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Toxoplasma gondii in animal and human hosts

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Declaration

I declare that the work presented in this thesis is the product of my own efforts, and the practical research on which it is based is my own except where stated in the text and in the acknowledgement section. This work has not been previously submitted for any other degree or professional qualification.

Alison Burrells

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Finally, I would like to dedicate this thesis to the man who inspired me to carry out this PhD, my father. It appears that there may be room for two Dr Burrells's in the family after all.

Abstract

The protozoan parasite *Toxoplasma gondii* (*T. gondii*) is an important zoonotic pathogen, which has the ability to infect all warm blooded mammals including humans, with approximately one third of the human population predicted to be infected. Transmission of the parasite to the foetus during pregnancy can result in miscarriage, however, a child infected during pregnancy may go on to develop clinical symptoms such as retinochoroiditis (ocular toxoplasmosis), hydrocephalus or learning difficulties in later life. Post-natally acquired infection in humans is generally asymptomatic, however, individuals who are immunocompromised may develop ocular toxoplasmosis or toxoplasmic encephalitis. *T. gondii* type II is reported to be the predominant genotype in Europe and the United States, but currently very little information exists about the prevalence and genotypes present within Great Britain. Consumption of *T. gondii* tissue cysts from raw or undercooked meat is a main source of infection for humans, with infected pork being considered a high risk. Currently the "gold standard" for assessing the viability of infective *T. gondii* tissue cysts is by an *in vivo* mouse bioassay. However, more recent ethical requirements to reduce, refine or replace experimental animals raises the question as to whether molecular technologies could be incorporated into these studies to reduce mouse numbers.

The main aims of this PhD were to: (i) determine the prevalence and genotypes of *T. gondii* within different wildlife populations and humans in Great Britain; (ii) determine whether vaccination of pigs with a live attenuated strain of *T. gondii* would reduce the load of viable *T. gondii* tissue cysts within this species; (iii) study the viability and dissemination of tissue cysts from oocyst and bradyzoite infected pigs and (iv) to compare mouse bioassay with molecular detection of *T. gondii* DNA from experimentally infected pigs.

The main findings of this work show that the prevalence of *T. gondii* within carnivorous wildlife varied from 6.0% to 44.4% depending on the host species with type II being the predominant lineage identified, however, type III and two alleles for type I were also present. In humans, serological detection of the parasite from a

group of Scottish blood donors from Glasgow and Dundee (n=1403) was determined at 13.0%, molecular detection of *T. gondii* in human brains (n=151) from the Sudden Death Brain Bank show a prevalence of 17.9%. A correlation between increasing age and an increase in the detection of parasite was identified from both study groups. *T. gondii* strain genotyping using DNA extracted from human brains identified alleles for type I and III, however, no direct link between cause of death and detection of parasite DNA could be made. Live vaccination and subsequent oocyst challenge of pigs showed a significant reduction in the establishment of viable *T. gondii* tissue cysts. Mouse bioassay clearly demonstrates this result, where 100% of mice that were inoculated with homogenised tissues from vaccinated/challenged pigs survived, compared to the survival of only 51% of mice, which received homogenised tissues from non-vaccinated/oocyst challenged animals. In addition, porcine tissues from pigs challenged with either oocysts or bradyzoites did not show a significant difference in mouse survival following bioassay of these tissues. Challenge with either stage of the parasite (oocysts or bradyzoites) showed a preference to form tissue cysts in brains and highly vascular muscles (tongue, diaphragm, heart or masseter) of pigs. The findings, comparing mouse bioassay with molecular detection of parasite DNA from homogenised porcine tissue (prior to inoculation into mice), showed similar levels of detection. However, mouse bioassay was more sensitive and also provides evidence of parasite viability.

In conclusion, this research not only provides current figures for prevalence and genotypes of *T. gondii* in both wildlife and humans in Great Britain, it also successfully answers the question as to whether live vaccination of pigs with the S48 strain can reduce the tissue cyst burden. These promising results show the potential of a vaccine against *T. gondii* in producing safer pork for human consumption. Although the mouse bioassay still remains the most sensitive method for the detection and viability assessment of tissue cysts, further research should be carried out in this area, perhaps incorporating a technique such as magnetic capture qPCR, to enable an effective *in vitro* technique to be developed.

Chapter 1: Introduction

1.1 History of *Toxoplasma gondii*

The apicomplexan protozoan parasite *Toxoplasma gondii* (*T. gondii*) is one of the most successful parasites worldwide due to its ability to infect all warm blooded animals including humans. The parasite was first discovered in 1908 by Charles Nicolle and Louis Manceaux at the Pasteur Institute in Tunis, who identified the parasite in a desert rodent, named a gundi (*Ctenodactylus gundi*), and initially presumed it was a species of *Leishmania*. In the same year a Brazilian scientist, named Alfonso Splendore, discovered the parasite in a rabbit (*Oryctolagus cuniculus*) from Sao Paulo and again mistakenly identified it as *Leishmania* (Splendore, 1908). However in 1909 following experimental infection and microscopic analysis the parasite was renamed to *Toxoplasma gondii* as described by Nicolle and Manceaux (Nicolle & Manceaux, 1909) due to the bow shaped morphology of the extracellular tachyzoite stage of the parasite; Toxo is derived from Greek for bow, plasma meaning life and gondii after the original host it was identified in (the gundi).

In 1939 the first identified case of congenital toxoplasmosis was reported from a 3 day old child who had developed seizures (Wolf *et al.*, 1939). The baby only survived for one month and following post mortem cerebral calcification, retinochoroiditis, and hydrocephalus were observed. This is now known as the classical triad of symptoms of congenital toxoplasmosis (Sabin, 1942).

In the 1950's *T. gondii* parasites were discovered in enucleated eyes (Wilder, 1952), this type of ocular toxoplasmosis (see Figure 1-1) was presumed to be a consequence of congenital transmission of the parasite, however, more recent studies have also described a greater number of cases than expected of ocular toxoplasmosis due to postnatally acquired infection (Burnett *et al.*, 1998; Gilbert *et al.*, 1999; Montoya & Remington, 1996).

It was not until 1970 that cats were identified as the definitive host for the parasite, when the first description of the sexual development of *T. gondii* in the small

intestine of cats was published (Frenkel *et al.*, 1970; Hutchison *et al.*, 1970). This discovery was important, as forty years on, felids are still presumed to be the only definitive host.

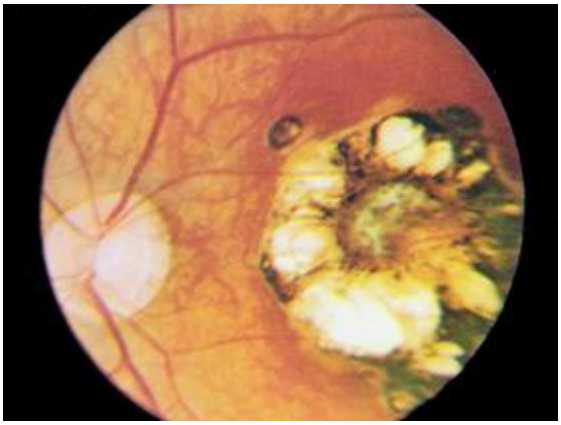

Figure 1-1: Image of lesions on the retina due to ocular toxoplasmosis. Scarring of the retina (retinochoroidal necrosis – the large white area in the right of the image) due to *T. gondii* can ultimately cause blindness as well as other visual complications such as blurred vision and sensitivity to light. Image source: http://wiki.ggc.edu/wiki/*Toxoplasma*_gondii

Another important part in the history of *Toxoplasma* was in the 1980's when AIDS patients were found to develop clinical symptoms of the parasite (Luft & Remington, 1988). *T. gondii* was identified as a major opportunistic infection for these immuno-compromised patients, where either newly acquired infection or recrudescence of latent infection would frequently cause toxoplasmic encephalitis (Luft & Remington, 1992).

The most recent developments for *T. gondii* are its possible effect on behaviour in both humans and animals. A study in 2000 showed that rats, which were infected with the parasite, were less fearful of cats (Berdoy *et al.*, 2000) and further research has also described how mice infected with the parasite are attracted to cat urine (Ingram *et al.*, 2013). These behavioural changes are presumed to increase the chance of predation by cats, and hence complete the parasite life cycle. Although the

link to *T. gondii* infection and behavioural problems in humans is not fully understood, several reports have linked infection to schizophrenia (Torrey & Yolken, 2003), increased risk taking and road traffic accidents (Flegr *et al.*, 2009) and an increased risk of suicide (Lester, 2012). Also, the emergence of genetically different strains (atypical strains) of the parasite have been linked to several fatal cases of acquired infection in immuno-competent individuals (Carme *et al.*, 2002; Carme *et al.*, 2009b), further highlighting the potential public health risk of the parasite (as further described in section 1.4.1.2).

1.2 Life cycle of the parasite

It was not until the 1970's that the life cycle of the parasite was fully understood with the discovery of *T. gondii* oocysts in cat faeces (Hutchison *et al.*, 1969; Hutchison *et al.*, 1971). This discovery was important as felids are presumed to be the only definitive host, and following primary infection can shed millions of *T. gondii* oocysts into the environment. There are three infective stages of the parasite; sporozoites (oocysts), bradyzoites (tissue cysts) and tachyzoites. These three stages also equate to the three main transmission routes which are involved in the life cycle: **1)** Horizontal transmission - ingestion of oocysts consumed from the environment; **2)** Horizontal transmission - consumption of bradyzoites (tissue cysts) from infected meat which is either raw or undercooked; **3)** Vertical transmission - *T. gondii* tachyzoites can be passed on to the unborn foetus via the placenta resulting in congenital toxoplasmosis (see Figure 1-2).

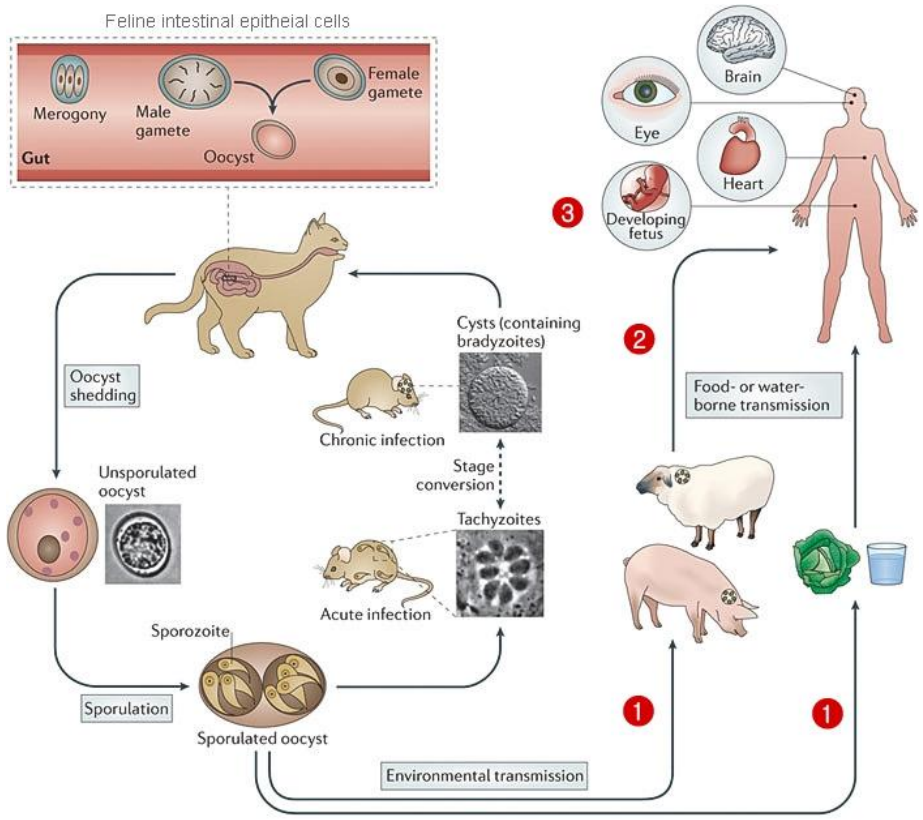

Figure 1-2: Life cycle of *T. gondii*. The three different stages of the parasite (oocysts, tissue cysts and tachyzoites) are transmitted by either: 1) - vertical transmission of oocysts from the environment; 2) vertical transmission from tissue cysts within intermediate hosts; and 3) horizontal transmission of tachyzoites from mother to foetus during pregnancy. Adapted from Hunter and Sibley. Nature Reviews Microbiology 2012 Nov; 10:766-778

Sexual replication of the parasite only occurs in the intestine of the definitive host (felids), whist asexual replication can occur within most likely all warm blooded intermediate hosts (Tenter *et al.*, 2000). Following ingestion of tissue cysts by the cat (for example by consuming an infected rodent when hunting), proteolitic enzymes in the gut and small intestine break down the cyst wall, releasing bradyzoites which then begin to replicate asexually (merogony) within the epithelial cells of the small intestine (Frenkel & Dubey, 1972). After successful replication, five morphologically distinct stages of the parasite are formed followed by gamete

formation and the sexual stage of the life cycle, which begins 2 days after ingestion of tissue cysts by the felid host. Fertilisation of the female macrogamete by the male microgamete results in the formation of a zygote which then develops into an oocyst (containing two sporoblasts). The unsporulated oocyst is released from the infected intestinal epithelial cell and excreted in the cat faeces along with thousands of other oocyts that have been produced in the same way. Once in the environment the sporoblasts within the oocyst begin to sporulate by endogeny, resulting in the development of four sporozoites per sporoblast. This development stage is vital as oocysts only become infective after sporulation in the environment, a process which takes approximately 1 to 5 days, however successful sporulation is dependent on the right environmental conditions, as both temperature and humidity are essential for this developmental stage (Ferguson *et al.*, 1979). Each sporulated oocyst measures approximately 11 to 13µm in diameter, it contains two ellipsoidal sporocysts (which measure 6x8µm), and within each of these structures four sporozoites can be found (Dubey & Frenkel, 1972). In soil sporulated oocysts are extremely resistant to environmental conditions and with a favourable mild moist climate they can remain infective for over 12 months (Frenkel *et al.*, 1975), whist in seawater they have been shown to survive for up to 54 months (Lindsay & Dubey, 2009).

