised of hundreds of different genotypes with no clear dominant strains.

The ever-increasing number of distinct genotypes has created a need for a new nomenclature. As the PCR-RFLP technique developed by Su and colleagues (2006, 2010) is favoured for its high resolution, ease of use and cost effectiveness, a database called ToxoDB has been created whereby users can upload the sequences of isolates types using multi-locus RFLP techniques (Gajria et al., 2008). Isolates in this database are typed using 10 markers: SAG1, SAG 2 (3/5'-SAG2 and alt.SAG2), SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1 and Apico (Su et al., 2010; Su et al., 2006). The 10-marker technique is sometimes described as an 11- marker technique as the 3/5' SAG 2 and alt.SAG2 markers can be considered to be two separate markers. With the addition of the Apico allele, this technique is able to differentiate between clonal Type II and Type II variant genotypes. In Type II clonal isolates the Apico allele is a Type II allele. However, the Type II variant genotype has a Type I allele at this locus. The most common genotypes and their corresponding ToxoDB genotype are presented in Table 1.1.

Other systems of genotype designations are described by Weiss and Kim (2011). In this thesis, the ToxoDB genotype system is used.

To date, the most frequently identified strains of *T. gondii* found worldwide include Type III (ToxoDB#2), Type II variant (ToxoDB#3), Type II clonal (ToxoDB#1), Type 12 (ToxoDB#5), Type BrI (ToxoDB#6), ToxoDB #7, Type BrIII (ToxoDB#8) and Type I (ToxoDB #10) (Shwab et al., 2014). The dominant strains on each continent vary; Type II variants and Type III were dominant in Africa, Chinese 1 and Type I in Asia, Type I, Type II and Type III in Europe, Type I, Type II, Type III and Type 12 were dominant in Northern America, while no dominant strains were identified in South America.

The clonal genotypes I, II, and III are responsible for most infections in humans and domestic animals in North America and Europe (Grigg and Boothroyd, 2001; Lehmann et al., 2006; Lindstrom et al., 2008). It is hypothesised that these three dominant strains are a result of a bottleneck event in which a single genetic cross occurred followed by radiation (Su et al., 2003). In South America, among wildlife in the United States and in some parts of Africa and Asia the rich diversity of atypical genotypes likely result from sexual recombination in the definitive host (Ajzenberg et al., 2004; Lehmann et al., 2006; Miller et al., 2004). Some of these atypical strains are clearly related to the three dominant strains, with mixtures of archetypal alleles and non-archetypal polymorphisms while others, referred to as "exotic" strains, have novel alleles at each locus (Khan et al., 2007).

Conventional genotype designation	ToxoDB PCR RFLP genotypes
Type I	#10
Type II (clonal)	#1
Type II (variant)	#3
Type III	#2
Type 12 (atypical, exotic)	#4
Type 12 (atypical, exotic, types A and X)	#5
Type BrI and Africa I	#6
Type BrII	#11
Type BrIII	#8
Type BrIV	#17
Chinese 1	#9

Table 1.1 The traditional name of common *T. gondii* genotypes and their corresponding ToxoDB genotype designation (table appropriated from Shwab et al., (2014)).

1.5.1 Strains and virulence

Different strains of *T. gondii* have characteristics that affect their virulence in different hosts. For example, sea otters, harbour seals, and sea lions are prone to developing encephalitis when infected with an atypical strain of Type X, while infections with this strain in terrestrial wildlife are usually not associated with clinical signs of disease (Dubey et al., 2011; Miller et al., 2004). Recent studies have focussed on the genetic variability of *T. gondii* and the effect of strain type on virulence. Experimental virulence is determined using a mouse model. It is often stated that Type I is the most virulent in mice; infections with Type II or Type III strains are usually subclinical, and other non-clonal strains are more likely to have increased virulence if they have a majority of Type I alleles (Ajzenberg et al., 2004; Darde, 2004; Luder et al., 1998; Mercier et al., 2010;

Saeij et al., 2005; Su et al., 2002b). Factors thought to affect virulence include variation in migration abilities, growth rate and ability to manipulate the host immune system.

The ability to migrate effectively across barriers such as the gut epithelium, blood-brain barrier or placenta, is essential for infection and dissemination of *T. gondii*. Certain subpopulations of Type I strains have superior migration abilities when compared to Type II and Type II strains. This enhanced ability is attributed to a long distance migration (LDM) phenotype found only in Type I parasites (Barragan and Sibley, 2002). Improved migration abilities are an effective and important component of new and reactivated infections as better migration results in better dissemination to multiple systems, which can enhance pathogenesis.

The ability of *T. gondii* to manipulate host immune responses makes it extremely successful. The parasite can use antigen-presenting cells as effective Trojan horses, essentially evading the immune system via down regulation of surface molecules. *T. gondii* decreases MHC II up-regulation of murine bone marrow-derived macrophages by 50%, although MHC I regulation is not affected (Luder et al., 1998).

Toxoplasma gondii can also transiently block the translocation of Nuclear Factor-kappa Beta (NF-κβ) in mice and human fibroblasts, thereby inhibiting the transcription of pro-inflammatory genes and preventing the production of cytokines after cell invasion (Butcher et al., 2001). Differences in strains with regards to this ability have been reported. For example, Type I and Type III do not induce NF-kB translocation in mice where Type II does (Dobbin et al., 2002).

The growth rate of a parasite is also a significant factor which can affect virulence. A faster growth rate results in an increased parasite load and an overstimulation of the

immune system, which results in increased inflammation and necrosis manifesting in severe clinical signs. The most virulent strain in mice Type I, is also the strain that induces a parasite load 1000 times greater than the less virulent Type II strain (Hill et al., 2012).

. The inoculum size and infectious phase of the parasite, the genetic background and immunological status of the host are also significant virulence factors of *T. gondii.*

There is, however, evidence to indicate that strains can have an important impact on the clinical outcome of an infection, specifically in immunocompetent hosts who normally develop a subclinical infection when infected with clonal strains. Moreover, association between atypical strains and severe clinical manifestations have been reported in human infections (De Salvador-Guillouet et al., 2006; Ferreira et al., 2011; Grigg et al., 2001; Stajner et al., 2013). For example, *T. gondii* strains isolated and genotyped from three of 16 immunocompetent human patients with severe primary toxoplasmosis in French Guiana were all atypical strains (Carme et al., 2002). The severity of toxoplasmosis is lower in regions where clonal strains are common, such as Western Europe, and Northern America (Howe and Sibley, 1995) and is comparatively high in regions where more atypical strains circulate, such as South America (Carme et al., 2002; Khan et al., 2006; Vallochi et al., 2005). In France, Type II strains are strongly associated with congenital toxoplasmosis and its various manifestations (Ajzenberg et al., 2002).

1.6 Detection methods

Toxoplasma gondii can be detected in biological samples using direct or indirect methods. Indirect serological methods used on live subjects are particularly useful in immunocompetent patients to indicate exposure; whereas direct methods are more definitive but require tissue or fluid samples. Because this thesis will focus on both direct and indirect molecular methods in both clinical and latent infections- a summary of both types of techniques is presented below.

1.6.1 Indirect

Once an animal is infected with *T. gondii,* tissue cysts persist for life. Infection stimulates a long-term humoral immune response, which is detectable using serological methods. Indirect detection methods use serological techniques such as the Sabin-Feldman dye test, indirect fluorescence antibody test (IFAT), Immunoglobulin G (IgG) /Immunoglobulin M (IgM) Enzyme Linked Immunosorbent Assay (ELISA), IgG avidity and agglutination tests. A combination of a number of these tests can be used depending on the information required (Montoya, 2002).

These tests utilize the fact that *T. gondii* stimulates a strong and persistent humoral response. Experimental infection of cats with *T. gondii* induces IgM production one to two weeks post-infection and IgG levels peak two months after infection and are detectable for life (Lappin et al., 1991). By measuring the levels of these two *T. gondii* specific antibodies, exposure to the parasite can be confirmed.

1.6.2 Direct detection methods

Direct detection methods confirm the presence of *T. gondii* in a biological sample by detecting parasitic DNA or isolating parasites. Examples of such techniques include bioassays (most commonly in mice or cell cultures), histological methods, hybridization, and amplification of characteristic genes by PCR (Montoya and Liesenfeld, 2004).

These methods can be divided two types: molecular identification and isolation of the parasite. Isolation of the parasite can be performed using bioassays and histological methods. Bioassays involve either inoculating or feeding potentially infected tissue to a test species- usually mice or *T. gondii* naïve cats.

When a mouse bioassay is performed to determine whether a sample is *T. gondii* positive, SPF mice are fed the sample or given an intraperitoneal injection of the sample. serology is carried out on mice serum and microscopic analysis of brain tissue for cysts is performed. In some cases, DNA is extracted from infected mouse tissues and PCR is performed (Dubey et al., 2003). If *T. gondii* antibodies are found, parasites or parasitic DNA are found in microscopic or molecular analysis of tissues or blood from the SPF mice then the presence of *T. gondii* in the sample is confirmed.

When naïve cats are used in a bioassay, the sample is fed to uninfected SPF cats and faeces are examined for oocyst shedding. Cats are also tested for seroconversion (Dubey, 1968). If oocysts are detected in faeces, if the cat becomes seropositive, or if parasites are detected on necropsy then the presence of *T. gondii* in a sample is confirmed. Not all cats will seroconvert immediately, or even within the study period, therefore seropositivity in cats should not be used as the sole indicator of exposure.

Molecular detection of *T. gondii* involves the amplification of characteristic genes using PCR to indicate the presence of *T. gondii* in a sample.

PCR was first used to detect *T. gondii* DNA by Burg and colleagues (1989). Burg and colleagues (1989) amplified the 35-fold-repetitive gene B1 and were able to detect as few as 10 parasites in 100 000 human leukocytes. Since then other characteristic markers have been used to indicate the presence of *T. gondii* in a biological sample and to characterize the genotype of the strain. The most common markers used are repetitive sequences B1 and a 529 bp sequence. Several other single copy genes such as the surface antigens SAG1, SAG 2, SAG4, SAG3, GRA4 and GR6 have been used as PCR targets for *T. gondii*.

The markers used depend largely on the aims of the analysis. If only identification is required, multiple copy markers are preferred due to increased sensitivity. However, if high-resolution fingerprinting or genetic characterization of the isolate is required, then more specific multi-locus markers or microsatellite markers are used.

A large number of studies use a standardized RFLP protocol designed by Su et al (2010) however, there are other methods such as microsatellite analysis that are gaining increasing popularity as these markers can be used to determine sources of outbreaks. Most studies that use this technique utilize 15 microsatellite markers as described by Ajzenberg and colleagues (2010).

2. Toxoplasmosis

2.1 Prevalence and population structure in cats

T. gondii is prevalent in cat populations throughout the world. Infection is usually subclinical. Feral and stray cats have a higher seroprevalence than domestic cats, especially those domiciled indoors (Nutter et al., 2004; Wu et al., 2011).

Recent studies have found a seroprevalence of 85.4% of feral cats sampled in Ethiopia (Tiao et al., 2013), 44.2% in Portugal (Waap et al., 2012), 21.3-85.4% in China (Tiao et al., 2013; Wu et al., 2011), 95.5%-97.4% in Egypt (Al-Kappany et al., 2011; Al-Kappany et al., 2010b) and 17.5% in Korea (Lee et al., 2011). While these figures demonstrate a generally high and varied prevalence worldwide, factors that affect seroprevalence are incompletely understood. Regional outdoor cat density, incidence of *T. gondii* infection in intermediate hosts, mean annual precipitation and climate have been suggested to play a role in feline *T. gondii* seroprevalence (Afonso et al., 2010; Masaaki et al., 2015).

A number of genotypes of *T. gondii* have been identified from latently infected cats with regional specific distribution. ToxoDB#9 is the most prevalent genotype found in the tissues of Chinese stray cats (80% of 35 cats sampled) (Qian et al., 2012; Tian et al., 2014; Yang et al., 2015) whereas Type II clonal genotypes (ToxoDB#1) are the most common genotypes found in European stray and household cats with 82% of 151 cats tissues sampled typed as Type II (Can et al., 2014; Montoya et al., 2008; Vilares et al., 2014).

2.2 Disease in felines

Clinical feline toxoplasmosis was first reported in 1942 from a four-month old cat in Middletown, New York, USA (Dubey, 2008). The occurrence of disease in cats has been well documented worldwide since this discovery (Dubey and Carpenter, 1993; Hartmann et al., 2013; Lappin et al., 1989). Although clinical disease is uncommon, severe systemic feline toxoplasmosis was reported in 3% of feline cases submitted for necropsy over a two or three year period in Europe (Henriksen et al., 1994; Jokelainen et al., 2012). Figures such as these indicate that toxoplasmosis is a significant cause of feline mortality.

It has been proposed that strain type, immune status, age of the individual, and concurrent infections can play a role in determining the severity of an infection in feline hosts (Dubey and Powell, 2013). The relative importance of each of these factors in determining the severity of feline toxoplasmosis remains to be determined.

Cats that are most severely affected by toxoplasmosis are those that acquire toxoplasmosis congenitally or are immunocompromised. The central nervous system (CNS), muscles, pancreas, lungs and eyes are areas commonly affected in cats, and widespread systemic disease can occur (Davidson et al., 1993b; Hartmann et al., 2013; Last et al., 2004; Nagel et al., 2013; Spycher et al., 2011, Dubey and Carpenter 1993).

Infections can be primary or recrudescent. Recrudescent infections occur when immunosuppression results in cyst rupture, bradyzoite dissemination and transformation into rapidly replicating tachyzoites. Common clinical signs in cats include but are not limited to neurological signs (eg seizures, ataxia), muscular

hyperaesthesia, dyspnoea, uveitis, icterus, diarrhoea, fever, lethargy, nasal secretions, anorexia and weight loss (Hartmann et al., 2013; Jokelainen et al., 2012).

3. *Toxoplasma gondii* in Australia

T. gondii is reportedly common in Australian wildlife and cats. Despite this, there is a surprising lack of recent information regarding the population structure of *T. gondii* in Australia and current rates of exposure in cats (Hartley and English, 2005; Hartley and Dubey, 1991; Jayamaha et al., 2012; Obendorf and Munday, 1983; Parameswaran et al., 2009a; Sumner and Ackland, 1999).

T. gondii seroprevalence rates in Australian marsupials range from 1.2% in Tasmanian Pademelons (Jakob-Hoff and Dunsmore, 1983) to 26.1% in wombats (Hartley and English 2005). While the seroprevalence of *T. gondii* indicates that toxoplasmosis is not invariably fatal, outbreaks of toxoplasmosis have been associated with declines and die-offs of Australian marsupials such as the Eastern Barred Bandicoot *(Perameles gunnii)* and the Woylie (*Bettongia penicillata)* (Obendorf et al., 1996, Smith et al., 2008).

There is a surprising amount of diversity among *T. gondii* genotypes in Australia, including variations and recombination of the three clonal genotypes prevalent Europe, North America and Africa and strains with atypical alleles (Pan et al., 2012; Parameswaran et al., 2010). Of 46 Australian marsupials screened by multi-locus PCR DNA sequencing at polymorphic genes, 67% were PCR positive and infected with non-archetypal/atypical strains (Parameswaran et al., 2010).

A possible explanation for this diversity can be seen in data collected from macropods in Western Australia. All 16 asymptomatic marsupial macropods tested using PCR-DNA

sequencing for *T. gondii* were positive, thirteen of these macropods had parasite DNA in at least two organs and 45 distinct genotypes were detected. Furthermore, 14 of the 16 macropods were co-infected by multiple genetically distinct *T. gondii* genotypes (Parameswaran et al., 2010). If this data represents the general trend in Australian wildlife then it is highly likely that feral cats, which hunt native wildlife, are exposed to multiple genotypes and the likelihood of sexual recombination between these strains is increased.

3. 1 *T. gondii* in Australian felines

Clinical disease has been reported in a number of Australian cats, however, the identity of genotypes infecting Australian cats is unknown (Foster et al., 1998; Lister et al., 1999; Barrs et al., 2007; Beatty and Barrs, 2003; Lindsay et al., 2010). Only one isolate from a cat from Tasmania, of unknown infection state (latent infection or clinical disease), has been genotyped (ToxoDB#10, n=1: Refer to Table 1.1 (Parameswaran et al., 2010). There is also a lack of recent information on how often owned domestic cats are exposed to *T. gondii* in Australia. The majority of serological surveys were performed in stray and feral cats over 30 years ago (Coman et al., 1981; Gregory and Munday, 1976; Jakob-Hoff and Dunsmore, 1983; Milstein and Goldsmid, 1997; Sumner and Ackland, 1999; Watson and McDonald, 1982).

More recently, 85% of feral and stray cats sampled in Tasmania were found to be seropositive, indicating that these cats are regularly exposed to the parasite (Fancourt and Jackson, 2014).

Surveys conducted to determine frequency of oocyst shedding in Australian cats have found approximately 0.1-1.2% of refuge and domestic cats were shedding in one time period, a similar rate to that reported elsewhere in the world (Herrmann et al., 2010; Palmer et al., 2008).

4. Rationale for the project or aims of this thesis

Currently, data on *T. gondii* prevalence and genotypes in Australia is sparse and knowledge about the impact of infecting genotype on disease outcome in feline toxoplasmosis is lacking. It is, therefore, important to expand the knowledge of genotypes present in definitive hosts and to determine prevalence in Australian cats.

In light of the lack of information on the of *T. gondii* infections in cats, to the aims of the research presented in this thesis are to:

1. Determine the seroprevalence of *T. gondii* in Australian domestic cats;
2. Determine potential risk factors which increase the likelihood of exposure to *T. gondii* in domestic cats;
3. Investigate the genotypes of *Toxoplasma gondii* present in Australian cats using high resolution techniques;
4. Identify the genotypes of *T. gondii* found in cats that have clinically diagnosed toxoplasmosis.

4.1 Thesis outline

Chapter two describes the materials and methods used to conduct a seroepidemiological survey to estimate the seroprevalence of *T. gondii* infections in domestic owned cats in Australia and the results of this survey. In addition to estimating seroprevalence of *T. gondii* in owned Australian cats, this chapter also evaluates lifestyle factors, which may play a role in exposure to *T. gondii* in these cats.

Chapter three describes, in detail, the materials and methods used to collect samples from cats with active and latent toxoplasmosis and the genetic analysis which typed isolates found in these cats.

Chapter four contains a manuscript which has been submitted to Veterinary Parasitology and is under review. This manuscript describes cases of feline toxoplasmosis and also the results of the genetic characterisation of isolates of *T. gondii* found in cats with active and latent infections. This chapter directly compares genotypes found in active and latent feline infections in order to determine whether the severity of *T. gondii* infections in feline hosts is dictated by infecting strain.

The final chapter concludes the thesis and discusses the main findings in accordance with the aim of the research. It also offers recommendations for further studies.

***Chapter 2:* Serological survey of *T. gondii* prevalence in owned Australian cats and risk factors for infection**

Toxoplasma gondii is a parasite of worldwide medical and veterinary importance. As the definitive host, cats are the only source of infectious oocysts which are shed in their faeces and are environmentally resilient. During primary infection, one cat can shed between three and 810 million oocysts (Dabritz and Conrad, 2010). Oocyst shedding is transient and usually ceases when a cat has mounted a sufficient immune response to the parasite, as evidenced by seroconversion (Dubey, 1995a).

In recent studies, the seroprevalence of *T. gondii* among owned domestic cats in Portugal, Tehran and the United States was between 30-40% (Haddadzadeh et al., 2006; Lopes et al., 2008; Vollaire et al., 2005).

In Australia, there is little recent information regarding *T. gondii* seroprevalence among owned domestic cats, despite an estimated pet cat population of 3.3 million (Animal Health Alliance, 2013). The last recorded survey of *T. gondii* seroprevalence in owned Australian cats was performed in Melbourne over 15 years ago. At that time, 39% of pet cats were seropositive to *T. gondii* (Sumner and Ackland, 1999). Data on the seroprevalence of *T. gondii* among stray and feral cats on mainland Australia is also not current, with most studies being performed over 25 years ago. Previously, 20%- 39% of stray and feral cats in Victoria (Coman et al., 1981), and 30% feral cats and cats in shelters in Western Australia (Jakob-Hoff and Dunsmore, 1983) were seropositive. More recently, in Tasmania, seroprevalence among feral and stray cats was found to range from, 85-96% (Fancourt and Jackson, 2014).

Access to the outdoors, consumption of raw meat and hunting have previously been identified as risk factors associated with *T. gondii* exposure in domestic cats (Must et al., 2015; Opsteegh et al., 2011). The relative impact of these factors on the prevalence of *T. gondii* in Australian cats has not been recently examined. However, in contrast to the United States, feeding of raw meat is common among pet owners and advocated over processed diets by some veterinarians (Lonsdale, 2001; Malik, 2015). Kangaroo meat is also a popular protein of choice to feed cats in Australia and 15.5 to 100% of kangaroos sampled have been found to be seropositive to or infected by *T. gondii* (Pan et al., 2012; Parameswaran et al., 2009a).

The aims of this study were to determine the seroprevalence of *T. gondii* among owned Australian cats and to determine risk factors for *T. gondii* infection.

2.1 Materials and methods

2.1.1 Sample collection

To determine *T. gondii* serological status, 1 mL of blood was collected from cats presenting to participating veterinary hospitals around Australia, with written informed consent of the owner and approval from the Animal Ethics Committee, University of Sydney April 9th 2015 (Protocol no. 2015/768). Samples were collected between 1st June 2015 and 30th September 2015.

Following centrifugation and separation of the serum, all the samples were stored at -20 °C until shipping on ice by courier.

2.1.2 Questionnaire

A questionnaire for owners of participating cats was designed to collect data for risk factor analyses. Information collected included signalment (age, sex, health status and source), environment (outdoor access, proportion of time spent outdoors, urban or rural area and toilet options) and diet (diet composition, raw meat in diet, types of raw meat fed, hunting habits, and prey types). The full questionnaire can be seen in Appendix 2.

2.1.3 ELISA serology to determine the exposure status of each cat

Samples were shipped for analysis to the Department of Clinical Sciences in the School of Veterinary Science, Colorado State University, Colorado, USA.

An enzyme-linked immunosorbent assay (ELISA) to detect toxoplasma specific immunoglobulin IgG was performed.

2.1.4 Plate preparation

The ELISAs were performed using 96 well MicroELISA plates and a previously published method (Brown et al., 2005). Briefly, to sensitise the plates, an optimal dilution of *T. gondii* RH strain tachyzoites was determined by titration. This was pipetted into wells of a microELISA plate (Immulon I; SeraCare, Marylands, USA) in in 100 µl 0.06 M carbonate buffer and the plate was incubated at 4°C overnight. Plates were sensitised in two batches. The optimal signal to noise ratio was calculated for each batch using log dilutions of known positive and negative controls. A checkerboard titration was used to determine the optimal secondary antibody concentration. When the optimal signal to noise ratio was determined for both known positives and negatives

and for the secondary antibody, plates from each batch were used in an optimised assay including 20 known positive and 20 known negative sera of varying titres to determine assay cut-off levels for positive results. All positive and negative test results were confirmed with a commercially available latex agglutination kit (TOXOTEST-MTEiken, Tanabe USA, Inc., San Diego, California, USA).

2.1.5 ELISA to determine the exposure status of each cat

Samples 1-295 were run using plates from Batch 1 and samples 295-425 were run using plates from Batch 2.

Plates and substrate (SureBlue TMB microwell peroxidase substrate; SeraCare, Marylands, USA) were removed from refrigeration (stored at 4°C) and allowed to equilibrate to room temperature.

Plates were washed three times with 200µl PBS-Tween 20 (PBS-Tw 20- Appendix 3) using a plate washer. Standardised duplicate controls of known positive IgG pooled cat sera and known IgG negative, pooled cat sera (cats that had never been exposed to the parasite) were included on each plate.

Negative and positive controls were assayed in quadruplicate and test sera were assayed in triplicate. Test samples and positive and negative controls were diluted 1:64 in PBS-Tw. 20 Negative controls and test sera dilutions comprised of 14 µl of sera and 882 µl of PBS-Tw 20. Positive controls consisted of 8 µl of pooled control sera and 504 µl of PBS-Tw 20. Diluted samples were vortexed for 30 seconds and 100 µl of each diluted sample were added to the microELISA plate. The top row was left empty to act

as blank for absorbance readings. Plates were covered and incubated at 37 °C for 30 minutes then washed with PBS Tw 20 as previously described.