Transmission of oocysts to intermediate hosts (including animals and humans) by ingestion results in another asexual cycle within its host. A combination of digestive enzymes and stomach acid in the gut rupture the oocysts, releasing sprozoites into the intestinal lumen. The parasite quickly invades and multiplies within surrounding host cells, transforming into the rapidly multiplying form of the parasite – tachyzoites (approximately 7x3µm in size). The tachyzoites replicate asexually within the host cell by a process called endodyogeny, which eventually causes the host cell to burst, and tachyzoites are released which invade other cells nearby. Tachyzoites are also disseminated throughout the host via the bloodstream, where tachyzoites can infect muscle and nervous tissue where they develop into much slower dividing bradyzoites. The bradyzoites are enveloped by a cyst wall (tissue cysts), and the cysts, of approximately 10 to 100µm, will remain within various host tissues throughout its lifetime (Dubey *et al.*, 1998a). Each tissue cyst may contain

hundreds of bradyzoites, and the life cycle of the parasite can continue following ingestion of infected tissues containing these parasitic cysts. The life cycle induced by the bradyzoite stage of the parasite replicates in a similar fashion within the host as the sporozoite stage, as once the digestive enzymes in the gut breakdown the tissue cyst wall, bradyzoites are released which invade nearby cells in the lamina propria and transform into rapidly multiplying tachyzoites.

The final route of transmission is via tachyzoites which are passed through the placenta from mother to foetus during pregnancy. In several hosts (including women, sows, ewes and goats) transmission of *T. gondii* to the foetus only occurs following primary infection during pregnancy, as infection prior to this results the host developing a protective immune response. However, in mice, congenital transmission can continue for several generations, due to either primary or persistent infection of the host (Beverley, 1959; Marshall *et al.*, 2004). Therefore rodents can also act as a reservoir for the parasite and hence an increased risk of exposure to the definitive host, the cat.

The life cycle of *T. gondii* can potentially continue indefinitely as even in the absence of the definitive host, transmission can occur between intermediate hosts from consumption of tissue cysts via carnivory and transplacentally following ingestion of tissue cysts during pregnancy (Tenter *et al.*, 2000).

1.3 Tissue cyst and bradyzoite development

Bradyzoites (brady = slow in Greek) are the slow replicating stage of the parasite and although they can develop in almost any visceral organ they are most commonly found in the brain and muscular tissues such as skeletal and cardiac muscle (Dubey, 1988). Because tissue cysts remain within an infected host throughout its lifetime, this stage of the parasite is critical for the transmission of *T. gondii* to carnivorous hosts. Tissue cysts can vary in size depending on their age, from 5µm in diameter for young cyst to 100µm for a mature cyst (see Figure 1-3).

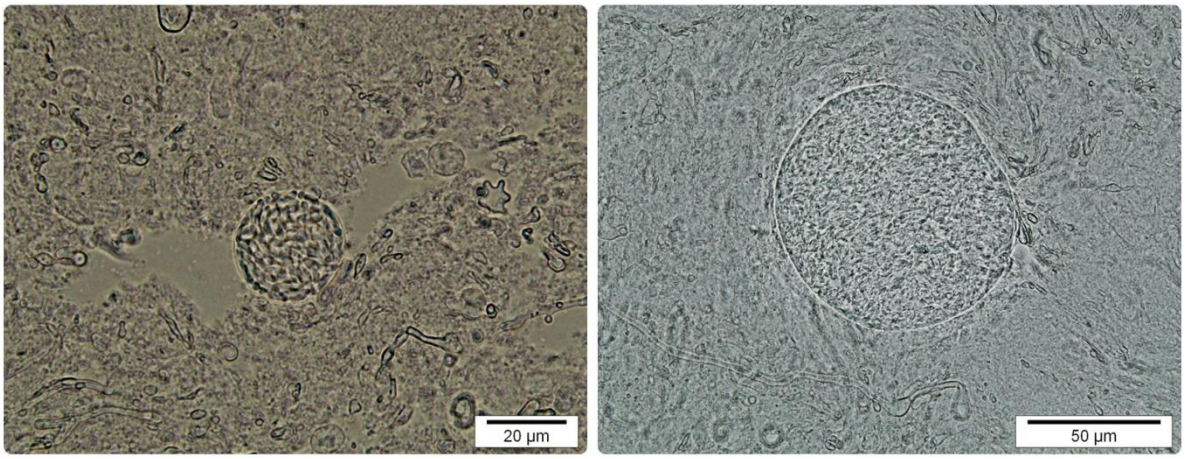

Figure 1-3: *T. gondii* tissue cysts from an infected mouse brain. Homogenised mouse brain (10µl), unstained, visualised using light microscopy. Individual bradyzoites can be seen within the cyst. (Image © A. Burrells 2013)

The larger the cyst the greater the number of bradyzoites it contains, older cysts may contain hundreds to thousands of bradyzoites whist in contrast a younger cyst may only contain a couple of bradyzoites, with each crescent shaped bradyzoite measuring approximately 7 x 1.5µm (Mehlhorn & Frenkel, 1980). After ingestion of a tissue cyst by a carnivorous host, proteolytic enzymes in the stomach and small intestine break down the cyst wall to release the bradyzoites. The morphological structure of bradyzoites means they are suitably adapted for transmission via the gut, as they are resistant to destruction by proteolytic enzymes and have been shown to be resistant to stomach acid and pepsin for up to 2 hours (Jacobs *et al.*, 1960a). Once bradyzoites are released the cell cycle continues with the formation of tachyzoites followed by the formation of new tissue cysts, as previously described (see section 1.2). Although the immune system generally controls parasite replication and maintains tissue cysts, causing no harm to the infected host, reactivation of latent infection can occur when the host's immune system is immunocompromised, with the rupture of the tissue cyst wall causing conversion of bradyzoites to tachyzoites and subsequent multiplication and establishment of the parasite within nearby cells.

1.4 The genome of *Toxoplasma gondii*

T. gondii has a 65Mb genome, comprising of 14 chromosomes which range from approximately 2Mb – 7.5Mb as shown in Figure 1-4 (Khan *et al.*, 2005b). The genome is closely related to another apicomplexan protozoan parasite, *Neospora caninum* (*N. Caninum*), and it is thought that around 28 million years ago the two parasites diverged from a common ancestor, due to the speciation of the definitive hosts (cats - *T. gondii*, dogs *N. Caninum*) (Reid *et al.*, 2012). In comparison to other apiocomplexan parasites, such as *Cyptosporidium parvum* (*C. Parvum*) and *Theileria parva* (*T. parva*), the genome of *T. gondii* is significantly larger, it contains more introns, more predicted genes and has a lower gene density (Ajioka & Soldati, 2001). One suggested reason for the difference in size compared to other apicomplexans could be due to the large number of secondary hosts which *T. gondii* is able to establish within (Roos, 2005).

In 2005, in order help facilitate forward genetic analysis, a composite genome map was derived from genetic crosses and linkage analysis of the three main archetypal *T. gondii* lineages (I, II, III) (Khan *et al.*, 2005b). The results from this work assisted researchers involved in mapping drug resistance genes and also to aided those studying the genes involved in transmission and infectivity. The genetic linkage map which was generated (see Figure 1-4) , identified 250 species specific markers, of which 12 are most commonly used for strain genotyping by PCR-RFLP (for further information see section 1.4.2). Figure 1-4 details each of these individual markers and also highlights markers which are strain specific and those which are present on all three archetypal strains.

The online genome database ToxoDB (http://toxodb.org/toxo/) provides further detailed information about the genome and the functional genomics of *T. gondii*. It also provides genome sequence information (including the facility to BLAST sequences), gene expression and proteomics data (Gajria *et al.*, 2008), which helps support research on the parasite. The database also contains detailed information for genetic maps and single nucleotide polymorphisms (SNPs), representing strains for the tree archetypal *T. gondii* lineages; GT-1 (type I strain), Me49 (type II strain) and

VEG (type III strain). Identification of SNPs between the three main lineages are used to define the different strains of the parasite by PCR-RFLP (Ajioka *et al.*, 1998; Sibley *et al.*, 1992) (see section 1.4.2).

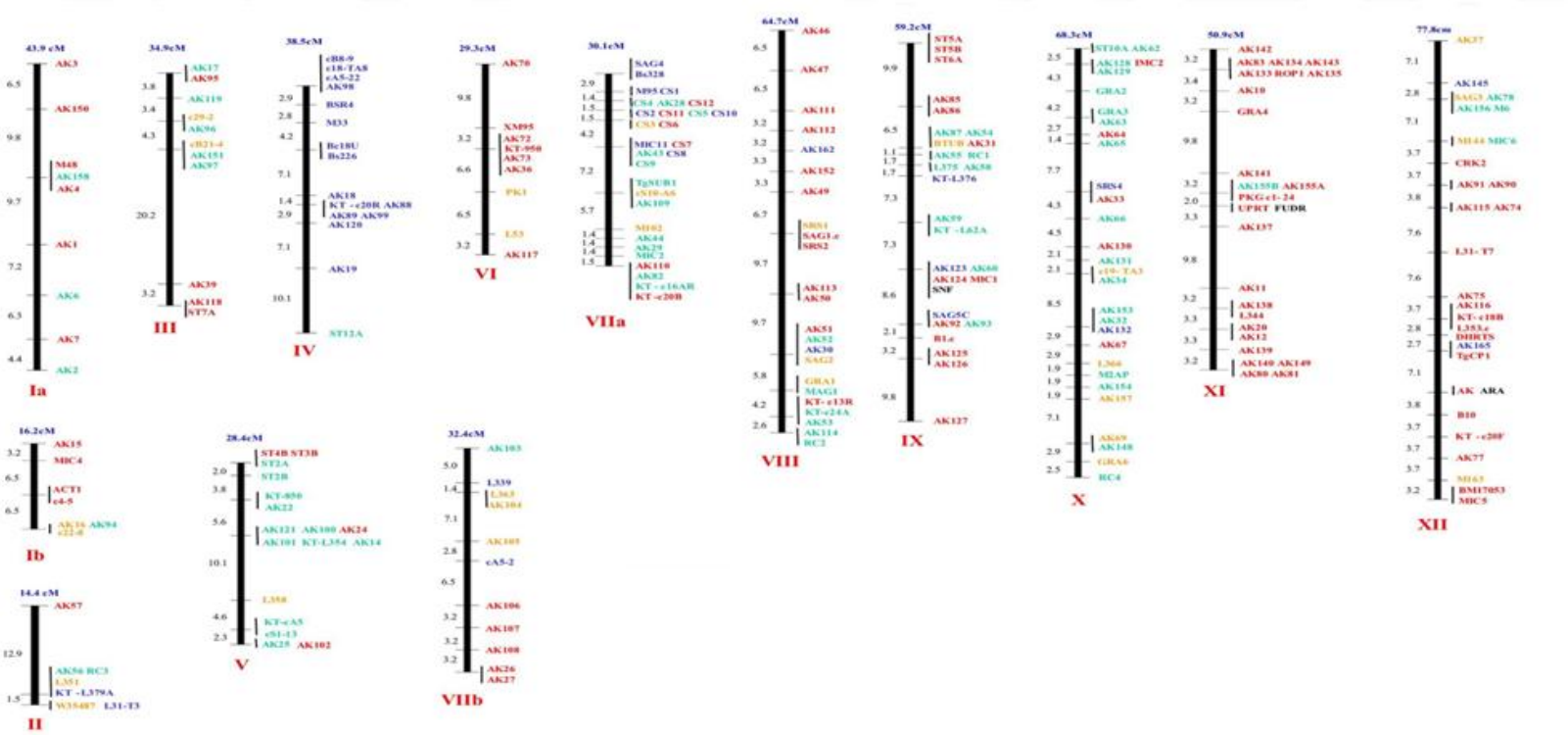

Figure 1-4: Genetic linkage map of *T. gondii*. The map shows the 14 different chromosomes with individual markers to the right of each chromosome. Adapted from Khan *et al* (2005b)

1.4.1 Genetic variation of *Toxoplasma gondii*

1.4.1.1 Predominant lineages

Research into the virulence of *T. gondii* in mice, and molecular characterisation of the parasite using multilocus restriction fragment length polymorphism (multilocus RFLP), from both animals and humans, identified the three predominant lineages of the parasite, which were designated as Type I, II or III (Howe & Sibley, 1995; Sibley & Boothroyd, 1992). From this research, and others, it has been demonstrated that Type I isolates have limited genetic diversity but are highly virulent in mice. This lineage is rarely isolated, with only 10% of strains collected in Europe and the USA being type I, however this lineage has been linked to reactivation of the parasite in immunocompromised individuals (Khan *et al.*, 2005a). Type II is found to be less virulent in mice however it is the isolate, which is predominant in human toxoplasmosis cases, and also the predominant lineage in sheep and pigs in the USA and Europe.

Type III isolates were originally deemed as non-virulent in mice, however more recent research states that they are "usually more virulent in mice than type II isolates" (Darde, 2004). Type III is relatively rare in humans but more common in animal hosts, with a study in chickens in North America exhibiting a higher prevalence of Type III compared to Type II strains (Dubey *et al.*, 2003).

Although early characterisation of the parasite studied its virulence in mice (Sibley & Boothroyd, 1992), the pathogenicity of the parasite can differ depending on its host species. The majority of infections in both animals and humans are asymptomatic, however, some host species can be vulnerable to initial infection, causing clinical symptoms and even death. Examples of this are evident in Australian marsupials (Canfield *et al.*, 1990; Dubey *et al.*, 1988), and also other animal species, such as lemurs, moved into zoos or wildlife parks (Dubey *et al.*, 2009; Hermosilla *et al.*, 2010; Juan-Salles *et al.*, 2011). This vulnerability is thought to occur due to susceptible animals being more geographically isolated from the definitive host (felids), where they have evolved in areas where, until relatively recently, cats have

been absent. Subsequently these animals could be more sensitive to *T. gondii* oocysts, with the immune system unable to deal with the infection effectively (Innes, 1997).

1.4.1.2 **Atypical lineages**

Molecular characterisation using multilocus RFLP has identified strains which have unique genotypes compared to the three predominant lineages (type I, II and III). Initially most of these atypical genotypes were isolated from exotic animals in geographically remote areas, however more recently multilocus RFLP has highlighted the presence of atypical alleles in poultry, pigs and sheep in Brazil (da Silva *et al.*, 2011; Dubey *et al.*, 2007a; Frazão-Teixeira *et al.*, 2011). Primary infection of atypical strains in humans has been linked to severe toxoplasmosis and ocular disease, with outbreaks reported in French Guiana, Brazil and Suriname (Carme *et al.*, 2009a; Demar *et al.*, 2007; Vaudaux *et al.*, 2010).

The main characteristics of these atypical strains is that they consist of novel alleles as well as shuffled combinations of alleles representing the three predominant lineages (Grigg & Suzuki, 2003).