	H	G	F	E	D	C	B	A	
			Substrate wells						1
		+ve control			- ve control				2
	PBS		25			26			3
	1	2	3	4	5	6	7	8	4
Samples									5
									6
	9	10	11	12	13	14	15	16	7
									8
									9
	17	18	19	20	21	22	23	24	10
									11
									12

Figure 2.1 A template for the plate setup used in this current study

2.1.6 Secondary Antibody and dilutions

Goat anti-cat *Toxoplasma* IgG (peroxidase labelled, heavy chain specific, SeraCare, Marylands, USA) was used as the secondary antibody to detect positive sera.

Secondary antibodies used in Batch 1 were diluted to a 1 in 1500 dilution and consisted of 6 μl of Goat Anti-cat toxoplasma IgG in 12 ml of PBS-Tw 20. Secondary IgG antibodies in Batch 2 were diluted 1 in 2000 and consisted of 8 μl Goat Anti-cat toxoplasma IgG in 12 ml of PBS-Tw 20. Once diluted the secondary antibody solutions were mixed and added to rows 2 to 12 in the corresponding plate. Row 1 was left empty again. Plates were incubated at 37°C for 30 minutes. After incubation, the plates were washed three times with PBS-Tw as described previously.

100 μl of the substrate (SureBlue TMB) was added to each row in the plate. Plates were incubated at room temperature for 10 minutes then 100 μl of 0.18M sulphuric acid (H_2SO_4) was added to each well to stop the initial reaction. Plates were read on a microELISA reader (Lab Systems Multiskan Ascent) using a 450nm wavelength.

2.1.7 Calculations

The mean absorbance for controls and each sample was calculated. The mean absorbance of each serum sample was compared to a standard curve developed from known negative and positive control sera and a titre was assigned. Positive cut-off absorbance values were defined as being greater than the mean plus two standard deviations of the positive results from 20 SPF cats. Positive titres in these cats were confirmed using a commercially available latex agglutination kit as per manufacturer instructions (TOXOTEST-MTEiken, Tanabe USA, Inc., San Diego, California, USA). Titres >1:64 were considered positive and titres <1:64 were considered negative. The multiplication factor for Batch 1 and 2 was 2.3.

A titre scale to determine the titre of each absorbance value on each plate was then created.

To create a titre scale, the average absorbance of the negative wells was multiplied by the multiplication factor to determine the cut off absorption for the 1:64 titre cut-off. The average absorbance for the positive control wells was calculated and placed at the corresponding titre.

A linear absorbance scale was made using the following equation:

$$\frac{\text{Average negative control absorbance X multiplication factor}}{\text{number of titres between the negative cut off and the positive titre}}$$

The value obtained from this equation was added to the 1:64 cut-off and then consecutively added thereafter to each titre bracket to give a value for each titre (an example of the calculations performed can be found in Appendix 4).

The titre of each test sample was determined by calculating the average absorbance of the four values obtained and then this value was aligned with a titre bracket calculated as above.

Statistical analysis

Data analysis was conducted on binary data: IgG > 0 (1) vs IgG = 0 (0). A logistic GLM was fitted to these binary data, with the same fixed and random effects structure as for the above model. Similarly, a backwards elimination procedure was applied for model selection. Both models were analysed using GenStat Release 17 (VSN International, Hemel Hempstead UK).

2.2 Results

Serum samples were collected from 425 cats from 18 different veterinary hospitals around Australia (Figure 2.2). Of these, 38% (n=162) had positive ELISA results for the detection of *Toxoplasma*-specific IgG antibodies. The overall prevalence in different geographical regions ranged from 16 to 48%. The highest seroprevalence was reported in the Australian Capital territory and the lowest in Western Australia (WA). The variables tested and the corresponding seroprevalence associated with them are listed in Table 2.1.

2.2.1 Statistical analysis

The REML analysis showed that the frequency of feeding raw meat to a cat was the only significant variable left after backwards elimination of variables. This showed a dose response with "no raw meat in diet" having the least predicted IgG value and "daily raw meat consumption" having the highest predictive value for a positive IgG titre. The variables that were significant in the logistic model were eliminated from the linear model due to collinearity among the variables so that their effects could not be estimated. These models are then complementary.

The general linear model found that meat related variables were a predictive value for a positive result. Cats that had raw beef (P=0.0016) or raw kangaroo (P=0.005) in their diet were significantly more likely to have a positive result than those that did not have this type of meat in their diet. Cats that had raw lamb in their diet were significantly less likely to be seropositive (P= 0.004). There was no significant difference between seropositivity rates in male and female cats. When age was treated as a continuous variable the likelihood of positive serology increased with age (P < 0.001). Cats that had

been observed by their owner to hunt rodents were significantly more likely (p=0.035) to be seropositive. Toilet options were not found to have a significant impact on the likelihood of a cat being seropositive (p=0.051) and the least significant difference test showed no significant difference between the options.

State had a significant impact on the likelihood of seropositivity. Cats which were from Western Australia were significantly less likely to be seropositive that cats from any other state studied. Cats sourced from New South Wales were significantly more likely to be seropositive than those from Victoria.
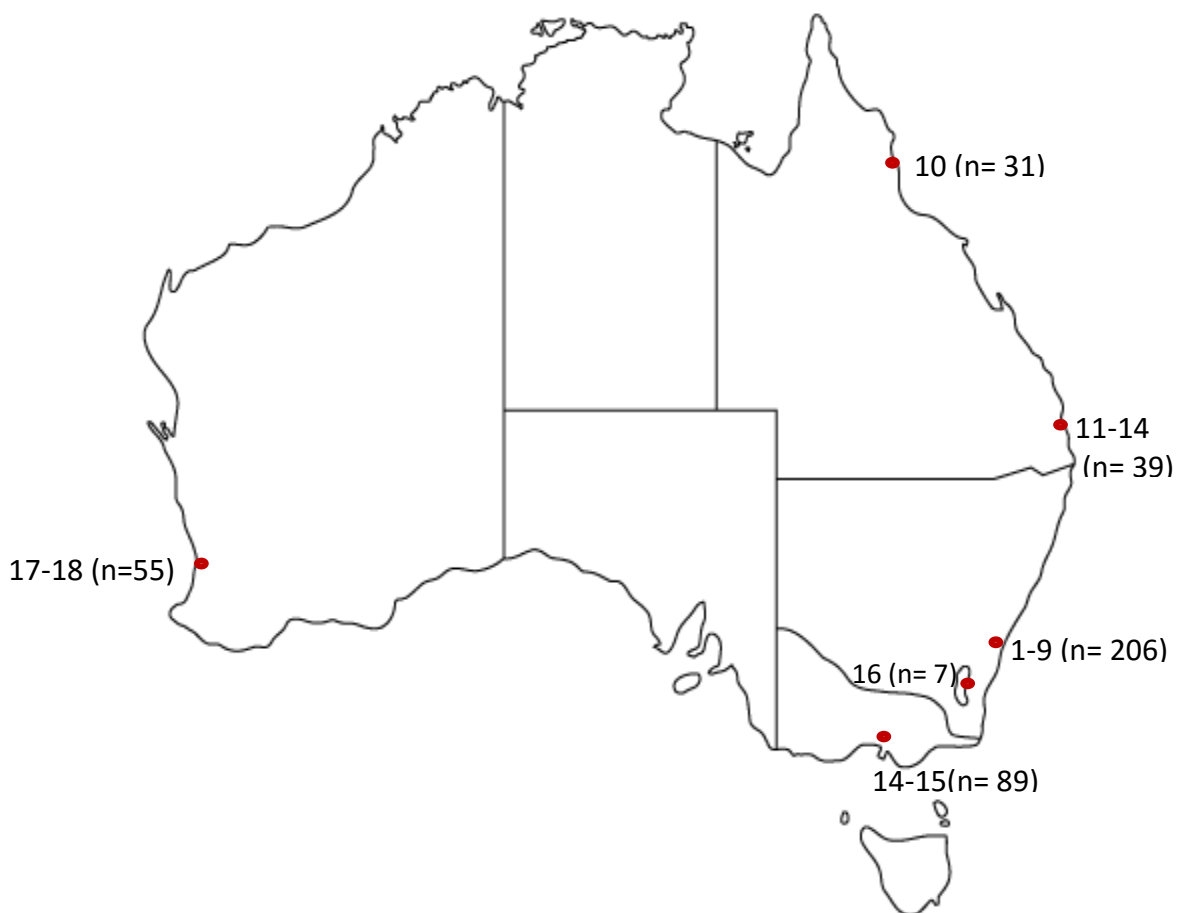
Fig 2.2 Distribution of veterinary hospitals that participated in the study and the number of sera collected from each site

Table 2.1 *Toxoplasma gondii* seroprevalence in Australian cats and lifestyle factors

measured

Variable		n	n seropositive	Seroprevalence (%)	p value
Total		425	162	38	
Age					
	<12 months	32	2	6	
	>12 months	383	153	40	
Sex					1.423
	male	208	78	38	
	Female	209	77	37	
Location					
	New South Wales	206	98	48	
	Queensland	69	25	36	
	Victoria	89	25	28	
	Australian Capital Territory	7	5	71	
	Western Australia	55	9	16	
Diet composition					0.799
	No raw meat in diet	266	101	38	
	Raw meat	248	139	56	
Type of raw meat					
	Beef*	155	69	45	0.061
	Chicken	150	68	45	0.298
	Kangaroo*	99	48	48	0.005
	Lamb	34	8	24	0.004
	Pork	15	5	33	0.454
	Other	11	3	27	0.353
	Rabbit	7	1	14	0.511
	Turkey	3	1	33	
Outdoor access					0.203
	Yes	288	110	38	
	No	137	53	39	
Toilet options					0.051
	Litter	225	82	36	
	Both	135	62	46	
	Outdoors	52	15	29	
Source					0.117
	Breeder	126	47	37	
	Friend	11	5	45	
	Online/other	93	23	25	
	Pet shop	37	18	49	
	Stray	125	50	40	

Vet	3	1	33	
Boarder				0.805
Yes	164	73	45	
No	243	85	35	
Other cats				0.124
Yes	232	95	41	
No	182	67	37	
Hunter				0.614
Yes	225	94	42	
No	176	58	33	
Prey type				
Birds	72	32	44	0.740
Lizards	111	48	43	0.395
Rodents*	75	37	49	0.035
Insects	101	43	43	0.549
Slugs/snails	5	3	60	0.398
Frogs	2	0	0	
Possums	2	2	100	0.948

Note: * signifies as statistically significant result as determined by the GLM analysis

2.3 Discussion

This study is the first to sample multiple states in Australia for feline exposure to *T. gondii* as indicated by seropositivity. It is difficult to compare the figures reported in the present study to past figures as there are vast differences in techniques used, sample sizes, and geographical location sampled and prior to the present study, location variability was unknown.   The highest prevalence in the present study was recorded in the Australian Capital Territory however due to low numbers this was not found to be statistically significant. The seroprevalence in New South Wales is similar to that found in cats previously sampled in Sydney (Watson and McDonald, 1982) and was significantly higher than the other states sampled. This could be due differences in commercial diets available, the mean age of cats sampled in the state and the proportion of cats fed raw meat or cats who had the opportunity to hunt rodents.

The seroprevalence of cats in the present study is lower than that reported in feral and stray cats in Australia, with the exception of reports from Queensland where no feral and stray cats tested seropositive (Coman et al., 1981; Cook and Pope, 1959; Fancourt and Jackson, 2014; Jakob-Hoff and Dunsmore, 1983). Overall, higher rates seroprevalence among feral cats compared to pet cats, has been reported worldwide (Al-Kappany et al., 2011; Fancourt and Jackson, 2014; Tiao et al., 2013).

The general linear model (GLM) found that inclusion of raw beef or kangaroo in a cat's diet was predictive value for a positive result. There is currently no data on the prevalence of *T. gondii* infection in beef cattle in Australia. Serological surveys conducted in Brazil and Thailand found 17.7% and 25.7% of cattle tested, respectively, had been exposed to the parasite (Santos et al., 2013; Wiengcharoen et al., 2012). Limited bioassay studies in the US, in which naïve cats were fed raw beef, did not isolate viable *T. gondii* from cattle slaughtered for consumption (Dubey et al., 2005a). As a result of these findings, beef is often described as a "low risk" meat with regards to *T. gondii* transmission to humans (Dubey et al., 2005a). However, despite this, similar to the present study, beef has been identified as a risk factor for *T. gondii* infection in US citizens (Jones et al., 2009).