Molecular genotyping of atypical strains found in South America, has shown a greater genetic diversity than the predominant lineages found in North American and European isolates, although with the development and further use of molecular genotyping, atypical genotypes are beginning to also be identified in North America and Europe (Dubey *et al.*, 2008a; Dubey *et al.*, 2008b; Herrmann *et al.*, 2010).

Other strains that cannot be classified into the three main predominant lineages also exist, these strains have been isolated on multiple occasions and appear to occur in specific hosts, or be linked to a specific geographical region. Type X is a genotype which has been predominantly been found in Californian sea otters (Conrad *et al.*, 2005; Sundar *et al.*, 2008). Microsatellite genotyping of French immunocompromised patients with toxoplasmosis identified new genotypes which were recovered from several patients; these were named Africa I, Africa II and

Caribbean I (Ajzenberg *et al.*, 2009). Further research in Gabon has identified the same African genotypes as well as an additional Africa III genotype (Mercier *et al.*, 2010)

Research from North America has identified a fourth clonal lineage, this lineage was initially classified as Type X and was originally isolated from Californian sea otters (Conrad *et al.*, 2005; Sundar *et al.*, 2008) (for further information see section 1.5). More recently this genotype has also been found in wild animals and in rare cases humans from North America, resulting in a defined, highly clonal, separate lineage belonging to haplogroup 12 (Khan *et al.*, 2011). At the time of writing, *T. gondii* is described to be composed of 15 different haplogroups, which defines six major clades (see Figure 1-5), this definition is based on analysis of the genetic diversity of 950 *T. gondii* isolates which were collected worldwide (Su *et al.*, 2012).

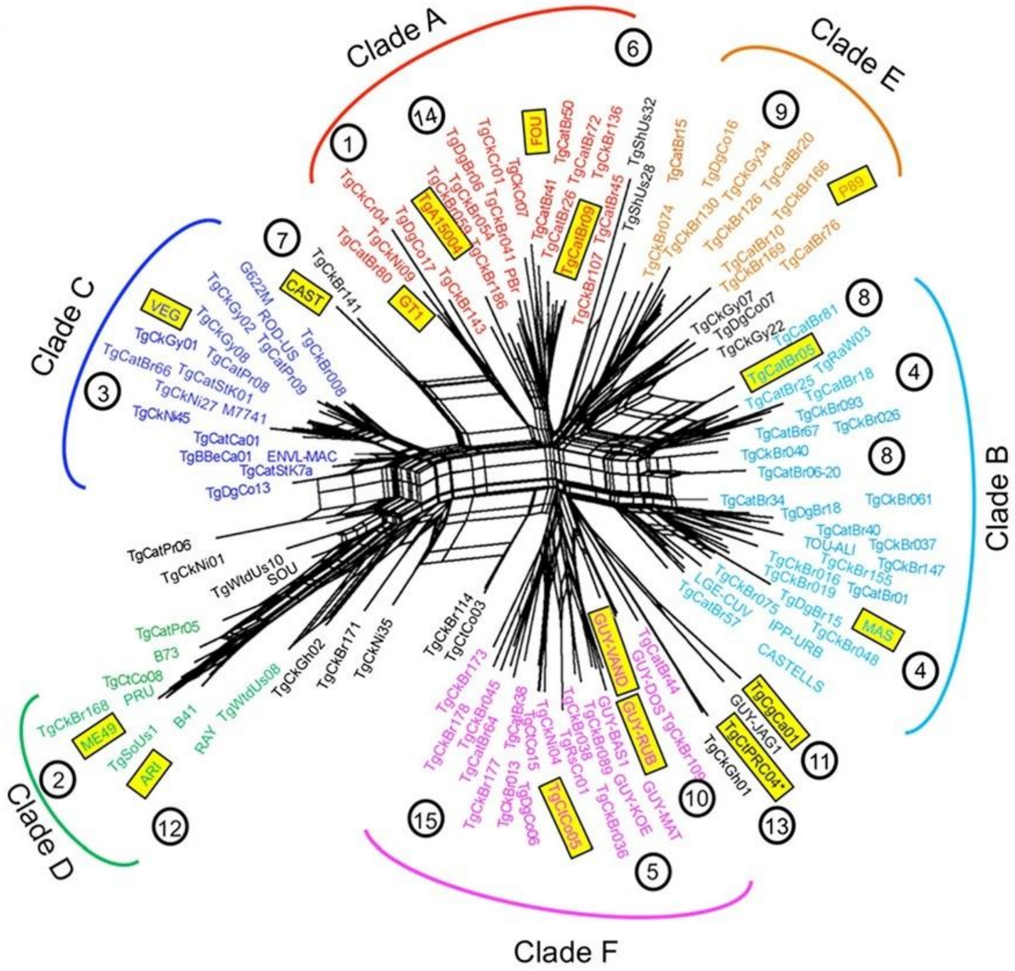

Figure 1-5: The fifteen haplogroups of *T. gondii* (1-15) defining six major clades (A-F). Strains typed in black to do not correspond with the major clades. Representative strains for each haplogroup are highlighted in yellow. Adapted from Su *et al* (2012)

Parasite strains which have undergone sexual recombination to generate mixed infections have also been identified by multilocus RFLP in both animal and human hosts (Aspinall *et al.*, 2003; Lindstrom *et al.*, 2008). Sexual recombination can only occur in the intestine of the felid host and its development must be due to infection with two strains of the parasite at the same time.

Despite the identification of atypical genotypes and mixed infections their occurrence is rare compared to the identification of predominant lineages, however with the development of new molecular tools, the sensitivity of detection has increased and therefore the possibility of discovering these unusual strains (if they are present) is becoming more likely.

1.4.2 Techniques for *T. gondii* strain genotyping

Genotyping of *T. gondii* is important for understanding the epidemiology and population genetics of the parasite, with different strains of *T. gondii* causing different clinical symptoms in both humans and animals (Grigg *et al.*, 2001; Wang *et al.*, 2013). Identification and tracking of these strains is important for understanding their virulence and their epidemiological impact. Initially, studies which focused on strain genotyping incorporated methodology based on multilocus enzyme electrophoresis (MLE) (Darde *et al.*, 1992), however, current research has focused on two typing methods; multi-locus nested PCR-restriction fragment length polymorphism (Mn-PCR-RFLP) and microsatellite sequence typing (MLST), with Mn-PCR-RFLP most commonly used in epidemiological studies. The markers currently used for Mn-PCR-RFLP only span specific regions of the *T. gondii* chromosome (see Table 1-1), therefore, it is possible that there may be an under estimation of the genetic diversity of *T. gondii* using this technique alone. Direct sequencing of PCR products, generated using either of these methods, will not only confirm the detection of SNP's, but will also identify insertions and deletions which may be present, providing additional information about genetic polymorphisms which may be present within an isolate (Sibley *et al.*, 2009). However, using sequence based technologies can be expensive and may not always be a cost effective option for large scale epidemiological studies.

1.4.2.1 **Mn-PCR-RFLP**

Mn-PCR-RFLP is based on digestion of PCR products by specific restriction enzymes which detect SNP's. The digested PCR products differ in size depending on the strain of *T. gondii* and the PCR marker used, resulting in bands of different sizes which are used to identify a particular allele for the parasite (see section 2.12.4, Figure 2-2). In order to detect not only clonal lineages (type I, II and III), but also atypical or mixed genotypes, a series of 10 to 12 markers are often used, which spans eight chromosomes and the plastid of *T. gondii* (see Table 1-1). This includes the following markers; BTUB, GRA6, SAG2 5', SAG2 3', SAG3, SAG1, altSAG2, c22-8, c29-2, L348, PK1, and Apico (Dubey *et al.*, 2014; Pena *et al.*, 2013), however, other studies which incorporate only five or six markers (BTUB, GRA6, SAG2 5', SAG2 3', SAG3 and Apico) have been reported (Boughattas *et al.*, 2010; Burrells *et al.*, 2013; Khan *et al.*, 2005a).

Table 1-1: Mn-PCR-RFLP primers and *T. gondii* chromosome number.

Mn-PCR-RFLP Marker	*T. gondii* chromosome number
SAG1, 5'SAG2, 5'SAG3, alt. SAG2	VIII
SAG3	XII
BTUB	IX
GRA6	X
c22-8	Ib
c29-2	III
L358	V
PK1	VI
Apico	Plastid

1.4.2.2 **MLST**

Genotyping by MLST is not as widely used as Mn-PCR-RFLP. The technique identifies nucleotide polymorphisms, which differ between different *T. gondii* strains, and generally incorporates five microsatellite markers (TUB2, W35, TgM-A,

B18, and B17) across five different chromosomes of the parasite (see Table 1-2) (Ajzenberg *et al.*, 2005). Primer pairs are used in a multiplex assay which incorporates fluorescent labelling of the 5' ends with fluorescein (6-carboxylfluorescein [6FAM] or hexchloro-6-carboxyfluorescein [HEX]). The PCR amplicons generated are accurately sized using an automatic sequencer and GeneScan analysis software (Fekkar *et al.*, 2011).

Table 1-2: Microsatellite markers and *T. gondii* chromosome number.

Microsatellite marker	*T. gondii* chromosome number
TUB2	IX
W35	II
TgM-A	X
B18	VII
B17	XII

1.5 *Toxoplasma gondii* infection in wildlife

All warm blooded wildlife species are intermediate hosts of *T. gondii*, which can become infected by ingestion of environmental oocysts, or in the case of omnivores and carnivores, via ingestion of both oocysts and tissues cysts. Therefore, it is important to consider this population when studying the epidemiology of the parasite. Although the prevalence in the majority of wildlife is high (Lopes *et al.*, 2011; Wendte *et al.*, 2011), clinical signs of disease are rarely reported. However, in animal species which have evolved in an environment relatively free of cats (and subsequently *T. gondii*), not only are these animals more likely to have a low parasite prevalence, but once infected they are more likely to show clinical signs of disease. Animals at risk include Australian marsupials, wildlife species imported into zoos (such as lemurs and flying foxes) and marine mammals (such as dolphins), where infection with the parasite in these species has proven to be fatal (Gonzales-Viera *et al.*, 2013; Juan-Salles *et al.*, 2011; Parameswaran *et al.*, 2010; Sangster *et al.*, 2012). Sea mammals can be particularly susceptible to *T. gondii* infection, such as sea otters, porpoises, dolphins and different species of whales and seals (Gibson *et al.*, 2011). Sea mammals can act as sentinel species, providing an indication of the levels of oocyst contamination in the marine ecosystem.

T. gondii infection in Californian Southern sea otters (*Enhydra lutris nereis*), is one of the best described and referenced examples. During 1997 – 2001 the mortality rate of this species of sea otter mysteriously began to increase, at the same time it was shown that the seroprevalence of *T. gondii* in these animals was also high, with 42% (n=116) of live sea otters being infected with the parasite and 62% (n=107) of dead otters were also found to be infected (Miller *et al.*, 2002). In one particular area of Californian coastline (Morro Bay) 87% (n=23) of sea otters tested positive for the parasite. Land-based surface run off or exposure to coastal plumes of municipal sewage was suspected to cause the high seroprevalence (and resulting mortalities) observed (Miller *et al.*, 2002). In particular, flushable cat litter, which may contain *T. gondii* oocysts, could readily enter the sewerage system and hence contaminate the marine environment. Further research into this outbreak identified two different strains of *T. gondii*; type II – which accounted for 40% if infections, and a novel

genotype, known as type X (now reclassified and referred to as haplogroup 12 - Khan *et al* (2011)), accounted for 60% of infections (Miller *et al.*, 2004). The authors also reported that sea otters from the Morro Bay area (where *T. gondii* seroprevalence was highest), were eight times more likely to be infected with type X. Several years later, further research identified wild felids from the same area of California to be infected with the type X strain, it was suspected that surface run off, which contained *T. gondii* oocysts, had resulted in contamination of one of the otter's main source of food - shellfish (Miller *et al.*, 2008).

Novel or atypical genotypes identified within different wildlife species are frequently described, ranging from a wild puma in Mexico (Dubey *et al.*, 2013a), to racoons in Wisconsin (Dubey *et al.*, 2007b), marsupials in Western Australia (Pan *et al.*, 2012) and capybaras in Brazil (Yai *et al.*, 2009). However, this diversity is not evident throughout all wildlife populations. European studies more commonly describe the type II archetypal lineage to be most prevalent, which has been identified in carnivorous wildlife from Great Britain (Burrells *et al.*, 2013), deer, fox, and mallards from France (Aubert *et al.*, 2010), arctic foxes from Norway (Prestrud *et al.*, 2008), and pigeons from Portugal (Waap *et al.*, 2008).

Despite type II being the predominant *T. gondii* lineage identified within Europe, worldwide their appears to be a greater degree of genetic diversity observed in wildlife populations, compared to those isolates identified from humans and livestock (Ajzenberg *et al.*, 2004; Wendte *et al.*, 2011), and as the molecular tools used to study parasite diversity advance, it is likely that an increased number of novel genotypes will be discovered within different wildlife species, perhaps even from animals in Europe.

1.6 *Toxoplasma gondii* infection in livestock

The following sections provide details of *T. gondii* in the four main livestock species; pigs, sheep, cattle and chicken. Livestock are intermediate hosts of the parasite and seroprevalence can vary depending on the species, how the animal is housed, feeding

habit and also whether cats or rodent are present on the farm. Comparisons between results are difficult, as the methodology between various studies tends to vary. However, some trends amongst the data can be clearly observed, for example the increased prevalence in free range animals such as pigs and chickens when they compared to their indoor reared counterparts (Dubey, 2010; van der Giessen *et al.*, 2007) .

1.6.1 Pigs (*Sus scrofa*)

1.6.1.1 Clinical signs of infection with *T. gondii*

Pigs are an important food source for humans and can acquire *T. gondii* infection by ingestion of oocysts from the environment or from consumption of tissue cysts from infected animals such as rodents. Although clinical signs or mortalities due to *T. gondii* infection in pigs is uncommon, outbreaks of severe disease have been reported. However, in the rare event where *T. gondii* has been reported to cause clinical signs, it has generally been referred to as a sporadic outbreak. One such reported outbreak occurred in China in 2010, where *T. gondii* infection proved lethal in fattening pigs from the Gansu province (Li *et al.*, 2010). Infection resulted in a morbidity of 57% (549/960) with a mortality rate of 2% (19/960), with pigs presenting signs of anorexia, depression and above average rectal temperatures (40 – 42^{o}C). *T. gondii* infection was confirmed by IgG and IgM ELISA and also by mouse bioassay from 2 infected pigs. Further investigation linked this outbreak to ingestion of contaminated feed, and cats were found to reside within the feed warehouse.

Porcine abortion caused by *T. gondii* infection has also been reported, although again a rare occurrence in this livestock species. In 1979 spontaneous abortion in five sows from Ontario, Canada were linked to the parasite. The foetuses aborted near term and upon histopathological examination *Toxoplasma* cysts were evident in the alveolar macrophages of the lung and intracellular parasites morphologically similar to *Toxoplasma* were also identified in myocardial cells. The sow had a high antibody titre against the parasite, and sections of placenta were also shown to be infected (Hunter, 1979). The farm described was known to have a rodent problem which can

increase the transmission of *T. gondii* to pigs (Kijlstra *et al.*, 2008). A more recent abortion outbreak occurred in Jeju Island, Korea, where pregnant sows showed clinical signs which included: fever, anorexia, depression, recumbency, abortion and in a few cases, death. Upon histopathological investigation including immunohistochemistry, *T. gondii* tachyzoites were observed in four adult sows and five aborted piglets from the same litter (Kim *et al.*, 2009). Infection of sows with *Toxoplasma* have also resulted in the birth of still born piglets (Thiptara *et al.*, 2006), similar to clinical signs observed in cases of ovine toxoplasmosis.

Although a number of other outbreaks of porcine abortion and severe clinical disease in naturally infected pigs have been reported, disease is generally subclinical. A more commonly observed symptom is a slight increase in rectal temperature during the initial stages of infection, which may persist for 1-2 days. Presentation of more acute clinical symptoms (although rare) are likely to be linked to age, immune status, and even breed of the animal (Dubey, 2009).

Clinical symptoms in pigs which have been experimentally infected with *Toxoplasma* are similar to those described above, but can prove fatal when pigs are inoculated with large numbers of oocysts ($\geq 4x10^4$) (Dubey *et al.*, 1998b; Garcia *et al.*, 2008).

1.6.1.2 Pigs and *T. gondii* tissue cysts

As the symptoms of clinical toxoplasmosis are not considered as a problem in pigs, the main reason to implement a control strategy to prevent or reduce the establishment of *T. gondii* tissue cysts in this species is to reduce the number of parasites entering the human food chain and their subsequent transmission. The ability of the parasite to infect all warm blooded mammals, combined with its cyst forming nature, *T. gondii* is readily found within the muscles and organs of infected pigs which are often used for human consumption (Bayarri *et al.*, 2012; Dubey *et al.*, 2005a; Wang *et al.*, 2012). Following ingestion of *T. gondii* oocysts or bradyzoites, infective tissue cysts can develop in the host within 6-7 days. The prevalence of the

parasite in pigs reared indoors is likely to be lower than those animals which are outdoor reared, due to an increased risk of exposure to environmental oocysts (Kijlstra *et al.*, 2004; van der Giessen *et al.*, 2007). The seroprevalence of *T. gondii* in pigs has been shown to increase with age (Dubey *et al.*, 1995b), and as seropositivity is likely to reflect the presence of infective tissue cysts within the animal (Dubey *et al.*, 1995a), it can be presumed that older pigs are more likely to harbour infective tissues cysts.

1.6.1.3 Increased risk of *Toxoplasma gondii* infection in outdoor reared pigs

The prevalence of *Toxoplasma* in pigs has declined in the past 30 years. This is most likely due to a change in farming practices where intensive well managed indoor farming methods have been introduced (Dubey, 2009; Edelhofer, 1994). Reports which describe current seroprevalence levels in pigs vary dramatically, they depend on the country (including regional differences), the farming method, the age of the animal and the test methodology used (see Table 1-3). However, the recent demand for "animal friendly" farming has led to a re-introduction of outdoor reared pigs. Transmission of *T. gondii* to pigs from the consumption of infected rodents has also been shown to be a direct source of infection, particularly in animals reared outdoors in animal-friendly production systems (Kijlstra *et al.*, 2004), where outdoor reared and organically raised pigs have generally been shown to have a higher prevalence of the parasite. A study by Dubey *et al* (2012) has shown the prevalence of *T. gondii* in an organic pig farm in Northern USA to be 90.9% (30/33), which is significantly higher than the figure reported for pigs raised in conventional indoor housing (2.7%) (Hill *et al.*, 2010a) (see Table 1-3). A study examining the parasite in Argentinean sows also showed a similar trend; animals which had been bred indoors compared to sows reared outdoors had a prevalence of 4.5% (4/88) and 40.2% (45/112) respectively (Venturini *et al.*, 2004). Although not such a significant difference, van der Giessen et al (2007) found the prevalence of *T. gondii* in conventionally raised pigs in the Netherlands to be 0.38% (1/265), whilst those animals which were outdoor reared had a prevalence of 5.62% (10/178), however when examining the

number of farms where pigs tested positive for *T. gondii*, those pigs which had been reared in a free-range system (where pigs have access to outdoor pens and can forage for food as well as obtaining commercial pig feed) were almost 16 times more likely (33.0%) to be infected with the parasite than indoor reared animals (4.0%) (van der Giessen *et al.*, 2007). These studies highlight an increased risk of *T. gondii* exposure to pigs which are reared outdoors.

The source of infection in pigs can either be via oocyst contamination from soil or contaminated feed, or from consumption of tissue cysts from infected rodents or other small mammals harbouring the tissue cysts stage of the parasite. Contamination with oocysts in either feed or soil is thought to be the main source of infection for pigs (Lehmann *et al.*, 2003). However, outdoor housing systems allow pigs to come into contact with rodents and other wildlife, and as pigs are omnivores, they will consume rodents or rodent cadavers as well as other small mammals and birds, which may be infected with *T. gondii* and harbour infective tissue cysts (bradyzoites). Consumption of other animals by pigs is probable, and several studies have demonstrated how rodent control programs can reduce *T. gondii* seropositivity in pigs (Hill *et al.*, 2010b; Kijlstra *et al.*, 2008). This research highlights the role that rodents and other small animals may have as a reservoir for direct transmission of the parasite to pigs.

It is clear that outdoor reared pigs are more at risk of direct exposure to *T. gondii* oocysts and bradyzoites compared to indoor reared animals, these animals are therefore at increased risk of *T. gondii* infection (Davies, 2011), and subsequent transmission to humans via undercooked or raw pork or pork products.

Table 1-3: Recent seroprevalence figures for *T. gondii* in pigs.

Country	Test	Number of animals	% Positive	Reference
Slovakia	IgG ELISA	923 slaughter 47 sows	2.06% 4.26%	(Turcekova *et al.*, 2013)
Mexico	IgG ELISA IgM ELISA	411 fattening 397 fattening	95.80% 92.50%	(Ortega-Pacheco *et al.*, 2013)
North Portugal	MAT	21 piglets 180 fattening 53 sows	42.90% 5.00% 13.20%	(Lopes *et al.*, 2013)
Ireland	LAT	317 slaughter	4.70%	(Halova *et al.*, 2013)
Switzerland	P-30 ELISA	20 finishing 120 adult 100 free range	14.00% 36.00% 13.00%	(Berger-Schoch *et al.*, 2011)
Czech Republic	IgG ELISA	511 slaughtered	36.00%	(Bartova & Sedlak, 2011)
Brazil	IgG ELISA	34 free range 27 indoor	20.60% 0.00%	(Frazao-Teixeira & de Oliveira, 2011)
The Netherlands	IgG ELISA	402 organic 178 free range 265 intensive	2.74% 5.62% 0.38%	(van der Giessen *et al.*, 2007)
USA	IgG ELISA MAT	6238 finishing indoor 33 organic	2.70% 90.90%	(Hill *et al.*, 2010a) (Dubey *et al.*, 2012)
Argentina	MAT	88 indoor 112 outdoor	4.50% 40.20%	(Venturini *et al.*, 2004)

Key: Finishing / fattening/ slaughter = 20- 24 months of age; piglets = 3 weeks to 3 months of age; intensive = intensive indoor reared; free range = permanent access to pasture, born outside then reared outdoors throughout their lives; organic = free range outdoor reared, with 80% of feed originating from organic agriculture.

1.6.1.4 Control of *T. gondii* infection in pigs

As clinical disease is not considered a problem in pigs, the main reason to implement a control strategy to prevent exposure to *T. gondii* in this species is to reduce the transmission of the parasite entering the human food chain. Pigs are known to harbour a greater number of tissue cysts compared to other animals such as sheep, goats, cattle and horses (Tenter *et al.*, 2000). Therefore undercooked or raw pork is

potentially a greater source of infection for foodborne toxoplasmosis in humans and has possible consequences for food safety (Djurković-Djaković *et al.*, 2013).

An effective way to reduce infection risk for humans from infected pork is to prevent infection and hence the formation of tissue cyst in the animal. Currently, the most successful control measures are those which involve good on-farm management, such as ensuring cats are not allowed to enter feed or grain stores and they have limited access to pig farms (Gamble *et al.*, 1999). A study examining a 4 month rodent control programme on three organic pig farms, followed by longitudinal analysis over seven months, showed that levels of *T. gondii* infection in pigs fell from 10.9% (10/92) to 3.3% (4/122). The prevalence of infected pigs rose again after the control campaign had stopped (Kijlstra *et al.*, 2008). Rodent control is of importance as pigs are omnivores which can consume rodents or rodent cadavers if they are present within their housing. Therefore any on-farm control strategies must concentrate on cat and rodent control, both within the farm and the area used for food storage. To further reduce oocyst viability it has been recommended that any cement floors be steam pressure washed between groups of pigs (Dubey, 1998b; Lehmann *et al.*, 2003).

Another control strategy is to vaccinate animals against *T. gondii* to prevent tissue cyst formation, hence reducing the transmission of the parasite from infected pigs into the food chain. Although a licensed vaccine for this purpose is currently unavailable, research has shown that vaccination using non persistent strains of the parasite has currently had more success than a killed vaccine approach (Innes *et al.*, 2011). Vaccination of pigs to reduce cyst number has been attempted using several different methods, such as using crude rhoptry proteins of *T. gondii*, a DNA vaccine comprised of *T. gondii* dense granules GRA1 and GRA7, and vaccination with live tachyzoites of the TS-4 and RH strains of *T. gondii* (Garcia *et al.*, 2005; Jongert *et al.*, 2008; Pinckney *et al.*, 1994). Although a reduction in tissue cyst burden has been reported by these publications (which are described in further detail – see section 1.11.2.2), there is still no vaccination strategy which can prevent or eliminate cyst formation in pigs.

1.6.2 Sheep (*Ovis aries*)

1.6.2.1 Transmission and clinical signs of infection

As sheep are herbivores, horizontal infection with *T. gondii* is acquired from the consumption of oocysts in the environment, either while the animal is grazing on contaminated pasture, from contaminated drinking/surface water, or from feed which has been contaminated with oocysts due to the presence of cats in the area where feed is stored (Innes *et al.*, 2009a; Skjerve *et al.*, 1998; Vesco *et al.*, 2007). Once the animal is infected it develops an effective immune response to control the parasite, despite being infected for life (as also described for all other intermediate hosts, including humans). Approximately 14 – 21 days after infection IgG antibody specific for *T. gondii* are detectable. Initial infection in sheep is generally asymptomatic, with little or no clinical symptoms, however, when infection occurs in a naïve pregnant ewe, congenital toxoplasmosis can have a devastating outcome. In these animals vertical transmission of the parasite across the placenta occurs and the parasite passes to the foetus. Infection in the first trimester usually results in abortion. The result of infection during the second trimester can lead to the birth of a stillborn or significantly weaker lamb, whilst when infection occurs during the third trimester the lamb may appear healthy when it is born, but it is likely to be persistently infected (Buxton, 1990). Another cause of congenital transmission which has been hypothesised, is from the reactivation of the parasite from a persistently infected ewe during pregnancy (Morley *et al.*, 2008; Williams *et al.*, 2005), this route of transmission is thought to be a very infrequent event, and therefore does not pose a significant risk (Buxton *et al.*, 2007a; Buxton *et al.*, 2007b; Rodger *et al.*, 2006).

It has been reported that ovine abortion caused by *T. gondii* affects approximately 1-2% of the UK's national flock (Blewett & Trees, 1987) which results in yearly losses of an estimated 0.5 million lambs, costing approximately £12 million annually in Great Britain alone (Nieuwhof & Bishop, 2005). Losses in other countries are also estimated to be high, for example in Uruguay the cost is predicted to be between

US$1.4 - 4.7 million (Freyre *et al.*, 1999). Although congenital infection results in neonatal loss for the pregnant ewe, sheep which are infected can potentially pass on the infection to humans, due to the consumption of undercooked or unfrozen meat harbouring infective *T. gondii* tissue cysts (Halos *et al.*, 2010).

Approximately 14 – 21 days after infection IgG antibodies specific for *T. gondii* are detectable and are thought to remain so throughout the lifetime of the animal, as with other species (Dubey & Jones, 2008). Detection of *T. gondii* specific IgG by ELISA is frequently used to assess the seroprevalence of the parasite, although latex agglutination is also another option to test for the presence or absence of IgG antibodies against the parasite. As the methodology used can vary from study to study, exact comparisons between countries or even different regions can be difficult. However, the results of such studies can give an indication of levels of infection.

The prevalence of *T. gondii* in sheep can vary from both different countries and regions, but the majority of research has highlighted an increase in seropositivity with increasing age. A study examining 125 Scottish sheep flocks (equating to 3333 sheep) found an overall prevalence of 56.6% (1619/3333) where an increase in seropositivity was related to age. This was particularly evident in sheep which were over six years of age, where 73.8% (104/141) tested IgG positive, in addition, this work has shown that within each flock (n=125) there was at least one *T. gondii* infected animal (Katzer *et al.*, 2011). A recent study investigating *T. gondii* IgG seroprevalence from sheep in Greece found that 53.71% (n= 458) of animals were positive by ELISA (Anastasia *et al.*, 2013), and similar to results by Katzer *et al* (2011) at least one animal from every flock (n=50) tested positive. Another study, which examined the prevalence of the parasite within sheep flocks in Great Britain, found an overall mean seroprevalence of 74.0% (2619/3539), and also described an increase in prevalence with increasing age (Hutchinson *et al.*, 2011). In the Netherlands a study into the seroprevalence of *T. gondii* in Dutch sheep at slaughter (n=1179) using a validated in-house IgG ELISA estimated the overall prevalence at 27.8%, this research also showed a higher seroprevalence (48.1%) in sheep that were over one year of age (Opsteegh *et al.*, 2010b). In Switzerland the prevalence of *T. gondii* based on an ELISA using the P30 antigen to detect *T. gondii* specific

antibodies from meat juice from sheep determined a prevalence of 61.6% (154/250) (Berger-Schoch *et al.*, 2011), and when the data was used to analyse seropositivity in adult sheep (excluding younger lambs from the analysis) 80.7% (121/150) of animals were positive.