Raw kangaroo meat in the diet was also a significant predictive variable for *T. gondii* exposure in cats in this study. *T. gondii* DNA was detected in one of 10 kangaroo mince samples sourced from a supermarket in Perth, however, no other studies have made an attempt to expand on this finding (Parameswaran et al., 2010). Wild macropods sampled from the Perth metropolitan area had a seroprevalence of 15.5% (Parameswaran 2009b). By contrast, *T. gondii* DNA was detected in 16 of 16 wild

macropods sampled in Western Australia, indicating that Australian macropods have variable exposure to *T. gondii* (Pan et al., 2012; Parameswaran et al., 2009a). In other areas where kangaroos have not been surveyed, grass-grazing species such as wombats in New South Wales have a *T. gondii* seroprevalence of 26.1% (Hartley and English, 2005). Further studies are warranted to determine the risk of *T. gondii* exposure after consumption of kangaroo meat in Australia.

Raw beef was the most popular type of raw meat fed to cats in this study (n=154), followed by chicken (n=150), then kangaroo (n=99). It is surprising that chicken was not identified as a significant risk factor for exposure to *T. gondii* in light of recent findings that 90% of free range chickens tested in Western Australia had serological evidence for exposure to *T. gondii* (Chumpolbanchorn et al., 2013). The reason for this could be that chicken sold for pet consumption is more commonly traditionally raised with no access to outdoor runs or exposure to oocysts in the environment. Free-range chickens with outdoor access have been shown previously to have high seroprevalence rates (Millar et al., 2012). The identification of raw beef and kangaroo as risk factors for infection in owned domestic cats indicates that regardless of whether cats have outdoor access they may still be exposed to the parasite if they are fed these types of raw meat. This finding is concerning, as these two meat types were among the most common fed to cats in this study.

Cats in this study that were observed by their owner to hunt and eat rodents were significantly more likely to be exposed to *T. gondii* (p=0.014). There is little recent data regarding infection rates in rodent species in Australia, however, in 1959, 30% of native and feral rodent species in Queensland had serological evidence for infection, indicating that over 55 years ago rodents were commonly infected with *T. gondii* (Cook and Pope,

1959). The significant difference between seroprevalence rates in cats that were seen to consume rats and those that didn't suggests that there may be high infection rates in rodents in Australia in comparison to other prey. In contrast to previous studies, there was no significant difference in seroprevalence rates between cats with or without outdoor access or between cats from single or multi-cat households. It is possible that rates of outdoor access reported by owners in this study were lower than actual outdoor exposure rates. In a recent survey of feline lifestyle factors conducted in Australia and New Zealand, 60% of cats that were reported to have no outdoor access had previously spent time outdoors (Johnston and McDonagh 2015). When this information was included, the proportion of cats that had outdoor exposure increased from 67% to 87% revealing a large difference between reported and actual outdoor exposure rates.

Similar to outdoor access, the number of "multi-cat households" may be under reported in the present study as the survey did not account for previously owned cats that had passed away or neighbourhood cats that visit. Good litter hygiene, that is, daily litter changing could also explain why cats from multi-cat households in this study were not more likely to be seropositive that cats from single cat households, however, this was not assessed in the questionnaire.

The results from the current study indicate that domestic, owned cats in Australia are commonly exposed to *T. gondii* and that raw meat, specifically beef and kangaroo and the predation of rodents are major risk factors for infection of owned Australian cats. Addressing these risk factors would decrease the likelihood of cats being exposed to *T. gondii* in Australia. For example, owners who feed their cats raw meat could freeze the meat for a minimum of 48 hours prior to feeding, which has been shown to destroy

tissue cysts (Djurkovic-Djakovic and Milenkovic, 2000; El-Nawawi et al., 2008; Gencay et al., 2013). Owners could reduce hunting opportunities by supervising or restricting outdoor access or investing in deterrents such as collar mounted pounce protectors or bells which have been shown to reduce mammal hunting (including rodents) by 55%, and mouse hunting by 63%, respectively (Calver et al., 2007; Gordon et al., 2010). Education with a focus on cat welfare would be an effective way to increase owner compliance as Australian cat owners have been shown to be more likely to implement change to cats lifestyles when welfare is advocated (Grayson et al., 2002).

Chapter 3- Methodology: Comparison of genotypes of *Toxoplasma gondii* in domestic cats from Australia with latent infection or fatal toxoplasmosis

Aims:

To determine the genotypes of *T. gondii* which are associated with clinical disease and compare these to those found in non-clinical individuals

3.1 Samples

The samples used in this study were sourced from individuals that went through the post mortem scheme at University Veterinary Teaching Hospital Sydney from March 2014-April 2015.

A 50g sample of muscle (usually gracillis), brain (from the occipital lobe), spleen, liver, kidney, lymph nodes, pancreas and lung were routinely collected from each individual. To prevent contamination during sampling, new gloves, scalpel blades and sterile tubes were used for each sample and instruments were wiped down with ethanol between each sample. If gross pathology was present- or in the case of previously unwell individuals, clinical signs suggested potential toxoplasmosis- then a 50 g sample of the associated organ was collected. These samples were stored at -80°C until analysed.

When possible blood was taken from individuals and a *Toxoplasma* IFA was performed to determine whether they had been exposed to the parasite.

As part of the routine post mortem process, tissues exhibiting pathology or tissues which potentially reflected ante-mortem signs were submitted for histology. If toxoplasmosis was suspected due to the presence of trophozoites and protozoal cysts *T. gondii* specific immunohistochemistry was performed on these tissues.

IHC staining for *T. gondii* antigen was performed using *T. gondii* epitome specific rabbit polyclonal antibody 1:1000 dilution according to previously reported techniques (RB-282-A; Thermo Fischer Scientific, Fremont, CA, US) (Lindsay et al., 2010)

3.2 DNA extraction

In order to minimise contamination between samples only one individual was analysed at a time.

DNA was extracted from each sample using DNeasy kit and a QIACUBE according to manufacturer instructions (DNeasy Blood and Tissue kit and automated processor QIACUBE; QIAGEN Germantown, MD). Three 25 mg samples of brain, muscle, lung and diseased tissue (if available and/or relevant) from each individual were weighed out and placed into sterile tubes. To reduce contamination, the extraction area was sprayed down with ethanol. A new scalpel blade was used for each 25 mg tissue and the petri dishes which were used to weigh them were changed between each organ. 180 µl of ATL and 20 µl of Proteinase K were added to each tube and also to an empty tube for a negative control. The solutions were mixed by vortexing and incubated at 56°C until the tissue was completely lysed (usually 5-6 hours). Once lysis was complete samples were transferred to a QIACUBE and the rest of the extraction process was carried out according to the DNeasy Animal Blood and Tissue program. DNA was eluted into 200 µl of AE buffer by the QIACUBE and the amount of DNA in each elution was quantified using a spectrophotometer.

To determine whether DNA extraction was successful spectrophotometry was performed using a (Nanodrop 2000, Thermo Fisher Scientific, Australia). One microliter of elution buffer (AE) was used as a blank and one µl of DNA elution was loaded onto a

cuvette to measure the quantity of DNA in each extraction. The concentration (ng/μl) was recorded as well as the purity of the DNA sample.

3.3 Polymerase Chain reaction

- Detection of housekeeping gene (GAPdH) to confirm that the correct DNA was extracted

To confirm that the correct DNA had been extracted a PCR which amplified the feline housekeeping gene GAPDH was used. Each 50 μl reaction contained 5 μl of 10x PCR buffer, 10mM dNTP, 75 mM $MgCl_2$, 10 pmol of each primer, 0.2 U of Taq QIAGEN DNA Polymerase (QIAGEN Germantown, MD) and 1 μl of template.

The products were visualised on a 1.5% agarose gel

-Detection of T. gondii in each sample

To detect *T. gondii* DNA in each non-clinical sample a nested PCR was used to amplify the 530bp B1 locus. Each 25 μl PCR reaction mixture contained 2.5 μl of PCR buffer, 15mM MgCl2, 5 pmol of each primer and 0.2 U of Taq QIAGEN DNA Polymerase (QIAGEN Germantown, MD) and 1 μl of DNA template. Cycle conditions for the two rounds of PCR were: initial 95°C for 4 min, followed by 35 cycles of 94°C for 30 s, 60°C for 1 min and 72°C for 2 minutes. PCR products from the first round were diluted by 1:20 before proceeding to the second round. This diagnostic test was performed on each of the four samples taken from each tissue four times (Appendix 1). The products of the PCR were visualised on a 1.5% agarose gel. The tissue sample which had the most consistent positive result was used in the next step.

3.4 Multiplex PCR

Strain typing was performed using a multiplex PCR which amplified the genetic markers SAG1, 5'SAG2, 3'SAG2, altSAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1, alt.SAG2, and Apico and was followed by a nested PCR as described previously (Su et al., 2006, 2010).

Multiplex PCR was performed on the samples that were consistently positive for *T. gondii* DNA according to the B1 diagnostic PCR. Each 25 µl reaction contained 12.5 µl of MyTaq Red Mix (Bioline, Australia), 0.38µl of each primer and 2 µl of template DNA. Cycle conditions for the multiplex were initial 95°C for 4 min, followed by 30 cycles of 94°C for 30 s, 55°C for 1 min and 72°C for 2 min.

Following the multiplex PCR, the products were diluted 1:1 using UltraPure Distilled Water (Invitrogen, California, United States) and a nested PCR was performed. Each 25 µl contained 12.5 µl MyTaq Red Mix (Bioline, Australia), 0.30 mM each of internal forward and reverse primer and 1.5 µl of multiplex PCR diluted product. The reaction mixture was then treated at 95°C for 4 min, followed by 35 cycles of 94°C for 30 s, 60°C for 1 min and 72°C for 1.5 min.

Once the amplification process was complete, PCR products were bi-directionally sequenced using amplification primers (Macrogen Inc., Seoul, South Korea). The results were analysed on CLC bio (version 7).

3.5 Sequence analysis

Chromatograms were visually verified using CLC Main Workbench Version 7.0.2 (QIAGEN, CLC bio, Aarhus, Denmark). Virtual RFLP was performed using NEB-Cutter[1]and results were compared to the same reference sequences (Gajria et al., 2008).

Double peaks were detected using the "secondary peak calling" function on CLC Main Workbench 7.0.2. A cut-off of 20% of the maximum peak height was chosen for both forward and reverse sequences prior to assembly to a reference sequence.

 Single nucleotide polymorphisms (SNPs) at the multiple copy B1 locus were identified by detection of double peaks at a single nucleotide position and then compared to the reference sequence (GenBank accession no. AF179871). Sequences were deposited in ToxoDB (EuroPathDB; http://toxodb.org/toxo/) and Genbank (NCBI, Genbank accession numbers KT881313 - KT881388).

When data from all 13 loci were available the samples were given a ToxoDB number (http://toxodb.org/toxo/). If data from all loci were not available a ToxoDB number or selection of numbers were given based on the data available.

The following measures were taken to prevent external and cross contamination:

1. **Sample collection**

At the time of sample collection, gloves were worn and the animal's fur was wet to prevent contamination from the fur. Gloves and scalpel blades were changed between

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[1] New England Biolabs Inc. See: http://tools.neb.com/NEBcutter2/index.php

each organ and the forceps used to hold tissue were washed with 70% Ethanol. All instruments and garments were changed if more than one animal was being sampled at a time.

The samples were stored in separate sterile containers stored at -80°C in a freezer which was located in an area away from PCR products.

2. DNA isolation

To prevent cross contamination, DNA was extracted from one animal at a time. Gloves and scalpel blades were changed between each individual 25 mg sample and a separate petri dish was used each time to weigh out the sample. The entire extraction process was carried out in an area away from PCR processes and gel stations. During the addition of Buffer ATL and Proteinase K, a new tip was used for each tube and the pipette was washed down with ethanol prior to use. This pipette was also one which had never been in contact with PCR products. Tubes were spun down prior to opening to prevent aerosolised contamination.

To confirm that no contamination had occurred during this process, a negative control which had Buffer ATL and Proteinase K added was also put through the program in the QIACUBE.

3. PCR preparation

To prevent the contamination of primers and the master mix, each reagent was aliquoted into smaller volumes (200-500 µl depending on the reagent) and spare aliquots were stored in a separate area.

All procedures prior to DNA addition were carried out in a sterile fume hood which was decontaminated for 20 minutes prior to work using UV light.

Pipettes used for PCR preparations were only ever used within the fume hood and, therefore never come into contact with any DNA.

New gloves were used for each preparation and UltraPure Distilled Water (Invitrogen, California, USA) was used in each reaction and dilution. Tubes were kept closed when nothing was being added to them.