In all of the reported seroprevalence studies highlighted above, there appears to be a positive correlation between seropositivity and increasing age of the animal, indicating high environmental contamination of *T. gondii* oocysts, and hence the majority of infections are likely to be acquired postnatally (Katzer *et al.*, 2011).

A licensed vaccine against ovine abortion (Toxovax®) is available in the UK, Ireland, New Zealand and France from MSD Animal Health. This live vaccine is composed of tachyzoites from the attenuated S48 strain of *T. gondii*, which was originally isolated in New Zealand from an aborted lamb foetus and following years of repeated passage in mice the strain lost its ability to differentiate into either bradyzoites or oocysts, and remained as the tachyzoite stage of the parasite (Buxton, 1993; O'Connell *et al.*, 1988). Efficacy trials of the vaccine were completed in Scotland at the Moredun Research Institute, showing that the vaccine was best administered 3 weeks prior to mating and that it was effective in protecting against *T. gondii* associated abortion for at least 18 months (Buxton & Innes, 1995). Although the S48 strain has the ability to produce a host immune response whereby sheep produce a cellular and humoral immune response which involves IFNγ, CD4 and CD8 T-cells (as reviewed by Innes *et al* 2009b), there is no information as to whether the vaccine prevents the formation of tissue cysts in sheep or indeed other livestock species (AMCSF, 2012).

1.6.3 Cattle (*Bos taurus*)

Natural infection in cattle does not appear to give rise to clinical symptoms or abortion of the foetus in pregnant cows as observed in sheep (Dubey, 1986) despite the seroprevalence in some studies being reported as quite high, for example 83.3% (n=504) in southern Spain (Garcia-Bocanegra *et al.*, 2013), 45.6% (n=406) in

Switzerland (Berger-Schoch *et al.*, 2011), 33.8% (n=74) in eastern Poland (Sroka *et al.*, 2010), and slightly lower 25.0% (n=995) in cattle over 12 months old from The Netherlands, where seroprevalence was determined by a cut off value. However, in The Netherlands this figure rose to 54.5% when prevalence was estimated using the frequency distributions (binormal mixture model) (Opsteegh *et al.*, 2011b). However, a recent publication describing the prevalence of *T. gondii* in different livestock species from north Portugal reported infection in cattle to be much lower at 7.5% (n=161) (Lopes *et al.*, 2013). Despite the high seroprevalence observed in the majority of studies, tissues cysts are rarely identified from seropositive animals. In fact, work by Opsteegh *et al* (2011) found results to the contrary, whereby 3% (2/66) of cattle which tested negative by ELISA (where the results tested negative by both the cut off value and the binormal mixture model) actually tested positive by MC-PCR using digested heart tissue. This result indicates that in this particular study, despite the animals being seronegative, *T. gondii* DNA was present within heart tissue of two animals. This result is interesting as it provides an indication that, in cattle, seroprevalence may not be directly linked to the presence of *T. gondii* DNA, and also raises the question as to whether detection of *T. gondii* DNA reflects the presence of infectious parasites (e.g. tissue cysts). However, evidence of viable infective tissue cysts from cattle which have been infected either experimentally or naturally infected is rare (Dubey, 1992; Esteban-Redondo *et al.*, 1999).

1.6.4 Chicken (*Gallus gallus domesticus*)

Although chickens can become infected with *T. gondii* it is very rare for infection to lead to any clinical signs of toxoplasmosis. The first reported case was reported from a hen in Germany (Hepding, 1939), where chorioretinitis, encephalitis and the identification of *T. gondii* like organisms were found in the retina of the animal. More recently only two cases of toxoplasmosis in chickens have been reported (Dubey *et al.*, 2007c; Goodwin *et al.*, 1994). In one of these cases three chickens from a group of 14 died suddenly, following post mortem examination on one animal, the brain appeared to show lesions, necrosis and perivascular cuffs, also

protozoa identified in the brain reacted positively with antibodies against *T. gondii*. The eleven surviving chickens were asymptomatic, however, when a mouse bioassay was completed using tissues from brain, heart and leg muscle, all eleven chickens contained viable tissue cysts (Dubey *et al.*, 2007c).

As outdoor reared free range chickens spend the majority of their time pecking at the ground or soil in search of food, this species is often used as a sentinel species, providing an indication to the amount of environmental contamination from *T. gondii* oocysts. Free range chickens are frequently used for epidemiological studies examining the prevalence and also the different *T. gondii* genotypes present (Dubey *et al.*, 2003; Tilahun *et al.*, 2013).

The prevalence of *T. gondii* infection in free range chickens varies dramatically, with seropositivity as high as 90% (n=20) in Australia (Chumpolbanchorn *et al.*, 2013), or even 100% (n=11) in the United States (Dubey *et al.*, 2007c). In contrast a prevalence as low as 1.6% (n=300) in Turkey (Inci *et al.*, 1998) was reported, and similarly, a lower prevalence of 18% (n=65) in free range chickens from Ireland has recently been described (Halova *et al.*, 2013). However, it should be noted (as it should be for other species), the seroprevalence can vary depending on the age of the animal and the methodology used. The prevalence of *T. gondii* from indoor reared chickens with no outdoor access is generally low (Hill & Dubey, 2013), further to this viable *T. gondii* could not be identified using mouse bioassay from chicken meat obtained from supermarkets in the United States (Dubey *et al.*, 2005b). As described, the prevalence of *T. gondii* in free range chicken appears to be relatively high and combined with the ability to detect viable tissues cysts within these animals highlights a potential public health risk for consumers of this meat is eaten undercooked.

As previously mentioned, chickens are a particularly useful sentinel species, and they play an important role for the isolation and identification of different *T. gondii* strains, providing valuable data about the diversity of the parasite worldwide (Lehmann *et al.*, 2006). Using this livestock species has been most useful for assessing the diversity of *T. gondii* within South America, where new genotypes and

atypical strains are frequently identified from free range chickens (Clementino Andrade *et al.*, 2013; More *et al.*, 2012; Pena *et al.*, 2013).

1.7 The presence of *T. gondii* tissue cysts in meat destined for human consumption

Ingestion of *T. gondii* tissue cysts from contaminated meat is a major route of infection for humans. In Europe a case control study, which focused on pregnant women from Switzerland, Belgium, Italy, Sweden and Denmark, found that between 30% to 63% of *T. gondii* infections within this group were linked to undercooked or cured meat products (Cook *et al.*, 2000). The detection of tissue cysts within food producing animals is known to vary depending on the species (Figure 1-6). Cattle rarely have detectable tissue cysts, even in animals which have been experimentally challenged (Esteban-Redondo *et al.*, 1999), whilst in outdoor reared sheep, pigs and goats, tissue cysts are more commonly identified (Dubey *et al.*, 1998a).

As reviewed in section 1.6 (*Toxoplasma gondii* infection in livestock), the parasite has a high prevalence within the main livestock species commonly farmed to provide meat for human consumption. The exact relationship between seropositivity and identification of viable *T. gondii* tissue cysts within meat for human consumption is not known.

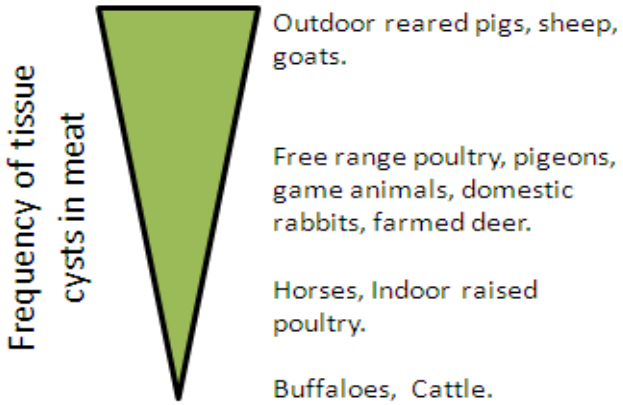

Figure 1-6: Tissue cyst detection in food producing animals.
Tissue cysts are detected more frequently in outdoor reared pigs, sheep and goats, whilst cysts are rarely detected in cattle (Tenter *et al.*, 2000*)*.

Once an animal is infected tissue cysts have a preference to establish in specific tissues of the host, such as liver, heart, brain, tongue, diaphragm, kidney and skeletal muscle (Dubey *et al.*, 1998a). A recent study has also further highlighted the importance of infected skeletal muscle and its role in the transmission of the parasite to humans from eating raw or undercooked meat (Swierzy *et al.*, 2013). Viable parasites and tissue cysts have been isolated from cuts of meat and meat products destined for human consumption from a variety of livestock species, such as pigs, sheep, goats and even cattle (Aspinall *et al.*, 2002; Dubey *et al.*, 1986; Kijlstra & Jongert, 2009; Opsteegh *et al.*, 2011b). Infected pork (in particular from outdoor reared pigs) is considered to be the major source of infection (Cook *et al.*, 2000; Davies, 2011; Djurković-Djaković *et al.*, 2013; Dubey, 2009).

In order gain a better understanding of possible sources of human infection due to meatborne contamination in The Netherlands, a quantitative microbial risk assessment (QMRA) model was designed to assess the number of bradyzoites in unprocessed portions of lamb, beef and pork products (Opsteegh *et al.*, 2011a). The results from the model predicted that beef was the most important source of infection for the Dutch population, with undercooked / underheated meat products identified as the main risk factor for infection. Interestingly, bradyzoites are not commonly isolated from cattle experimentally infected with *T. gondii* (Esteban-Redondo *et al.*,

1999), but a typical Dutch dish consumed in The Netherlands (Filet Americain), consists of a raw beef spread seasoned with herbs and spices, as this dish is uncooked the potential risk from infection is high. In other countries traditional or typical dishes also consist of raw beef, such as, steak tartar in France, yukhoe in Korea, and kitfo in Ethiopia, all of which carry a greater risk of infection from bradyzoites (Vitale & Di Marco Lo Presti, 2013).

In France a study which was undertaken in 2010 identified ovine meat to be contaminated with significantly high levels of *T. gondii* (Halos *et al.*, 2010), and to assess the viability of tissue cysts incorporated a mouse bioassay of ovine hearts (n=433) collected at slaughter. Viable parasites where identified from 5.4% of sheep, however this increased dramatically when the age of the animals was taken into account, with 42% of adult animals containing viable tissue cysts.

In Brazil, viable tissue cysts were identified by mouse bioassay in 8.7% (n=149) of fresh pork sausage samples collected from eight different factories (Dias *et al.*, 2005), and research by Belfort-Neto *et al* (2007) detected *T. gondii* DNA from porcine tongue collected from local abattoirs in 66.0% (n=50) of animals. This study did not include a mouse bioassay, therefore, the viability of infective tissue cysts could not be determined from these animals.

Detection of parasite DNA was also carried out using commercially available meat samples from the UK, the results found 38% (n=71) of samples tested where positive for the presence of *T. gondii* DNA (Aspinall *et al.*, 2002). As detection was carried purely by PCR, with no information available regarding the viability of the parasite, it is not possible to conclude whether consumption of these meat samples would have resulted in infection. Ideally, research which incorporates molecular detection of the parasite in conjunction with a mouse bioassay would help to address the problem between presence of parasite DNA within meat for human consumption and its ability to cause infection.

Despite the evidence provided on the presence of tissue cysts within meat and meat products, transmission can be reduced if the meat is thoroughly cooked or frozen for

at least 24 hours in a household freezer, as an internal temperature above 67^{o}C or below -12^{o}C will render tissue cysts non-viable (Dubey *et al.*, 1990; Kotula AW *et al.*, 1991). Generally methods used for salting and curing meat reduce the viability of the parasite (Hill *et al.*, 2004), however, there is also recent evidence that *T. gondii* tissue cysts can be resistant to these methods (Bayarri *et al.*, 2012; Pott *et al.*, 2013).

Finally, the development of a vaccine which could prevent tissue cyst formation in food producing animals would dramatically reduce foodborne transmission of *T. gondii* in humans. From a public health perspective, the disease burden associated with foodborne transmission of *T. gondii* in humans is high, and is further described in section 1.8.2 – Disease burden.

1.8 Toxoplasmosis in humans

Human *T. gondii* infection is a problem which spans worldwide, with seroprevalence estimates ranging from 0 – 100% depending on the origin of the population studied (Tenter *et al.*, 2000). The overall global trends for the seroprevalence of *T. gondii* were published in 2009 (Pappas *et al.*, 2009), which examined published literature on the seroprevalence of the parasite in women of child bearing age (and pregnant women) for a period of ten years (January 1999 – December 2008).

A global map highlighting the seroprevalence of *T. gondii* in humans, generated from the publication by Pappas *et al* (2009), is shown in Figure 1-7.