4. DNA addition/ dilution for PCR

To prevent contamination of the workspace, tubes were spun down prior to opening and the surface was wiped down with ethanol and exposed to UV light prior to work. The pipette tip used was changed between each addition of DNA and pipettes were exposed to UV light for 20 minutes and wiped down with ethanol after each addition. Only one animal was analysed using the multiplex and nested protocol at a time and a new diagnostic PCR was left until these analyses were complete.

Once the DNA had been added to each tube the tube remained sealed until gel analysis was required.

5.   Diagnostic gel

Gel analysis was carried out in a space separate to PCR thermocyclers. Gel specific pippetes were used and the tips were changed between each tube. Tubes were kept closed at all times unless they were being used.

Gels were only run if no further PCRs were to be carried out that day due to the significant contamination risk that they pose.

Chapter 4: Comparison of genotypes of Toxoplasma gondii in domestic cats from Australia with latent infection or fatal toxoplasmosis

Anthea Brennan, Shannon L. Donahoe, Julia A. Beatty, Katherine Belov, Scott Lindsay, Katherine A. Briscoe[1], Jan Šlapeta, Vanessa R. Barrs*

Faculty of Veterinary Science, University of Sydney, NSW 2006, Australia

[1] Present address: Animal Referral Hospital 250 Parramatta Rd, Homebush West NSW 2140

*Author for correspondence: Tel: + 61 2 9351 3437, Fax: +61 2 9351 4261,

vanessa.barrs@sydney.edu.au

 Abstract

Associations between genotype and virulence of *Toxoplasma gondii* have been detected in experimental infections in mice. Little is known about whether genotype is associated with severity of disease in naturally occurring toxoplasmosis in domestic cats, or about the population structure of *T. gondii* in Australia. The aim of this study was to compare genotypes of *T. gondii* in latently infected cats with those from cats with fatal toxoplasmosis.

A diagnostic PCR targeting the B1 gene was performed to detect *T. gondii* DNA and was positive in 11 (65%) cats, of which four had severe active toxoplasmosis and seven had latent infection, as confirmed by histologic findings and immunohistochemistry.

Parasite DNA concentrations were adequate for genotype determination in all four cats with toxoplasmosis and in three of the latently infected cats using multi-locus PCR-RFLP including 11 loci (*SAG1, 5'-SAG2* and *3'-SAG2*, *alt.SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1* and *Apico*) and direct sequencing of a multi-copy B1 marker. A non-archetypal type II *T. gondii* strain (ToxoDB genotype #3) was detected in all seven cats. In the other latently infected cat, due to low DNA concentration, partial genotyping using six loci was performed and it identified a Type II-like strain. Novel B1 gene polymorphisms were present in two strains and B1 gene polymorphisms unique to Australia were identified in another. One cat was co-infected with two or more strains. In natural feline infections in Australia, the genotype of *T. gondii* does not appear to be a major determinant of disease progression.

Keywords: Feline; Genotype; *Toxoplasma gondii*; Toxoplasmosis; Australia

4.1. Introduction

Toxoplasma gondii infections in owned domestic cats are usually subclinical and severe toxoplasmosis is uncommon, as reflected by high serological exposure rates of approximately 30% among healthy pet cats (Haddadzadeh et al., 2006; Liu et al., 2014; Vollaire et al., 2005). In Finland and Denmark, 3.1% (6/193) and 3.2% (5/155), respectively of cats undergoing necropsy were diagnosed with disseminated toxoplasmosis (Henriksen et al., 1994; Jokelainen et al., 2012) on the basis of histologically detectable disease. Congenitally infected kittens and immunocompromised cats are most susceptible to fatal toxoplasmosis (Barrs et al., 2007; Davidson et al., 1993b; Dubey, 1982). Rarely, healthy cats with no detectable immune deficits or comorbidities develop severe clinical toxoplasmosis. Predisposing factors in these cases are not well understood.

The severity of clinical disease in an infected host can be influenced by *T. gondii* genotype, however, little is known about whether specific genotypes are over-represented in cats with naturally occurring clinical disease (Su et al., 2002; Carme et al, 2002; Miller et al., 2004). Experimental infections in mice have shown differences in virulence between clonal types of *T. gondii.* Type I infections are fatal while infections caused by Types II and III are less severe (Howe et al., 1996; Sibley and Boothroyd, 1992; Su et al., 2002). Microsatellite DNA analysis, multilocus PCR-RFLP genotyping and genome analysis have demonstrated high genetic diversity of *T. gondii* with striking geographical hotspots of diversity such as South America (Shwab et al., 2014). Healthy cats have been found to carry a number of genotypes of *T. gondii* with regional specific distribution: a non-clonal genotype termed Chinese 1 (ToxoDB PCR-RFLP genotype #9)

was most prevalent in the tissues of stray cats in China (28/35 cats sampled) (Qian et al., 2012; Tian et al., 2014; Yang et al., 2015) while in European stray and pet cats, clonal Type II (ToxoDB genotype #1) was most common (82%, 124/151 cats sampled) (Can et al., 2014; Montoya et al., 2008).

The aim of this study was to compare the genotypes of *T. gondii* in latently infected cats with those from cats with fatal toxoplasmosis. We applied multi-locus PCR-RFLP genotyping based on a previously established panel of 11 single locus markers (Su et al., 2010) and the multi-copy *B1* marker (Grigg and Boothroyd, 2001). We utilised recent cases of clinical and latent infections of *Toxoplasma gondii* in Australian cats undergoing a post-mortem examination and histopathological investigation.

2.Materials and methods

2.1 Sample collection

Samples were sourced prospectively from cats undergoing post-mortem examination at the University Veterinary Teaching Hospital Sydney (UVTHS) (n=28) from March 2014 to April 2015, with owner consent and approval from the University of Sydney Animal Ethics Committee (N00/7-2013/3/6029). Archived frozen tissues from a cat euthanased due to systemic toxoplasmosis in 2011 were also included. Multiple tissue samples including appendicular skeletal muscle, forebrain, liver, and lung were collected in 10% neutral buffered formalin for histopathological examination and also frozen at -80 °C for subsequent DNA extraction. Serology to detect *T. gondii* specific IgG was performed after collection of heart blood from cats of unknown *T. gondii* serostatus

(IDEXX NSW, Australia). Histological examination of formalin fixed paraffin embedded tissue specimens was performed after routine haemotoxylin and eosin (H&E) staining. Immunohistochemical (IHC) staining for *T. gondii* antigen was performed using *T. gondii epitope-specific* rabbit polyclonal antibody at 1:1000 dilution (RB-282-A; Thermo Fischer Scientific, Fremont, CA, US) (Lindsay et al., 2010). Data was extracted from the medical records of each patient including signalment, medical history and serological test results for feline immunodeficiency virus (FIV) and feline leukaemia virus (FeLV) (IDEXX SNAP Combo) (Table 4.2).

2.2 Definitions

For each case, *T. gondii* infection was classified as latent or active (clinical toxoplasmosis). Active infection was confirmed if protozoal cysts and/or tachyzoites and associated inflammation were identified on histological examination of one or more tissues and if parasite identity was confirmed as *T. gondii* by positive IHC labelling. Latent infection was defined as a positive anti-*T. gondii IgG* titre and the absence of inflammation-associated protozoal cysts or zoites on histopathological and immunohistochemical examination of multiple tissues.

2.3 DNA extraction

DNA was extracted from muscle, lung, liver and brain tissues (DNeasy Blood and Tissue kit and automated processor QIACUBE; QIAGEN Germantown, MD). To increase the likelihood of *T. gondii* detection, DNA was extracted from three tissue aliquots (25 mg) of each tissue type. All reactions included two negative controls: molecular grade water

(Life Technologies, Scoresby, VIC, Australia) (25 µl) and a DNA negative extraction control. DNA from each tissue sample was quantified and checked for purity using a spectrophotometer (Nanodrop 2000, Thermo Fisher Scientific, Australia). For each tissue, the presence of amplifiable feline genomic DNA was confirmed using a GAPDH PCR (Beatty et al., 2014).

2.4 B1 Diagnostic PCR and genotyping

For detection of *T. gondii* DNA, a diagnostic PCR to amplify the 530 bp B1 locus was performed on the three DNA samples extracted from each tissue type (liver, lung, muscle and brain,) as described previously (Parameswaran et al., 2010). In order to minimise the potential for cross-contamination, genotyping was performed on only one case at a time. The genotype of *T. gondii* DNA detected in the diagnostic *B1* PCR was determined using a multi-locus nested PCR to amplify the genetic markers *B1, SAG1, 5'SAG2, 3'SAG2, altSAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1, alt.SAG2,* and *Apico* (Grigg and Boothroyd, 2001; Su et al., 2010). PCR products were sequenced bi-directionally using amplification primers (Macrogen Inc., Seoul, South Korea). Forward and reverse sequences were aligned to reference clonal Type strains I (GT1; ToxoDB genotype #10), II (Me-49; ToxoDB genotype #1) and III (VEG; ToxoDB genotype #2) (Gajria et al., 2008). Chromatograms were visually verified using CLC Main Workbench Version 7.0.2 (QIAGEN, CLC bio, Aarhus, Denmark). Virtual RFLP was performed using NEB-Cutter and results were compared to the same reference sequences (Gajria et al., 2008). Dinucleotide peaks were detected using the "secondary peak calling" function on CLC Main Workbench 7.0. 2 with the default cut-off of 20% of the maximum peak height for both forward and reverse sequences prior to assembly to a reference sequence.

Single nucleotide polymorphisms (SNPs) at the *B1* locus were identified by detection of dinucleotide peaks at a single nucleotide position and then compared to the reference sequence (GenBank accession no. AF179871). Sequences were deposited in ToxoDB (EuroPathDB; http://toxodb.org/toxo/) and Genbank (NCBI, Genbank accession numbers KT881313 - KT881388).

3. Results

3.1 Cats with *T. gondii* infection

Tissue samples were collected at post-mortem from 28 cats, especially targeting cats for which *T. gondii* serostatus was known or where toxoplasmosis had been diagnosed antemortem. Seventeen cats were seropositive for *T. gondii*. In three cats with active toxoplasmosis, only liver or lung or brain was available for genotyping.

Table 4.1 B1 PCR results for each tissue tested in triplicate and visualised on a 1.5% agarose gel.

Cat	Active/Latent	Muscle	Lung	Liver	Brain
1	Latent	---/+-+/+++	0	0	+--/-+-/+---
2	Latent	---/---/--+	-+-/+--/--+	0	---/+--/---
3	Latent	0	0	++-/+++/---	0
4	Latent	+++/+++/---	+++/+++/+++	++-/+-+/+++	-++/+++/+--
5	Active	n/a			+-+/+++/+-+
6	Active	n/a		+++/+++/+++	n/a
7	Active	+++/+++/+++	+++/+++/+++	+++/+++/+++	+++/+++/+++
8	Active	n/a	+++/+++/+++	n/a	
9	Latent	0	0	0	-+-/---/---
10	Latent	0	+--/---/---	0	0
11	Latent	+--/+--/+--	0	0	0

Note: "+" indicates the presence of a band, "-" indicates the absence of a band, "0" indicates no *T. gondii* DNA found in that tissue and each triplicate is separated by a "/". Cats 1-8 were used for further genotyping however cats 9-11 had insufficient *T. gondii* DNA to perform high resolution genotyping.

Diagnostic PCR for the *T. gondii* B1 gene was positive in tissue samples from 11 of the 17 seropositive cats tested (65%) including seven cats with latent infection and four cats with active toxoplasmosis (Tables 4.1 and 4.2). Four seropositive cats with negative *B1* PCR results were excluded from further study.

Of the four cats with active infections, toxoplasmosis was diagnosed antemortem in two cats. In one of these, an FIV positive (not vaccinated for FIV) nine year old male castrated domestic shorthair cat, that had presented with a 4-day history of lethargy, anorexia and jaundice, tachzyoites were detected in cytological preparations of liver from an ultrasound-guided fine-needle aspirate (Cat 7, Tables 4.1 and 4.2). Despite treatment with clindamycin (Dalacin, Pfizer 12.5 mg/kg IV q 12 h) and pyrimethamine (Daraprim, Aspen Pharmcare 1 mg/kg PO q 12 h) the cat developed respiratory distress after two days and was euthanased. Post-mortem examination findings confirmed severe disseminated toxoplasmosis. In the other case, a four-year old male castrated Burmese cat receiving immunosuppressive therapy (prednisolone 1mg/kg q 24 h PO, Apex Laboratories; cyclosporin 6 mg/kg q 24 h PO, Atopica, Novartis) for a severe chronic inflammatory polyarthropathy, was presented for an acute onset of inappetence, lethargy and fever. Tachyzoites were detected in cytological preparations of pleural fluid and lung (Cat 8, Tables 4.1 and 4.2). Fungal hyphae were also detected in lung and pleural fluid cytology. Culture of lung yielded a phaeohyphomycosis, *Cladophialophora bantiana* and concurrent bacterial pneumonia due to *Salmonella infantis,* identified at the National Mycology Reference Laboratory Adelaide, and the Salmonella Reference Laboratory, Adelaide, respectively. The cat was euthanased. Post-mortem findings included mycotic encephalitis and pneumonia and disseminated

toxoplasmosis with involvement of lung, cerebellum, adrenal gland and mesenteric lymph nodes.