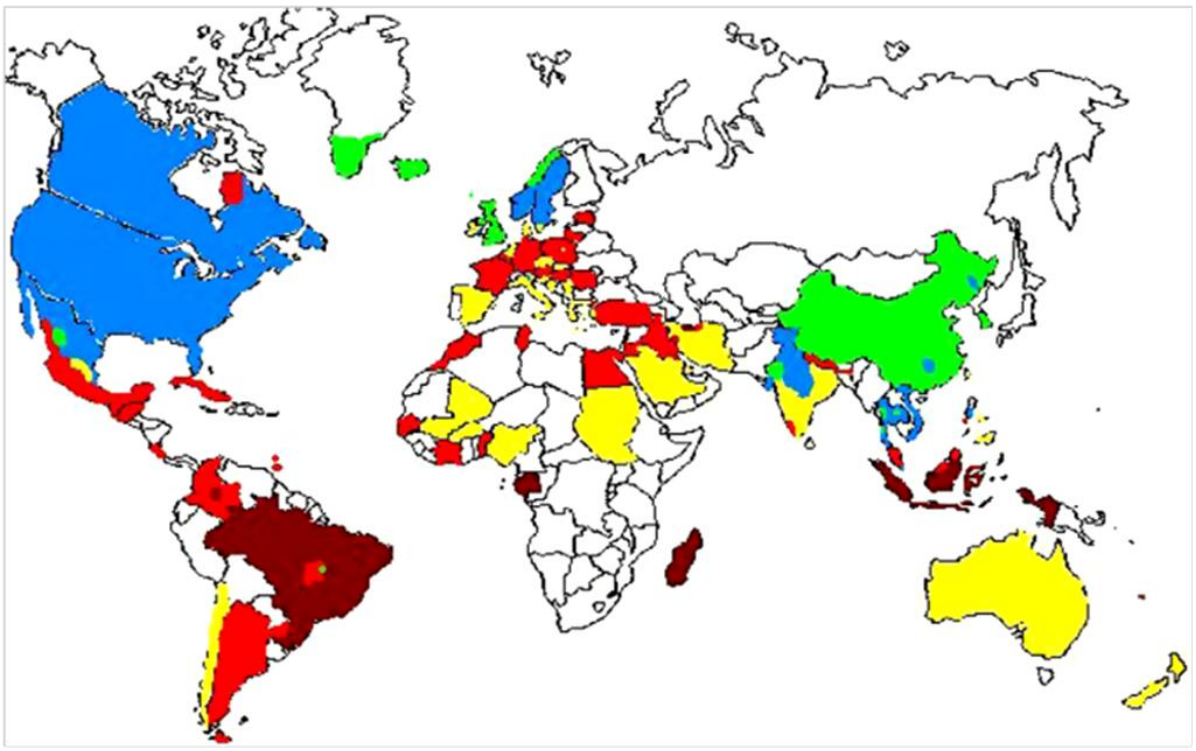

Figure 1-7: Worldwide seroprevalence map of *T. gondii*. Seroprevalence is indicated using the following colours: Dark red = above 60%, light red = 40 – 60%, yellow = 20 – 40%, blue = 10 – 20%, green = less than 20%, white = unknown. The map shows a high prevalence in South America, South East Asia and mainland Europe. Adapted from Pappas *et al* (2009)

A high prevalence was reported in humans from South American countries such as Brazil and Argentina where seroprevalence has been reported at 77.5% (n=503) and 53.4% (n=650) respectively (Porto *et al.*, 2008; Rickard *et al.*, 1999), whilst a lower prevalence was noted in the majority of European countries, such as the United Kingdom (9.1% n=1,897), Denmark (27.8% n=89,873) and the Czech Republic (19.8 n=1,053) (Kankova & Flegr, 2007; Lebech *et al.*, 1999; Nash *et al.*, 2005). However data on the seroprevalence in Germany and France have shown higher levels of *T. gondii* infection (59.0% n=4854, and 54.0%, n=13459 respectively) compared to their European counterparts (Fiedler *et al.*, 1999; Tenter *et al.*, 2000). Despite the high seroprevalence reported in France, a recent publication reports a

reduction in the incidence of *T. gondii* infection, where levels in pregnant women have fallen from 7.5/1000 in 1980 to 2.4/1000 in 2010 (Nogareda *et al.*, 2013). This reduction is presumed to be due to changes in meat production practices, hygiene and food habits, with the study also describing a 30% decline in the consumption of ovine meat (a food source known to have a high level of contamination - (Halos *et al.*, 2010)) as reported by the French Ministry of Agriculture.

Although the data obtained provides an overview of the seroprevalence of *T. gondii* within the human population, the results described above focus on a particular group of the population, pregnant women or women of childbearing age. Studies which include a broad range of the population, including both males and females across all age ranges, could provide a better indication of *T. gondii* seroprevalence in humans.

1.8.1 Clinical symptoms

Despite a high seroprevalence in humans, the immune system generally keeps the parasite under control and most healthy individuals are asymptomatic, however patients may experience mild flu like symptoms such as fever, myalgia or asthenia (Montoya & Liesenfeld, 2004). There is also recent evidence to suggest that the strain which has caused initial infection can also affect the severity of clinical disease. This has been documented from cases of toxoplasmosis from Brazil and French Guiana, where primary infection linked to atypical or genetically distinct strains of the parasite have resulted in severe toxoplasmosis and ocular disease (Demar *et al.*, 2012; Ferreira *et al.*, 2011). This majority of these cases are thought to be due to consumption of raw or undercooked meat from an infected animal. One such report where severe toxoplasmosis in an immunocompetent individual from France (symptoms included; weight loss, night sweats, severe rectinochoroiditis and pulmonary toxoplasmosis), was linked to a primary infection of an atypical strain of *T. gondii*, with consumption of raw horsemeat imported from Argentina presumed to be the cause of infection (Sobanski *et al.*, 2013). Although, these cases are relatively rare and tend to only be associated with outbreaks from particular geographical areas.

It is immunocompromised individuals that are most at risk. This includes individuals who are receiving chemotherapy for the treatment of cancer, those taking immunosuppresive drugs following organ transplantation, and AIDS patients. Primary infection with the parasite when these individuals are immunocompetent may not result in any clinical symptoms, however, when an adequate T-cell response is not mounted in times of immunodepression, reactivation of the parasite can occur, which can prove to be life threatening (Porter & Sande, 1992). Clinical symptoms are often a result of CNS involvement, in particular toxoplasmic encephalitis, causing seizures, cranial nerve disturbances, movement disorders, mental status changes, speech abnormalities and focal motor deficits (Montoya & Liesenfeld, 2004). Although the brain is most commonly involved, dissemination of the parasite to the eyes (ocular toxoplasmosis), and heart (pulmonary toxoplasmosis) have also been reported (Kovari *et al.*, 2010; Rothova, 2003).

In both immunocompetent and immunocompromised women primary infection during pregnancy with *T. gondii* can result in the transmission of the parasite to the foetus via the placenta. The risk of transmission and the severity of disease depends on the gestational stage at which the mother first becomes infected. The risk of infection being passed to the foetus increases as gestation progresses, however, inversely, the severity of disease decreases with an increase in gestation period (Dunn *et al.*, 1999). For example, a foetus which becomes infected during the first trimester often leads to abortion, stillbirth or a child born with severe abnormalities of the brain and eyes, such as hydrocephalus, deafness, mental retardation, seizures, retinochoroiditis, and even blindness (Robert-Gangneux & Darde, 2012; Swisher *et al.*, 1994). Transmission to the foetus in the third trimester is less likely to cause such severe clinical manifestations, but may result in subclinical disease which can result in retinochoroiditis or learning difficulties in later life (Weiss & Dubey, 2009). The incidence of congenital toxoplasmosis varies depending on country and region. In some areas of Brazil (such as the state of Minas Gerais) the rate of infection from November 2006 to May 2007 was been reported to affect 1.3/1000 live births, with infection as high as 76.9/1000 live births in one particular region (Jequitinhonha) (Carellos *et al.*, 2013). In addition, infants in Brazil with congenital toxoplasmosis

more frequently show signs of severe toxoplasmic retinochoroiditis, compared to infants with congenital toxoplasmosis in Europe (Gilbert *et al.*, 2008). The severity of disease is presumed to be linked to the high genetic diversity of *T. gondii* identified in animals from this country, and recently this increased parasite diversity has been reported from congenitally infected Brazilian babies (Carneiro *et al.*, 2013). In the UK, a study conducted between 2002 and 2004 in England and Wales estimated congenital infection to affect 3.4/100,000 live births (Gilbert *et al.*, 2006). Although this figure is relatively low compared to some other countries, the symptoms of disease can have a lifelong effect on the child and the resulting disease burden of such cases is further described in section 1.8.2.

Ocular toxoplasmosis which has been acquired postnatally (as opposed to congenital infection) is now thought to account for up to half of all reported cases in England and Wales (Gilbert *et al.*, 2006). In 1995, an outbreak of toxoplasmosis in humans from Canada resulted in infection of 110 individuals (Bowie *et al.*, 1997), with 20 cases of ocular toxoplasmosis linked to the outbreak the following year (Burnett *et al.*, 1998). As previously mentioned, acquired infection has also potentially been linked to schizophrenia (Yolken et al., 2009), Alzheimer disease (Kusbeci *et al.*, 2011), traffic accidents (Flegr *et al.*, 2002a), self-directed violence (Pedersen *et al.*, 2011) and suicide (Lester, 2010). It is not completely clear how the parasite can cause these disorders, but it is thought that it may be due to an increase in dopamine and a decrease in tryptophan within the brain, which, in intermediate hosts, increases the chance of predation by the definitive host and hence transmission and completion of the parasite's life cycle (Flegr, 2013).

1.8.2 Disease burden

Symptoms of toxoplasmosis can be lifelong, in particular, children born with congenital toxoplasmosis who suffer from learning difficulties or individuals with ocular toxoplasmosis (whether acquired postnatally or congenitally) which, at its

most severe, can cause blindness. A small number of studies within Greece, The Netherlands and the USA have analysed the disease burden of foodborne pathogens, comparing organisms such as *Campylobacter* spp (*Campylobacter*), *Salmonella enteric* (*Salmonella*), *Listeria monocytogenes* (*Listeria*), *Cryptosporidium parvum* (*C. parvum*), *Escherichia coli* O157:H7 (*E. coli* O157:H7) with *T. gondii* (Batz *et al.*, 2012; Gkogka *et al.*, 2011; Havelaar *et al.*, 2012; Hoffmann *et al.*, 2012). This research has taken into account the annual cost or disability of illness linking it to either quality-adjusted life-years (QALYs) or disability-adjusted life- years (DALYs). From these results *T. gondii* ranked third to *Salmonella* and *Campylobacter*, based on QALYs in the USA, and from representative data taken in 2009 from The Netherlands, *T. gondii* ranked highest among 14 other foodborne pathogens when DALY's were used to classify these pathogens (see Figure 1-8). In Greece the parasite was ranked 5th out of a possible 15 other foodborne pathogens using DALY's for classification. The high disease burden reported in these publications highlights the lifelong effects which *T. gondii* can have on infected individuals.

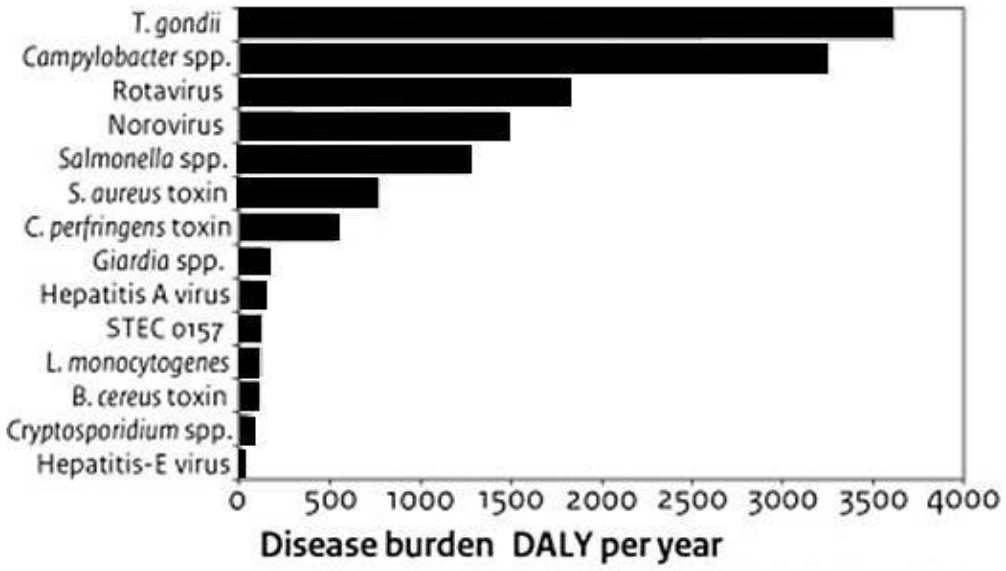

Figure 1-8: Disease burden (DALY's) of *T. gondii* against other foodborne pathogens in The Netherlands from 2009. Adapted from Havelaar *et al* (2012)

1.9 Serological detection of *T. gondii*

1.9.1 Sabin Feldman dye test

There are several different techniques available for serological detection of *T. gondii* antibodies against the parasite from both human and animal samples. Historically, the oldest method used for detection of antibodies to *T. gondii* is the Sabin Feldman Dye Test (SFDT) which was first described in 1948 (Sabin & Feldman, 1948), and is still considered the "gold standard" for the serological detection of *T. gondii* in humans (Reiter-Owona *et al.*, 1999). This method is based on complement mediated cytolysis and requires live tachyzoites. The tachyzoites are mixed with heat inactivated test sera and also human seronegative test sera (which acts as an accessory factor), and following an incubation period, methylene blue is added and results visualised using a light microscope. Methylene blue will stain live intact tachyzoites, therefore, a result is deemed positive if ≥55% of tachyzoites are not stained with the dye, and negative if ≤55% of tachyzoites are stained with the dye (Udonsom *et al.*, 2010). Although this test is very sensitive in detecting low levels of *T. gondii* antibodies, it requires the use of live tachyzoites, and therefore maintenance of this stage of the parasite in either mice or cell culture. A similar method to the Sabin Feldman dye test, which utilises *T. gondii* tachyzoites, is the indirect immunofluorescent antibody test.

1.9.2 Indirect immunofluorescent antibody testing

An indirect immunofluorescent antibody test (IFAT), was first reported to be suitable for diagnostic use by Fletcher *et al* (1965). This method consists of cell culture derived whole tachyzoites (which can be cultured and then frozen for long term storage) which are fixed onto a microscope slide and incubated with dilutions of test serum. An appropriate secondary antibody coupled with fluorescein isothiocyanate (FITC) is used which allows visualisation of the result using a fluorescent microscope. This method is particularly useful for detecting antibodies against *T.*

gondii at the early stages of infection and (along with the Sabin Feldman dye test), is thought to be more sensitive and specific compared to methods such as the agglutination test and ELISA (Piergili, 2004).

1.9.3 Enzyme linked immunosorbent assay

The most commonly used technique in diagnostic laboratories for the detection of *T. gondii* infection (in particular IgG and IgM) is the enzyme linked immunosorbent assay (ELISA) (Robert-Gangneux & Darde, 2012). A description of the methodology for the detection of *T. gondii* IgG from human serum is described in section 2.10.1. However an ELISA can also be optimised to detect IgA or even IgE antibodies specific to *T. gondii*. An avidity assay is an adaptation to the ELISA, which incorporates a denaturing agent (such as urea) within serum dilutions to test for antibody avidity. Avidity values are lower during the acute phase of infection and increase over time, which can be used for "dating" the infection, to determine when seroconversion has occurred, and is particularly useful in cases of congenital toxoplasmosis (Lappalainen *et al.*, 1993).