In two cats, including a seven year female neutered domestic longhair cat that presented with jaundice, active toxoplasmosis was diagnosed post-mortem,. The cat had been treated for four months with multiagent chemotherapy for hepatic lymphoma and was thought to be out of remission and euthanased. The owner consented to a limited post-mortem to obtain a liver biopsy. Histological findings confirmed recurrent hepatic lymphoma and severe toxoplasmosis (Table 4.2). In the other cat, a four year old male castrated domestic shorthair cat with a three-month history of progressive neurological findings including ataxia, circling and difficulty swallowing, neurological examination revealed the presence of multiple cranial nerve deficits. Diffuse or multifocal CNS disease was suspected, and due to disease severity and financial constraints the cat was euthanased. On post-mortem examination, the cat was found to have severe non-suppurative encephalitis associated with *T. gondii* infection and no co-morbidities. Protozoal cysts with associated inflammation and necrosis were detected in brainstem, cerebrum, cerebellum, cervical spinal cord and kidney (Cat 5, Tables 4.2 and Figure 4.1).

Table 4.2 Patient characteristics of cats seropositive for *T. gondii* with latent infection or active clinical toxoplasmosis

No.	Signalment	Immuno-histochemical labelling for *T. gondii*	Infection type	Tissue genotyped	FIV serology	FeLV serology	Immuno-suppressive medications	Diagnoses and Post-mortem findings
1	12 y MN Oriental shorthair	No *T. gondii* detected	Latent	Muscle	Negative	Negative	None	Diabetic ketoacidosis Severe chronic pancreatitis Lymphoplasmacytic enteritis Chronic rhinosinusitis
2	11 y FS DSH	No *T. gondii* detected	Latent	Lung	Negative	Negative	Multi-agent chemotherapy	Nasal and retrobulbar lymphoma, out of remission
3	6 y MN DSH	No *T. gondii* detected	Latent	Liver	Positive	Negative	None	Pancytopenia Acute myeloid leukemia
4	11 y MN DSH	No *T. gondii* detected	Latent	Liver	Negative	Negative	None	Chronic pancreatitis
5	4 y MN DSH	Positive labelling of protozoal tissue cysts in CNS and kidney	Active	Brain	Negative	Negative	None	Multiple cranial nerve deficits and ataxia. Severe necrotising non-suppurative diffuse encephalitis (brain-stem) with free and intracellular protozoal cysts. Protozoal cysts also detected in cerebrum, cerebellum, cervical spinal cord and kidney Disseminated toxoplasmosis
6	7 y FS DLH	Positive labeling of protozoal cysts and tachyzoites in liver associated with severe necrosis and inflammation	Active	Liver	Negative	Negative	Vincristine Cyclophosphamide Prednisolone	Extrahepatic portosystemic shunt Hepatic lymphoma Hepatic toxoplasmosis

7	9 y MN DSH	Positive labeling of protozoal cysts and tachyzoites in tissues stained (liver and brain).	Active	Liver	Positive	Negative	None	Multi-organ inflammation and necrosis, including liver, heart, brain, kidneys and pancreas Disseminated toxoplasmosis
8	4 y MN Burmese	Positive labeling of protozoal cysts and tachyzoites in lung, colon and adrenal gland in association with necrosis and inflammation.	Active	Lung	Negative	Negative	Prednisolone Cyclosporine	Severe inflammatory polyarthritis Mycotic pneumonia (*Cladophialophora bantiana*) Disseminated toxoplasmosis
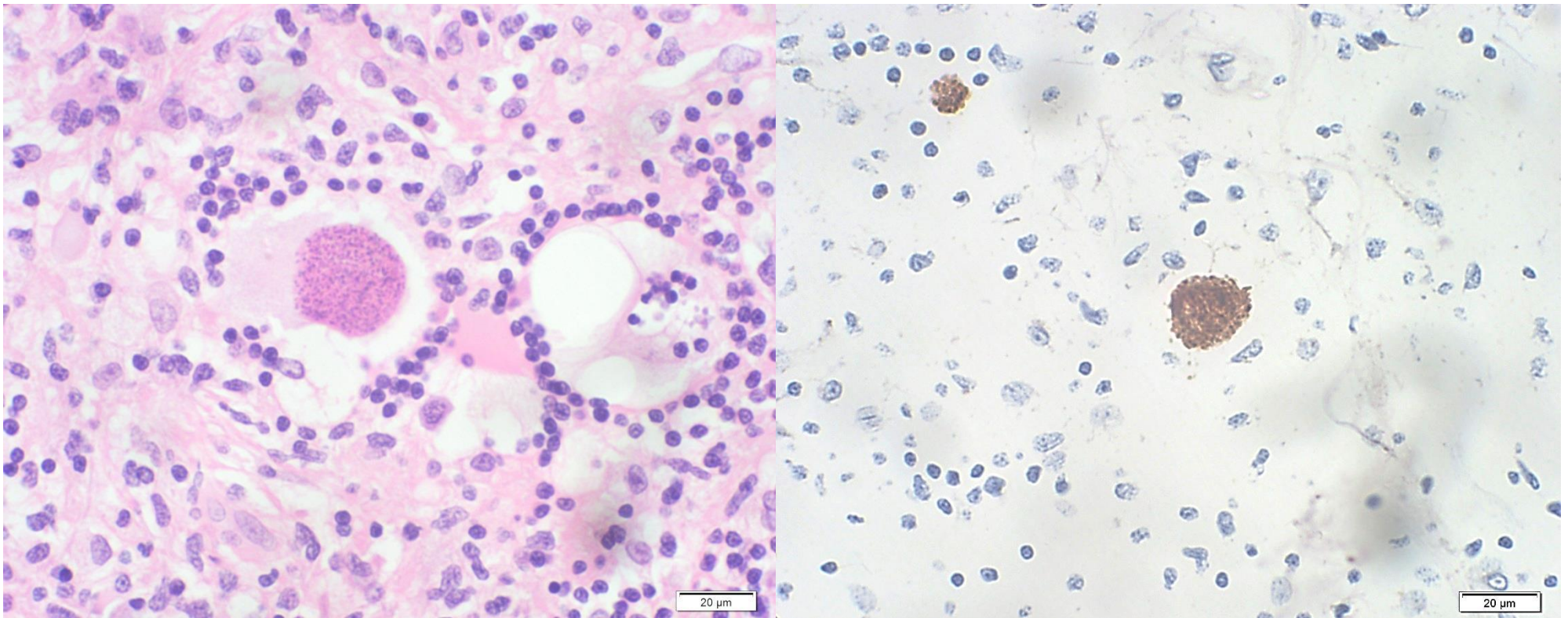
Figure 4.1 Lesions in cat 5, an active case infected with *Toxoplasma gondii* nonarchetypal type II-like strain. A) Protozoal cyst and inflammatory infiltrate associated with necrosis in the brain stem (haemotoxylin and eosin stain). B) Immunohistological staining of aggregated *T. gondii* tachyzoites in the brain stem. Scale bars A, B and C- 20μm

3.2 *T. gondii* genotypes in cats with active and latent infection

Genotyping of *T. gondii* strains by PCR-RFLP was performed in eight of the 11 cats that tested positive on the B1 diagnostic PCR. *T. gondii* DNA concentrations in tissues from the four remaining cats were inadequate for PCR-RFLP despite repeated attempts. Based on serological, histopathological and IHC results, four cats were latently infected with *T. gondii* (Cats 1-4) and four had active toxoplasmosis (Table 4.2). In seven cats, Type II alleles were identified at *SAG1, 5'-SAG2, alt.SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358* and *PK1* loci and a type I allele was found at the *Apico* locus. Accordingly, these isolates were assigned the genotype ToxoDB genotype #3 (Type II variant), which differs from ToxoDB # genotype #1 (clonal Type II) only at the *Apico* locus (Table 4.3). In five cats, Type II alleles were present at the B1 locus, while in three cats non-archetypal alleles were detected (Table 4.4).

 In one cat, sequences were obtained for six loci. Due to the presence of type II alleles at 3'-5'-SAG2, alt SAG2, L358 and SAG 3 possible genotypes includeToxoDB#1, #3, #128 or #129 (Cat 4, Table 4.3).

Table 4.3 Summary of the genotypes found in the cats in the present study.

	B1	C22-8	GRA6	C29-2	L358	PK1	alt.SAG2	SAG1	3'SAG2	5'SAG2	SAG3	BTUB	Apico	Genotype
Type I (GT1*)	I	I	I	I	I	I	I	I	I	I	I	I	I	ToxoDB#10
Type II (ME49*)	II/III	II	II	II	II	II	II	II/III	II	II	II	II	II	ToxoDB#1
Type III (CTG*)	II/III	III	III	III	III	III	III	II/III	III	III	III	III	III	ToxoDB#2
1	II/III	II	II	II	II	II	II	II/III	U-1[a,c]	II	II	II	I	ToxoDB#3
2	II/III	II	II	II	II	II	II	II/III	II	II	II	II	I	ToxoDB#3
3	U-X[c]	II	II	II	II	II	II	II/III	II	II	II	II	I	ToxoDB#3
4	U-Y[c]	nd	nd	nd	U-1[c]	nd	II	nd	U-2[c]	II	II	nd	nd	ToxoDB#1
5	II/III	II	II	II	II	II	II	II/III	II	II	II	II	I	ToxoDB#3
6	U-6[b]	II	II	II	II	II	II	II/III	II	II	II	II	I	ToxoDB#3
7	II/III	II	II	II	II	II	II	II/III	II	II	II	II	I	ToxoDB#3
8	II/III	II	II	II	II	II	II	II/III	II	II	II	II	I	ToxoDB#3

Note: *Genotype determined from ToxoDB v11 sequences (Gajria et al. 2008)
a- A mixed infection was found at this allele with two or more similar type II-like strains
b- B1 gene dinucleotide calling parameters: fraction of maximum height peak = 0.2 in CLC Main Workbench v7.0.2 and visual confirmation (see text, Fig. 3)
c- novel alleles unique to this study
nd- no data

3.3 Polymorphisms and dinucleotide peaks found

Single nucleotide polymorphisms (SNPs) were identified in the single copy locus 3'SAG2 in two cats (Table 4.4). Cat 1 at nucleotide position 1222 and 1275 in muscle tissue (GenBank accession no. AF249697.1) and Cat 4 at nucleotide position 1205 in liver. Cat 4 also had a SNP in the L358 at allele at nucleotide position 199 from liver tissue (Genbank accession no. KM246835.1) (Table 4.4). Dinucleotide peaks were detected at the single copy 3'SAG2 locus at nucleotide position 1222 and 1275 in Cat 1 from the same muscle sample, consistent with the presence of more than one non-archetypal Type II strain (Table 4.4). PCR-RFLP was repeated for this locus on two subsequent muscle sections from Cat 1 and the same results were obtained for nucleotide position 1275 but not for 1222. The SNPs in Cat 1 and Cat 4 were not at restriction sites and did not change the fragment length when analysed with NEBCutter.

Table 4.4 Polymorphisms found in the B1, 3'SAG2 and L358 locus

Marker	B1						3'SAG2				L358	
Nucleotide	233	366	378	504	595	**Allele**	1205	1222	1275	**Allele**	199	**Allele**
Consensus	C	C/T	G	G/C	T		A	G	G		T	
GTI (Type I)	G	T	.	G	.	I	G	G	.	I	.	I
ME-49 (Type II)	.	.	.	.	.	II	.	.	.	II	.	II
CTG (Type III)	G	.	.	.	.	III	.	.	.	III	.	III
Cat 1	.	.	.	.	.	II/III	.	G/C	A/G	U-1[a,c]	.	II
Cat 2	.	.	.	.	.	II/III	.	.	.	II	.	II
Cat 3	.	T	.	.	.	U-X[c]	.	.	.	II	.	II
Cat 4	G	C	.	G	C	U-Y[c]	G	.	.	U-2[c]	C	U-1[c]
Cat 5	.	.	.	.	.	II/III	.	.	.	II	.	II
Cat 6	.	.	G/A[b]	.	.	U-6	.	.	.	II	.	II
Cat 7	.	.	.	.	.	II/III	.	.	.	II	.	II
Cat 8	.	.	.	.	.	II/III	.	.	.	II	.	II

Note: Sequence residues correspond to reference sequence from Genbank: AF179871, AF249697.1 and KM246835.1 respectively. Sequence residues for samples from this study can be found with the Genbank accession numbers

a -A mixed infection was found at this allele with two or more similar type II-like strains

b- B1 gene dinucleotide calling parameters: fraction of maximum height peak = 0.2 in CLC Main Workbench v7.0.2 and visual confirmation (see text, Fig. 3)

c-novel alleles unique to this study

When compared to the reference B1 sequence (Genbank accession number AF179871), SNPs were identified in Cat 6 at nucleotide position 378 and in Cat 4 at positions 233 and 595 (Table 4.4). Dinucleotide peaks were detected in the B1 locus at position 366 in all cats except Cat 3 and Cat 4, at nucleotide position 504 in all cats except Cat 4 and at nucleotide position 378 in Cat 5 (and Table 4.4).

4. Discussion

This is the first study to characterise the genotypes of *T. gondii* in Australian cats where disease status (active versus latent infection) was definitively identified. Type II variant (ToxoDB genotype #3) was identified in cats with asymptomatic latent infection and in cats that died or were euthanased due to severe active toxoplasmosis. While the findings in this study do not rule out the possibility that certain genotypes may be more virulent than others in cats, they suggest that factors that influence host susceptibility are more common determinants of clinical disease severity. Of the four cats with active clinical toxoplasmosis, three had immunosuppressive diseases or had been administered immunosuppressive medications, including one cat with lymphoma on chemotherapy, one cat treated with cyclosporine and prednisolone for an immune-mediated polyarthropathy, and one cat with FIV infection (Table 4.2). Only one cat with active toxoplasmosis had no identifiable co-morbidities. It is possible that, as in humans, variations in immune genes such as the major histocompatibility complex (MHC) could be associated with increased susceptibility to toxoplasmosis in cats. The frequency of the MHC class II human leucocyte antigen gene DQ3 was significantly higher in congenitally infected infants with hydrocephalus than in infected infants without

hydrocephalus or than in the general US population (Mack et al., 1999). Transgenic murine studies confirmed these findings, mice with the human DQ3 gene had a higher tissue cyst burden when infected orally with *T. gondii* tissue cysts than mice with the related human DQ1 gene (Mack et al., 1999).

Although experimental studies in cats have found associations between *T. gondii* genotype and disease severity, differences among studies in the inoculum dose, infecting stage (tachyzoites, oocysts or bradyzoites) and route of infection could be confounding variables. Experimental studies designed to investigate disease pathogenesis often involve parenteral inoculation of tachyzoites or oocysts and are not representative of natural infection. Correlations between virulence and genotype are likely to be most applicable in experimental studies where the route of inoculation, life-stage of the parasite and inoculum dose mimics natural infection (Lappin et al., 1996; Omata et al., 1994; Powell and Lappin, 2001).

Congenital or lactational infection of kittens by Type II strains (Maggie, Mozart, and ME-49) is consistently associated with the development of ocular lesions (chorioretinitis), of which the Mozart strain is most oculotropic (Powell et al., 2001; Powell and Lappin, 2001). Ocular lesions with few or no other clinical signs have also been induced in cats experimentally by intra-carotid inoculation with Type II strain tachyzoites (ME-49) (Davidson et al., 1993a; Davidson et al., 1993b). However, the Type II strain ME-49 has also been documented to cause occasional fatal disease in cats after oral inoculation of tissue cysts (Dubey, 1995b; Powell and Lappin, 2001). Intravenous inoculation of cats

with Type I tachyzoites (RH strain) into the jugular vein, consistently resulted in acute disseminated fatal infections (Parker et al., 1981).

The infecting strain of *T. gondii* has been genotyped in only eight other cases of clinical toxoplasmosis in cats (Dubey and Powell, 2013; Jokelainen et al., 2012; Spycher et al., 2011). In contrast to this study where ToxoDB genotype #3 was identified in all cats with active infections, this same genotype has only been detected in one other case of naturally occurring feline disease, in a cat from Switzerland (Spycher et al., 2011). In that case, similar to Cat 5 in this study, concurrent disease was not identified and immunosuppressive drugs had not been administered. Other genotypes that have been detected in cats with toxoplasmosis include Type 12 clonal (ToxoDB genotype #4, n=1) in one cat in the United States and clonal Type II (ToxoDB genotype #1) in six cats in Europe (Dubey and Powell, 2013; Jokelainen et al., 2012).The infecting strains in these cases were of a genotype endemic to the area which the cats had come from and had also been detected in healthy feline hosts without signs of toxoplasmosis (Herrmann et al., 2010) and other non-feline species (Dubey et al., 2011; Herrmann et al., 2013; Jokelainen et al., 2011).

The high prevalence of the ToxoDB#3 genotype in this study suggests that it is endemic in Australia. It has also been identified in feline hosts worldwide and was highly prevalent among latently infected feral cats (51%) in Egypt and in pet cats with primary infections shedding oocysts (79%) in Germany (Al-Kappany et al., 2010a; Herrmann et al., 2010). The ToxoDB #3 genotype is among the dominant genotypes found in Europe,

Africa and North America and is the second most frequently identified genotype of all hosts worldwide after the clonal Type II genotype (ToxoDB#1) (Shwab et al., 2014).

Few other Australian isolates of *T. gondii* have been genetically characterised using high resolution molecular techniques (Figure 4.2). In contrast to the findings here, the most common genotype identified in other Australian studies was ToxoDB#1(clonal Type II) from a seal, dog, goat, wallaby and two wombats (Al-Qassab et al., 2009; Donahoe et al., 2014; Donahoe et al., 2015; Parameswaran et al., 2010). ToxoDB#10 (clonal Type I) was also identified in a cat and a goat (Parameswaran et al., 2010). Type III strains were reported in another Australian study, however since only 3 genetic loci (B1, SAG2 and SAG3) were used to type these strains (Pan et al., 2012), it is not possible to determine whether isolates had clonal Type III alleles at all loci or were recombinant strains (Chumpolbanchorn et al., 2013).
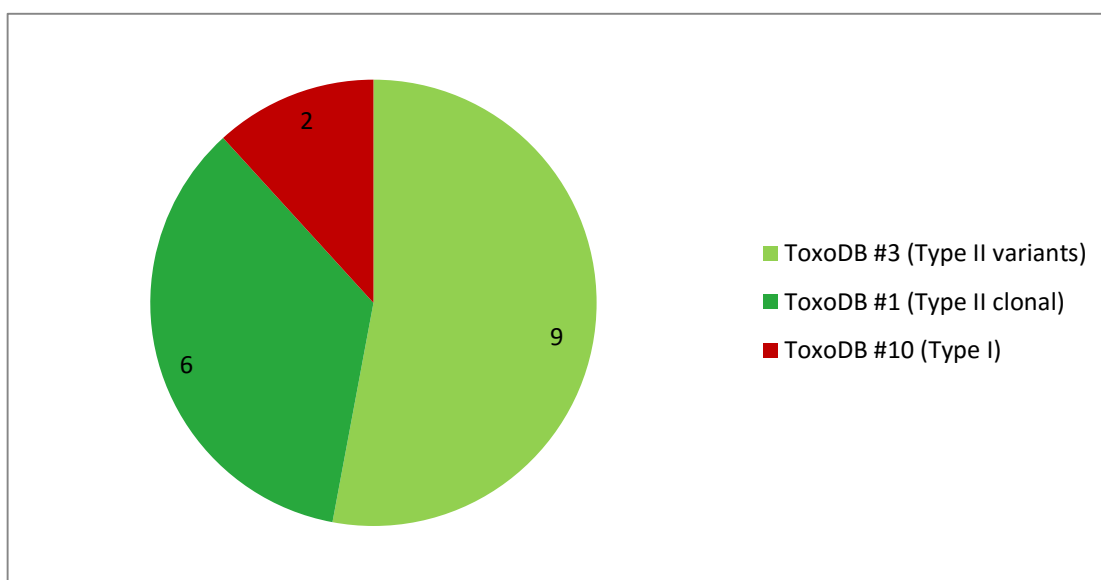
Figure 4.2 The total number of Australian isolates of *T. gondii* for which genotyping of 11 loci as presented in this study have been performed. (Al-Qassab et al., 2009; Donahoe et al., 2014; Donahoe et al., 2015; Parameswaran et al., 2010; Shwab et al., 2014) and the present study)

The ToxoDB genotype #3 has been reported in Australia previously in two intermediate hosts, a wombat and a human (non-AIDS) patient (Donahoe et al., 2015; Shwab et al., 2014; Sibley and Boothroyd, 1992). The wombat had active clinical toxoplasmosis with severe neurological signs. The true prevalence of this genotype in Australia is unknown since genetic characterisation of other Australian isolates did not include the Apico locus (Pan et al., 2012; Parameswaran et al., 2010). Thus, strains previously reported as "archetypal Type II" could be either ToxoDB#1 or ToxoDB#3 since these genotypes only differ at the Apico locus (Su et al., 2010; Su et al., 2006).

The addition of the highly polymorphic B1 locus to the commonly used 11 multi-locus DNA markers is important for genotyping of Australian isolates of *T. gondii* because non-archetypal and Type II-like alleles have been found at this locus frequently (Donahoe et al., 2014; Donahoe et al., 2015; Pan et al., 2012; Parameswaran et al., 2010). Dinucleotide peaks at position 366 and 504 in the majority of cats are characteristic for Type II/III strains whereas the SNP at position 378 found in Cat 6 and at position 233 in Cat 4 are inconsistent with Type I, II or III strains (Table 4.4). The polymorphism found at 378 np at the *B1* locus in Cat 6 represents a non-archetypal polymorphism which has been described previously in several Australian mammals (Parameswaran et al., 2010).

This study found less diversity in *T. gondii* strains in owned domestic cats than has been previously reported among infected Australian wildlife, although it is possible this is a reflection of small sample size. Only two unique alleles were identified at the *B1* locus in the cats in this study, whereas diversity in this allele was high in wildlife samples from

Western Australia (Pan et al., 2012; Parameswaran et al., 2010). Whether this reflects host differences or geographical region is not known and highlights the need for further investigation into the population structure of *T. gondii* in Australia.

Mixed infections, as detected in Cat 1 (Table 4), were also found to be common among Australian wildlife (Pan et al., 2012). Cats can become multiply infected when they ingest prey infected with more than one strain, or when they ingest multiple infected prey over a short period during oocyst shedding. In feline hosts with two (or more) strains, there is potential for sexual reproduction to occur between strains. The non-archetypal polymorphisms found in Cat 1 at *3'SAG2* represent novel polymorphisms at nucleotide positions 1222 and 1275 (Table 4.4) that are unique to this study.

Cat 4 (Tables 4.2- 4.4) could only be partially genotyped, presumably due to low *T. gondii* DNA concentrations. Although this isolate could be one of four possible ToxoDB genotypes, it is most likely genotype #3 or #1, given the genotypes identified in the other cats here and previously in Australia. Similar difficulties genotyping latently infected cats using multi-locus PCR-RFLP have been reported previously (Tian et al., 2014). A high degree of diversity was found in the loci that could be genotyped in Cat 4 including novel SNPs at the *B1, L358* and 3*'SAG2* loci (nucleotide positions 233 and 595, 199 and 1205, respectively Table 4.4). When comparing the number of samples for which *T. gondii* DNA was consistently detectable by diagnostic PCR between cats with active or latent infection, overall *T. gondii* was detected most frequently and consistently in the tissues of cats with active infection (Table 4.1). Thus multi-locus

PCR-RFLP is well suited to detect and genotype *T. gondii* in cats with active infection, but can be problematic in latent infections. It is possible that the low sensitivity of the technique used for *T. gondii* DNA extraction in the present study may create a bias towards sampling genotypes which have a high parasite burden such that the true diversity of *T. gondii* may be underestimated (Costa et al., 2011). Genotyping of other seropositive latently infected cats in this study may have been more likely using techniques to concentrate *T. gondii* DNA such as mice bioassay.