1.9.4 Modified agglutination test (MAT)

The modified agglutination test (MAT) was first described in 1959 by Fulton and Turk (Fulton & Turk, 1959). The test incorporates formalin fixed whole tachyzoites and is used to detect IgG antibodies raised against *T. gondii*. The addition of 2-mercaptoehtanol (2ME) is used to suppress any non-specific binding from natural IgM antibody (Desmonts & Remington, 1980). The benefit this test has over other detection methods is that it does not require a species specific conjugate, therefore, enabling detection of IgG antibodies against *T. gondii* from a variety of different hosts, including livestock, wildlife and humans. The test is also available as a commercially available kit, which has been shown to perform as successfully as ELISA when testing porcine serum samples, however, MAT was more sensitive at detecting *T. gondii* IgG from porcine tissue fluid (meat juice) (Forbes *et al.*, 2012).

1.10 Detection of *T. gondii* parasites

1.10.1 Molecular detection of parasite DNA

Although molecular detection of parasite DNA by the polymerase chain reaction (PCR) can be used for the diagnosis of *T. gondii* infection, it is more commonly used in scientific research. The DNA targets which are most frequently reported in the literature include the 110 copy ITS1 region of *T. gondii* (Hurtado *et al.*, 2001; Tenter *et al.*, 1994), the 35 copy B1 region (Burg *et al.*, 1989) and the 300 copy 529bp repeat element (Homan *et al.*, 2000). To improve the sensitivity of these markers, nested PCR (nPCR) or quantitative PCR (qPCR) methodologies have been developed and successfully used to improve detection thresholds (Jauregui *et al.*, 2001; Reischl *et al.*, 2003).

Despite the sensitivity of the markers used for detection of the parasite, they are based within regions of the genome which are highly conserved, which has made them unsuitable for strain genotyping. Research which has recently been published describes the ability of the B1 gene to discriminate between the three main *T. gondii* lineages (Costa *et al.*, 2011). This method incorporates high resolution melting curve analysis (HRM) for detection of SNPs, and looks promising for future research whereby detection and genotyping can be completed using a single reaction.

Magnetic capture PCR (MC-PCR) for the detection of *T. gondii* in meat samples has recently been described (Opsteegh *et al.*, 2010a), with molecular detection based on the 529bp repeat element. The process requires digestion of a 100g tissue sample to form a crude extract, and with the removal of free biotin (using streptavadin sepharose to bind the biotin), biotin labelled *T. gondii* specific capture-oligos (based on the 529bp repeat element) are added to the extract which hybridise to any *T. gondii* DNA present. The addition of streptavidin beads enables magnetic capture of *T. gondii* DNA which has bound to the sequence specific capture-oligos. A qPCR incorporating primers designed to target the 529bp repeat element is then carried out on DNA isolated from the technique. This method may be useful as an alternative to

mouse bioassay, however DNA detection of *T. gondii* tissue cysts, unlike the bioassay, does not reflect how viable the tissue cysts are.

1.10.2 Mouse Bioassay

The mouse bioassay has been described as the gold standard to test for the viability of *T. gondii* tissue cysts from animal tissues (Ware *et al.*, 2010). The method dates back to the 1960's when it was used to isolate viable tissue cysts from swine, cattle and sheep (Jacobs *et al.*, 1960b). The method has been adapted since this time (Dubey, 1998a), and is fully described in section 2.8.1. Briefly, the method involves an acid / pepsin digest of homogenised animal tissue, which is then neutralised and inoculated by the intra-peritoneal (IP) route into mice. After approximately 4 to 6 weeks, if viable tissue cysts were present in the inocula this can be confirmed by either; microscopic identification of parasite tissue cysts from the mouse brain; or detection of parasite DNA from the mouse brain. The technique is sensitive enough to detect 1 cyst / 100g of tissue (Rothe *et al.*, 1985), however this does not take into account the possible inhomogeneous nature of the distribution of tissue cysts in a sample. Although this method is time consuming and requires an *in vivo* mouse model, it is still currently the only way to test both the presence of *T. gondii* and also the viability of the parasites detected.

1.11 Vaccination strategies

1.11.1 Cats

As oocysts, shed by the definitive host (the cat), are the primary source of infection (Dubey & Beattie, 1988), a vaccine which prevents oocysts shedding would have a dramatic effect on the environmental contamination with oocysts (Innes 2009). A reduction in the amount of oocysts in the environment would subsequently reduce *T. gondii* infections in both animals and humans.

One of the most successful vaccines shown to reduce oocyst shedding in cats incorporated a mutant strain of *T. gondii* named T-263. When this strain was used to vaccinate kittens, prior to challenge with a non-mutant strain of *T. gondii*, 84% (31/37) of the animals were reported to have not shed any oocysts (Frenkel *et al.*, 1991). During a field trial with the same mutant strain (T-263), results showed that vaccination of cats on 8 commercial swine farms resulted in an overall reduction of the seroprevalence of the parasite in pigs (Mateus-Pinilla *et al.*, 1999). Despite the success of this strain in reducing oocyst shedding in cats, it has never been manufactured as a commercial vaccine. The main reason behind this is due to the process in which the strain is produced and maintained in mouse brains and also requires a cold storage chain. In addition, the bradyzoites which are used for vaccination are also infective to humans, making administering the vaccine potentially hazardous. A more desirable approach would be a vaccine which incorporates recombinant DNA, however studies using this methodology are few in number and their success has been limited. For example, research by Mishima *et al* (2002) using recombinant feline herpesvirus-1 expressing the ROP2 antigen of *T. gondii* did not reduce oocyst shedding in cats. Whilst Garcia *et al* (2007) and Zulpo *et al* (2012) noted partial protection against oocyst shedding when cats were vaccinated intranasally with crude rhoptry proteins of *T. gondii* combined with Quil-A as an adjuvant. Another approach used by Omata *et al* (1996), where ^{60}Co-irradiated *T. gondii* tachyzoites of two different strains of the parasite (RH and Beverley), were used to vaccinate cats, however only partial protection was observed, with oocysts not detected in 21.4% (3/14) of infected cats.

1.11.2 Livestock

1.11.2.1 Sheep

Ovine toxoplasmosis is one of the major causes of ovine abortion in both the UK and worldwide (as further described in section 1.6.2). Currently the only commercially available vaccine against the disease is Toxovax®, which is licensed by MSD Animal Health (Milton Keynes, UK). As previously described in section 1.6.2, the vaccine

consists of the tachyzoite stage of the S48 strain of *T. gondii*, which has lost the ability to differentiate into bradyzoites or oocysts, and remains as the tachyzoite stage of the parasite (Buxton, 1993; O'Connell *et al.*, 1988).

Although this vaccine has proved to be successful, a recent study across Scottish sheep flocks highlighted that only 24.7% (22/93) of farmers used the vaccine (Katzer *et al.*, 2011). The relatively low percentage of farmers vaccinating their ewes may be due to a failure in the manufacture of the vaccine in recent years, resulting in reduced availability of the vaccine within Europe.

1.11.2.2 **Pigs**

As clinical signs of toxoplasmosis in pigs are rare (as described in section 1.6.1.1). From a public health perspective, a vaccine which could reduce the number of viable tissue cysts (bradyzoites) in the edible tissues of pigs would be desirable.

Various vaccination strategies have been employed, but, as yet, no vaccine has been shown to provide complete protection against tissue cyst formation. Initial vaccination trials in the 1990's incorporated either the tachyzoite or oocyst stage of a non-persistent strain of the parasite (RH), however, following mouse bioassay of porcine tissues, only partial protection from tissue cyst formation was obtained (Dubey *et al.*, 1994; Dubey *et al.*, 1998b; Dubey *et al.*, 1991; Pinckney *et al.*, 1994). In 2004, Kringel *et al* also used the RH strain of *T. gondii* for vaccinating pigs, however the vaccine also included an immunostimulating adjuvant by incorporating CpG-oligodeoxynucleotides (which enhances the Th-1 immune response). This vaccine provided partial protection against tissue cyst formation following oocyst challenge with the VEG strain of *T. gondii*, with detection of tissue cysts in only 26.7% (16/60) of mice (following mouse bioassay of porcine tissues). As further discussed in section 5.6, two similar studies incorporated crude rhoptry proteins from tachyzoites of the RH and either immunostimulating complexes (ISCOMS) (Garcia *et al.*, 2005) or Quil-A (Cunha *et al.*, 2011) as an adjuvant. When porcine tissues from this experiment were assessed for the absence of tissue cysts (using mouse bioassay) only partial protection was observed. Another approach, which has had some success, is a DNA based vaccine. In 2008 a vaccine containing a cocktail of

two of the dense granule proteins of *T. gondii*, GRA1 and GRA7, was shown to produce a strong humoral and type I cellular immune response in pigs against *T. gondii* infection (Jongert *et al.*, 2008). A mouse bioassay on porcine heart tissue from the vaccinated pigs in this experiment found two out of a total of three animals to be tissue cyst free.

Although the S48 strain of *T. gondii* is used in the commercial vaccine against ovine toxoplasmosis (Toxovax®), it has never been reported whether this strain of the parasite has any protective response in the formation of tissue cysts in either infected pigs or indeed lambs. In a recent document produced for the Food Standards Agency (AMCSF, 2012), it was stated that one of the gaps in the current knowledge relevant to a UK risk assessment was: "*Vaccines based on live attenuated strains of tachyzoites are effective in reducing morbidity in sheep but it is not known whether vaccination has any effect on the formation of tissue cysts"*. Therefore, to address this knowledge gap and potentially improve food safety, future research into vaccination of pigs to reduce tissue cyst formation should look at the S48 strain of *T. gondii*.

1.11.3 Humans

A vaccine against toxoplasmosis in humans is, as yet, unavailable. A vaccine for humans would be most beneficial for those in vulnerable risk groups such as women of childbearing age who are known to be *T. gondii* seronegative and immunocompromised individuals who are again known to be seronegative. The vaccine would also be of use in countries such as Brazil, where not only is the seroprevalence of the parasite high in humans, but the frequency of atypical genotypes, which can cause ocular toxoplasmosis in non-immunocompromised, is also greater. The only vaccine which has had success against toxoplasmosis is comprised of live tachyzoites of the attenuated S48 strain of the parasite, and licensed for veterinary use only (primarily breeding ewes). It is therefore unlikely that this live vaccine (or any other vaccine comprised of live tachyzoites) will be considered safe for human use, whilst DNA or subunit vaccines are considered safer

alternatives, particularly for parasitic infections (Ivory & Chadee, 2004), however, these may not be as effective as live vaccines (Innes *et al.*, 2011).

Research which has focused on DNA and subunit vaccines for humans, has been carried out mainly in mice and rats, with the main vaccine candidates comprised of crude antigen, recombinant protein or DNA from surface antigens of *T. gondii* (such as SAG1) (Couper *et al.*, 2003), rhoptry proteins (such as ROP2), dense granules (such as GRA1, 4, 5 and 7) (Desolme *et al.*, 2000) and combinations of these antigens (Martin *et al.*, 2004; Vercammen *et al.*, 2000).

Recent research using BALB/c mice immunised intranasally with recombinant *T. gondii* actin (rTgACT) has had some success in protecting mice against challenge with the RH stain of the parasite (which is fatal in mice) (Yin *et al.*, 2013). Intranasal immunisation resulted in a 50% increase in mouse survival, with parasite burden on the brains of chronically infected mice reduced by 60% compared to control animals.

Another study from 2013 where mice were vaccinated with DNA from *T. gondii* eukaryotic initiation factor-2α (TgIF2α- which is responsible for parasite viability (Joyce *et al.*, 2010)) reported a 44.1% reduction in the formation of tissue cysts in the brains of vaccinated mice when compared to control animals (Chen *et al.*, 2013). The results from this research also described a strong cell mediated immune response following vaccination, with a significant increase in IFNγ and IL-2 from these animals.

In the majority of these experiments mice are used as a model for infection, but just how these vaccines will perform in humans is not understood. It has been reported that rats may serve as a better model for *T. gondii* vaccine research, as in this species resistance to the parasite is the same as observed in humans (the RH stain of *T. gondii* is not lethal in immunocompetent humans or rats, but is lethal in mice) and may be more useful for studying human acute toxoplasmosis, chronic toxoplasmosis and disseminated toxoplasmosis in AIDS patients (Zenner *et al.*, 1999).

From published research, it is clear that a vaccine which incorporates antigens from the different stages of the life cycle is important. $CD4^+$, $CD8^+$ and IFNγ are involved in protective immunity to *T. gondii* and if a greater number of antigens are present in a single vaccine, it is more likely that they will be processed and presented to the immune system (Innes *et al.*, 2011). Other factors, which appear to be important and should be considered for inclusion for future vaccines, are immunostimulating adjuvants and also the route in which the vaccine is administered.

1.12 Aims of the PhD

The research carried out within this thesis aims to address some of the knowledge gaps regarding the prevalence and genotypes of *T. gondii* from animal and human hosts in the UK. The work will also examine whether a live attenuated strain of the parasite (S48) is effective at reducing viable tissue cysts in pigs, additionally, this *in vivo* experiment is designed to enable analysis into which tissues the parasite preferentially disseminates to following either tissue cyst or oocyst challenge.

The research will focus on the following main points:

- Determine the prevalence of *T. gondii* in wild mammalian carnivores by molecular detection of parasite DNA.

- Determine *T. gondii* lineages present within carnivorous wildlife in Great Britain and whether mixed or atypical genotypes exist in this study population.

- Examine the prevalence of *T. gondii* infection from two different study groups; Scottish blood donors (seroprevalence) and human brain samples from the Sudden Death Brain Bank in Edinburgh (molecular detection).

- Discover the rate of seroconversion (or reversion) within Scottish blood donors and whether any recent infections are a result of oocyst or tissue cysts infection.

- Determine the genotypes present from *T. gondii* positive human brain samples.

- Evaluate the effectiveness of a live attenuated strain of *T. gondii* (S48) in its ability to reduce viable *T. gondii* tissue cysts within porcine tissues by mouse bioassay.

- Using a mouse bioassay, determine which porcine tissues the parasite preferentially disseminates to following challenge with either oocysts or tissue cysts.

- Investigate whether detection of parasite DNA using homogenised porcine tissues, prior to inoculation into mice, will be as sensitive as mouse bioassay.

Chapter 2: Materials and Methods

2.1 Experimental animals

All experimental animals were used in accordance with Home Office regulations, and prior to the study commencing experimental design approved by Moredun Research Institute's experiment committee.

2.1.1 Cats

Two male cats (*Felis catus*) aged approximately six months old were obtained from a *Toxoplasma gondii* free colony originating from Spain. Cats were used for the production of oocysts of the Moredun M4 strain. Animals were fed using a commercial cat food that was supplemented with olive oil to prevent constipation, which can commonly occur in cats following infection with *T. gondii*. Both animals were re-homed after use.