5. Conclusions

We have genetically characterised the strains of *T. gondii* in eight naturally infected cats from Sydney, Australia. There were few differences in the genotypes and diversity of infecting strains between cats with latent or active infection. The results confirm that host factors are of major importance in determining whether cats develop active toxoplasmosis.

Conflicts of interest: The authors declare that they have no conflict of interest.

Chapter 5: Further discussion and future directions

The research presented in this thesis represents an important step in our understanding of *T. gondii* in Australia and also in feline infections generally.

Chapter two describes a seroepidemiological study which measured the seroprevalence of *T. gondii* in owned domestic cats in Australia. This was the first study to evaluate cat lifestyle factors which increase the risk of exposure to *T. gondii* in Australian owned cats*.* Of the cats surveyed, 38% were seropositive, indicating that Australian cats are commonly exposed to the parasite. Raw beef and raw kangaroo in the diet and also rodent hunting were identified as risk factors for exposure. Surprisingly, outdoor access was not identified as a risk factor, however, this could be due to survey design and under reporting of outdoor access in pet cats. Future studies should consider this when designing a survey to collect data on cat's lifestyles.

Studies which aim to further investigate the seroprevalence of *T. gondii* in owned Australian cats should focus on expanding on the areas that are sampled as not all states are represented in the current study. The samples tested in Chapter two were sourced from veterinary hospitals in capital cities with the exception of 31 samples from Clinic 10 in Townsville. Cats from rural areas in Australia may have different lifestyles to urban cats and are more likely to come into contact with feral cats which are a population that have high rates of exposure to *T. gondii* (Fancourt and Jackson, 2014). As such, investigation of the seroprevalence of *T. gondii* in rural cats is warranted as is likely to differ from rates found in urban cats.

Educating owners about the risk factors which were identified in Chapter two and encouraging them to address these risk factors with a focus on cat welfare could decrease the likelihood of cats being exposed to *T. gondii* in Australia.

Currently there is no data on the prevalence of *T. gondii* in Australian cattle and very little is known about the prevalence of *T. gondii* in kangaroos from populations which are hunted for the meat industry. Given that these meats were identified as risk factors for *T. gondii* exposure in Australian cats in Chapter two, investigation into the prevalence of *T. gondii* and viability of *T. gondii cysts* (if present) in beef and kangaroo is needed to determine the exact risk not only to cats but also other species which consume raw beef and kangaroo, such as dogs and humans.

Chapter Four describes cases of feline toxoplasmosis and also the results of the genetic characterisation of isolates of *T. gondii* found in cats with active and latent infections. In this chapter direct comparisons were made between genotypes found in active and latent feline infections in order to determine whether the severity of *T. gondii* infections in feline hosts is dictated by infecting strain. The research presented in this chapter represented the first case of feline toxoplasmosis in Australian cat to have the infecting strain genotyped.

The non-archetypal type II *T. gondii* strain (ToxoDB genotype #3) which was detected in seven of the eight cats appears to be endemic to Australia and has been previously identified in Australian hosts. Naturally infected cats with active and latent infection were both infected with the same strain, indicating the infecting strain is not the major determinant of disease progression and that other host factors may be of greater importance. Future studies which assess the determinants of disease severity should

assess potential host immune factors which may have a greater impact than infecting strain.

The research presented in Chapter Four added an additional seven isolates to the total number of isolates typed using high resolution techniques in Australia (n=17- Figure 4.2). While this is a significant contribution, further investigation into genotypes which infect different host species and also felines from different areas is required to obtain a more robust picture of the population structure of *T. gondii* in Australia.

Detection and characterisation *T. gondii* isolates in latently infected hosts is limited by the sensitivity of the technique presented in Chapter Four and also the availability of multiple organs as *T. gondii* cyst distribution is not uniform or organ specific. Mouse bioassay could assist in concentrating DNA in these hosts. There is also potential for magnetic bead DNA capture to assist in *T. gondii* detection in latently infected hosts however currently there is no protocol which combines magnetic bead capture and the 11 multi-locus RFLP markers exists.

Appendices

Appendix 1. A schematic which demonstrates the aliquots taken from each tissue (pink boxes) and the triplicate diagnostic PCR which was performed on each aliquot

DNA Extraction

Tissue aliquot 1

Tissue aliquot 2

Tissue aliquot 3

B1 diagnostic
PCR

B1 PCR products
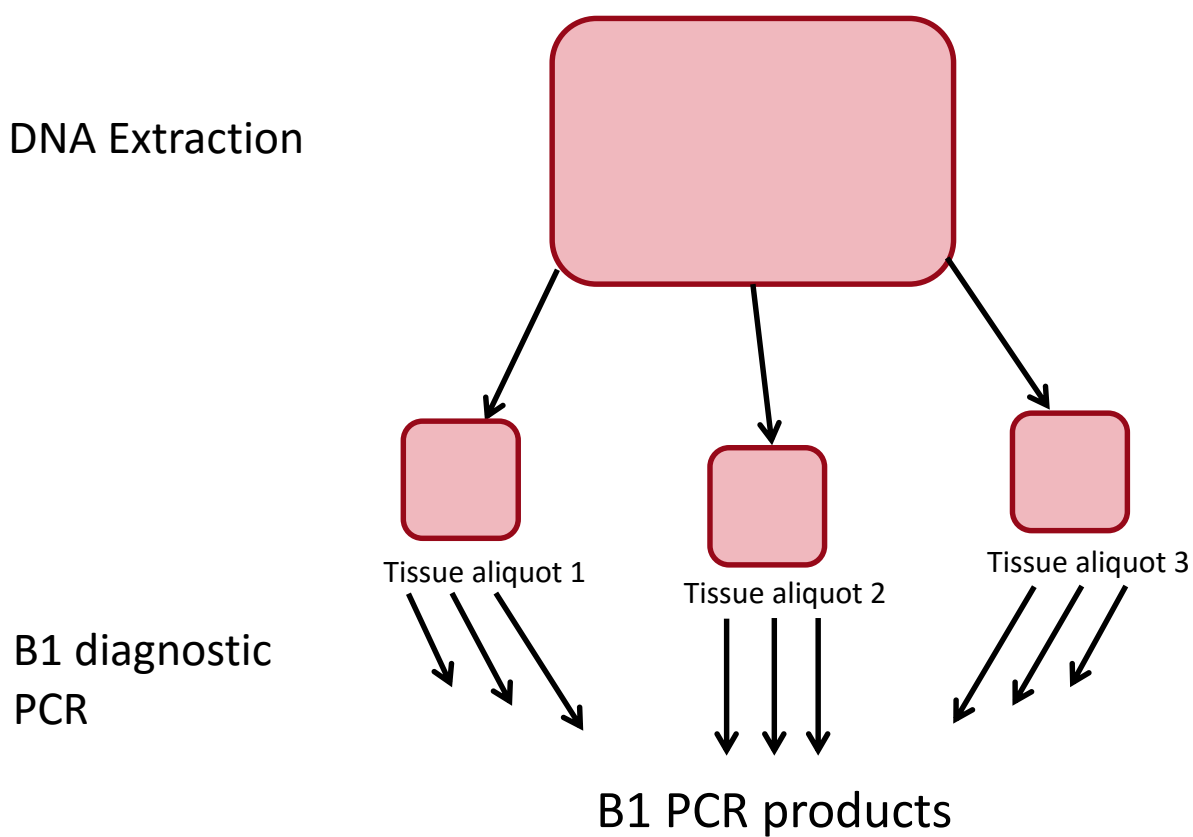
Risk Identification Survey for toxoplasmosis in cats

Please take a few minutes to fill out this survey that will help identify potential environmental risk factors for exposure to *Toxoplasma gondii* in cats. As you are aware, this is a parasite which can cause disease in cats however in most cases cats show no signs of being infected. Our group is performing research understand the risk factors that contribute to exposure to *Toxoplasma gondii* in Australian cats and also to determine what proportion of Australian cats have been exposed to this parasite.

Date: _____

Vet Clinic name and address: _____

1. Information on your cat

Age	_____
Breed	_____
Sex	☐ Male ☐ Female
Is your cat desexed?	☐ Yes ☐ No
How old was your cat when you first got it?	_____
Where did you get your cat from?	☐ Breeder ☐ Pet shop ☐ Shelter ☐ Vet ☐ Online/other
Post code where your cat lives	_____
Does your cat have any current illnesses?	☐ Yes ☐ No
List illnesses	_____

2. Environment

Does your cat have any access to outdoors?	☐ Yes ☐ No
Regarding outdoor access how would you describe it:	☐ Indoors only ☐ More indoors than outdoors ☐ 50% indoors, 50% outdoors ☐ More outdoors than indoors ☐ Outdoors only

If your cat has or had outdoor access is/was it	☐ Enclosed run, courtyard or enclosed yard			
	☐My cats outdoor access is not restricted to my property boundaries			
If your cat has/had outdoor access is/was it	☐ Daytime only	☐ Night time only	☐ Day and night time	
Area lived in:	☐ Rural	☐Semirural	☐Urban	
Is your cat most active	☐ Daytime	☐ Night-time	☐ Unknown	
Toileting habits	☐ Litter box only	☐ Both litter and outdoors	☐ Outside only	

3. Diet

The following statement best describes my cat's diet (if applicable):

☐ Totally (100%) wet food from cans or pouches
☐ Totally (100%) dry food
☐ A combination of commercial wet and dry food only
☐ A combination of raw meat and commercial food
☐ A combination of cooked meat and commercial food

Do you ever feed your cat raw meat?	☐ Yes	☐ No		
If yes, how often?	☐ Daily	☐ Weekly	☐ Monthly	☐ Occasionally

What types of raw meat have you fed your cat – tick all that apply

☐ Chicken	☐ Beef	☐ Lamb	☐ Pork	☐Rabbit
☐ Kangaroo	☐ Other			

Does your cat hunt?	☐ Yes (often)	☐ Sometimes	☐ Never
If yes, have you ever seen your cat eat the prey?	☐ Yes	☐ No	

What prey have you seen your cat catch? Tick all that apply

☐ Insects	☐ Slugs or snails	☐ Lizards	☐ Rodents	☐ Birds

Does your cat ever go to boarding facilities?	☐ Yes	☐ No
Do you have any other cats?	☐ Yes	☐ No

If yes, how many? _____

If you would like to be contacted with the results of this study please let your veterinarian know

Comments: _____

_____

_____

Please be advised that your information will be stored securely and your identity/information will be kept strictly confidential, except as required by law. Study findings may be published, but you will not be individually identifiable in these publications.

Appendix 3. Standard Recipes used in the Toxoplasma specific IgG and IgM ELISA

0.01M PBS:	85g NaCL
	21.75g $Na_2HPO_4 \bullet 7H_2O$
	3.5 g $Na_2HPO_4 \bullet 1H_2O$
	QS made up to 10 L with deionised water

PBS-Tween: 0.5 ml Tween 20 is added to 1000 ml PBS (0.01M) to make up a 0.05% solution

0.18 M H_2SO_4 (stop solution): Stock solution used is 8N H_2SO_4

44.8 ml of 36N H_2SO_4 was added to 175.2 ml deionised water to obtain 8N H_2SO_4

The working solution is 0.18M H_2SO_4

To make this 4.5 ml stock solution (8N H_2SO_4) was added to 95.5 ml deionised water

Appendix 4. Creating a titre scale

An example of the calculations necessary to create a titre scale is given below

Firstly, the 1:64 titre cut off value is determined by calculating the average negative control absorbance and multiplying it by the multiplication factor.

For example, if the average absorbance of the wells with the negative control was 0.258 and the multiplication factor for that plate was 2.3 then:

$$0.258 \times 2.3 = 0.593$$

To create the titre scale this value is subtracted from the average of the positive control wells (eg 1.730) and divided by the number of titres between 1:64 and the predetermined positive titre (eg 512)

$$\frac{\text{Average negative control absorbance X multiplication factor}}{\text{number of titres between the negative cut off and the positive titre}}$$

So in this example;

$$\frac{0.593}{3} = 0.197$$

The value obtained from this equation was added to the 1:64 cut-off and then consecutively added thereafter to each titre bracket to give a value for each titre as below

0.593 (1:64)
0.972 (1:128)
1.351 (1:256)
1.730 (1:512)
2.109 (1:1024)
2.488 (1:2048)

The titre of each suspect sample was determined by calculating the average absorbance of the four values obtained and then this value was aligned with a titre bracket calculated as above.

For example, if the average absorbance of the triplicate for a particular sample is 0.677 that sample would be given the titre 1:64, if the average absorbance was 0.332 then that sample would be considered negative and if the average absorbance was 1.82 the titre would be 512.

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