2.1.2 Pigs

Twenty three, six week old Large White/Landrace cross bred pigs (*Sus scrofa*) of mixed gender, were obtained from Scottish Agricultural College, Easter Howgate. Animals were separated into five groups depending on experimental challenge (see section 5.2.1 for further details of the experimental design). Animals were fed using a commercial pig feed and water was available *ad libitum*.

2.1.2.1 **Temperature monitoring and health status scoring**

Daily rectal temperatures of all pigs were monitored 14 days post vaccination. At day 0 prior to challenge all animals were micro-chipped, iDENTICHIP® with Bio-Thermo (Animalcare Ltd., York, UK), and temperature monitored for 14 days post challenge.

Daily monitoring of pigs was completed throughout the experiment. Animals were scored on their health status as described in Table 2-1. Any animal which obtained a score of 6 or above were to be treated and removed from the experiment.

Table 2-1: Pig health status scoring system.
Defined end points: 1) any animal scoring 6 or above, 2) an animal that reaches the top score in any of the parameters except for temperature, where temperature below 37°C and above 41.5°C would be defined as the endpoint, or two consecutive days above 41°C but below 41.5°C.

Rectal Temperature (°C)	38 – 40 (0)	37 – 38 (1)	40 – 41 (1)	<37 – 41* (2)
Demeanour	Normal (0)	Depressed (1)	Recumbent (2)	Moribund (3)
Body Condition	Normal (0)	Thin (1)	Anorexic (2)	
Faecal Consistency	Normal (0)	Soft (1)	Fluid (2)	Fluid and Copious (3)
CNS	Normal (0)	Poor coordination, circling, head tilt (1)	Any signs of onset of paralysis (2)	

2.1.2.2 **Blood sampling**

Blood sampling was carried out weekly at days 0, 7, 14, 12 and 28 days post vaccination, then at days 0, 7, 14, 21, 28, 35 and 42 post challenge. Blood was collected using 2.7ml S-monovette serum tube with an S-monovette 20G x 1.5" safety needle (Sarstedt, Leicester, UK). Blood serum was left to clot overnight at 4°C, tubes centrifuged for 10 minutes at 2000g and serum transferred to a sterile 1.5ml Eppendorf. Serum samples were stored at -20°C until required.

2.1.2.3 **Post mortem and collection of samples**

All pigs were culled six weeks post challenge by electrical stunning followed by severing of the jugular vein and exsanguination. At post mortem tissues and body

fluids were collected for pathology, PCR and mouse bioassay (as described in Table 2-2).

Table 2-2: Porcine tissues collected at post mortem.

*** indicates total brain material = 50g, ** indicates 50g of each tissue collected**

Sample No	Sample Type	Fixative			
1	**Brain**	**10% BF**	**4% Para**	**Frozen 5g**	**Bioassay***
A	CNS 1 (internal capsule + cortex)	✓	-	✓	✓
B	CNS 2 (corpus callosum + cortex)	✓	✓	✓	✓
C	CNS 3 (midbrain + hippocampus)	✓	-	✓	✓
D	CNS 4 (hippocampus + cortex)	✓	✓	✓	✓
E	CNS 5 (pons + cerebellum)	✓	-	✓	✓
F	Cervical spinal cord	✓	✓	✓	✓
G	Thoracic spinal cord	✓	✓	✓	✓
H	Lumbar spinal cord	✓	✓	✓	✓
3	**Lymphoreticular system** (slices from the central area)	**10% BF**	**4% Para**	**Frozen 5g**	**Bioassay**
A	Prescapular LN	✓	✓	✓	-
B	Distal jejunal LN	✓	✓	✓	-
C	Ileon (Peyer's patches)	✓	✓	✓	-
E	Mesenteric LN	✓	-	✓	-
F	Retropharyngeal LN	✓	-	✓	-
G	Spleen	✓	-	✓	-
4	**Muscles**	**10% BF**	**4% Para**	**Frozen 5g**	**Bioassay****
A	Heart (interventricular septum)	✓	✓	✓	✓
B	Chop (Longisimus dorsi, 7 toracic)	✓	-	✓	✓
C	loin (middle)	✓	-	✓	✓
D	Fore right limb (triceps)	✓	-	✓	-
E	Fore left limb (triceps)	✓	-	✓	✓
F	Hind right limb (semitendinosus)	✓	-	✓	-
G	Hind left limb (semitendinosus)	✓	-	✓	✓
H	Neck (Trapezius)	✓	-	✓	-
I	Diaphragm	✓	✓	✓	✓
J	Tongue	✓	-	✓	✓
K	Masseter	✓	✓	✓	✓
5	Lung (caudal lobe)	✓	✓	✓	-
6	Liver	✓	✓	✓	-
7	Kidney	✓	✓	✓	-
8	Right Eye	✓	-	✓	-
9	Left Eye	✓	-	✓	-
10	**Body Fluids**	**10% BF**	**4% Para**	**Frozen 5g**	**Bioassay**
A	Blood serum	-	-	✓	-
B	Right Humor Vitreo	-	-	✓	-
C	Right Humor Acuoso	-	-	✓	-
D	Left Humor Vitreo	-	-	✓	-
E	Left Humor Acuoso	-	-	✓	-

2.1.3 Mice

Porton mice, which are a minimally inbred stain, were used for both bioassay, tissue cyst production (sections 2.8 and 2.7 respectively), and used to maintain the Moredun M4 isolate of *T. gondii* (see section 2.4.1). Food and water was supplied *ad libitum.*

2.1.3.1 Health status scoring

Mice inoculated with *T. gondii* were observed twice daily and scored on their health status as described in Table 2-3. Any animals that showed signs of infection i.e. obtained a health status sore of three or above, were removed from the experiment.

Table 2-3: Mouse health status scoring system. Total score = A + B. Animals were killed using a Schedule 1 procedure if an animal reached a maximum score in either category (A or B), or if an animal has a total score of 3 for three consecutive days.

Category	Description	Score
A	Sleek/glossy coat	0
Coat Condition	Ruffled coat	1
(max score 2)	Stary stiff coat	2
B	Bright and active	0
Demeanour	Hunched	1
Scoring cumulative (max score 3)	A reluctance to move	1
	Tottering gait	1

2.1.3.2 Post mortem and collection of samples

Mice that showed either signs of infection or that had survived until the end of the experiment were culled by cervical dislocation (Schedule 1 procedure). Blood samples were taken and brain and heart tissue were collected. Each tissue sample was stored separately in a sterile vial containing 1ml PBS (see Appendix 8.1.6).

2.2 Human samples

All human samples were obtained with appropriate ethical consent.

2.2.1 Blood serum

Serum samples originated from Scottish National Blood Transfusion Services (SNBTS) blood donors, from two cohorts located in both Glasgow (n=1102) and Dundee (n=301). Serum had previously been collected for a Health Protection Scotland *Cryptosporidium* study, and was obtained from Dr Kevin GJ Pollock (Health Protection Scotland, Glasgow, UK) and Dr Claire Alexander (Scottish Parasite Diagnostic Laboratory, Glasgow, UK). Samples were collected over four donation periods which spanned from 24th April 2006 until 09th February 2009 (2006 – 2009). The number of serum samples tested using the *T. gondii* human IgG ELISA (see section 2.10.1) totalled 3274, which included repeat donations from individuals across all collection periods. Each donor completed a questionnaire, which included basic information such as age, gender, and location.

2.2.2 Brain tissue

Brain tissue was obtained from the Medical Research Council Sudden Death Brain and Tissue Bank (MRC Sudden Death Brain and Tissue Bank, Edinburgh, Scotland). The Brain Bank has local Research Ethics Committee approval (LREC 2003/8/37) and works within the legal framework of the Human Tissue (Scotland) Act 2006. Brain tissue was taken from 151 individuals who had died of sudden death (no

known underlying illness or disease). To access these samples a research application was submitted using the Integrated Research Application System (IRAS – myresearchproject.org.uk). The application was reviewed and subsequently approved by the South East Scotland Research Ethics Committee (NHS Lothian, Edinburgh, Scotland), with the following reference numbers; REC number 11/AL/0113, IRAS project number 69852 (see Appendix 8.4). From each individual (n = 151), approximately 1g of tissue from three specific regions of hind brain; pons, cerebellum and medulla (see Figure 2-1) were collected. DNA extraction (see section 2.11.2) and detection of *T. gondii* (see section 2.12.2) was carried out using a pooled sample of three brain regions per individual.

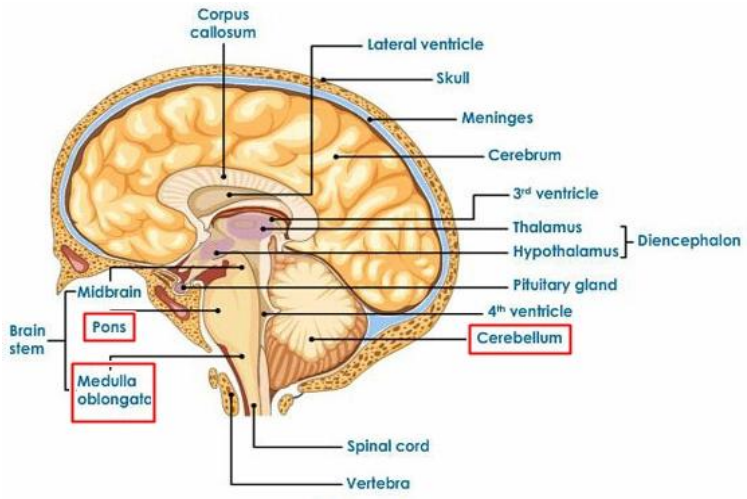

Figure 2-1: Regions of the human brain. Regions pooled and processed for DNA extraction per individual are highlighted in red (pons, cerebellum and medulla).

2.3 Wildlife samples

Tissue samples were collected from six different wildlife species, Eurasian badger (*Meles meles*), ferret (*Mustela furo*), Red fox (*Vulpes vulpes*), American mink (*Neovison vison*), European polecat (*Mustela putorius*), and stoat (*Mustela erminea*), from various locations throughout Great Britain, as described in Table 2-4. Badger and stoat tissue samples were collected following fatal impacts with motor vehicles (badgers were collected with the permission of Scottish Natural Heritage). Where possible full necropsy was performed on the carcasses with tissue samples collected under sterile conditions. All other samples were kindly provided by The University of Edinburgh, The National Museums Scotland and The Food and Environment Research Agency (see Table 2-4). All tissues processed showed no visible signs of decay or putrefaction. Samples were stored at -20^{o}C prior to DNA extraction and PCR amplification.

Table 2-4: Samples collected for detection of *T. gondii* by PCR analysis.

Species	Provider	Locations of samples	Tissue samples collected	No. of animals
Ferret (*Mustela furo)*	National Museum of Scotland	Scotland	brain	99
Red Fox (*Vulpes vulpes*)	University of Edinburgh & Moredun Research Institute	Scotland	brain	83
European Polecat (*Mustela putorius*)	National Museum of Scotland	England, Scotland, Wales	brain	70
American Mink (*Neovison vison*)	The Food and Environment Research Agency	Scotland	brain, tongue, neck muscle	65
Eurasian Badger (*Meles meles*)	Moredun Research Institute	Scotland	brain, tongue, heart, lung, liver, kidney, spleen, spinal cord, clotted blood, submandibular lymph node, neck muscle, hind leg muscle	64
Stoat (*Mustela erminea*)	Moredun Research Institute	Scotland	brain, tongue, heart, lung, liver, kidney, spleen, clotted blood, neck muscle, hind leg muscle	9

2.4 *Toxoplasma gondii* strains

Five different strains of *T. gondii* were used thought the PhD as described in sections 2.4.1 and 2.4.2.

2.4.1 M4, S48 and RH

The Moredun M4 strain was initially isolated at Moredun Research Institute from a case of ovine toxoplasmosis in October 2006. Genotyping using PCR-RFLP has identified M4 as a type II strain.

S48 is an 'incomplete' strain of the parasite which was originally isolated from a case of ovine abortion in Wallaceville, New Zealand (Hartley & Marshall, 1957; Wilkins *et al.*, 1987). This strain is used in the commercial vaccine against ovine toxoplasmosis, Toxovax®, and has been genetically classified as a type I strain of the parasite. Due to repeated passage of the parasite in mice (over 3000 times), this strain has lost the ability to differentiate into the two infectious stages of the parasite, bradyzoites and oocysts, (Buxton & Innes, 1995; O'Connell *et al.*, 1988)

The RH strain was initially isolated in 1937 from a case of acquired toxoplasmosis in a six year old boy (Dubey, 2008; Sabin, 1941). According to the *Toxoplasma* database (ToxoDB), this strain has been genetically classified as a type I.

During this research M4, S48 and RH were cultured in the laboratory (see section 2.5) from tachyzoites which had been stored in LN_2 at Moredun Research Institute.

2.4.2 NED and B1

Tachyzoites from both NED and B1 strains were purchased from the *Toxoplasma* Biological Resource Centre (BRC *Toxoplasma*, Limoges, France). The B1 strain was originally isolated in 2001 from an aborted bovine foetus in Portugal (Canada *et al.*, 2002) and has been classified by BRC *Toxoplasma* to have a type I genotype. The NED strain of the parasite was isolated in 1989 from a case of human toxoplasmosis in France (Darde *et al.*, 1992) and has been classified by BRC

Toxoplasma as a type III strain. On arrival both strains were immediately stored to LN_2. When required, tachyzoites were cultured in the laboratory as described in section 2.5.

2.5 Growth and maintenance of tachyzoites in cell culture

2.5.1 Initial growth

For initial growth of the parasite, tachyzoites, which had been cryopreserved in LN_2 at approximately $1x10^7$, were thawed quickly (no longer than two minutes in a 37°C water bath) and the outside of the vial decontaminated with 70% ethanol. Contents of the vial were transferred to a 15ml tube and washed with 10mls 5% FCS IMDM (see Appendix 8.1.4) at 900g for ten minutes and the pellet resuspended in 10mls 2% FCS IMDM (see Appendix 8.1.5). Five milliliters of the parasite resuspension were added to two T25 flasks containing Vero cells (ATCC No. CCL-81), which, 24 hours previously had been cultured at a concentration $1x10^5$ per flask with 2% FCS IMDM (see Appendix 8.1.5). Flasks were incubated at 37°C, 5% CO^2.

2.5.2 Maintenance

Maintenance of tachyzoites was achieved using Vero cells in a similar fashion to the initial growth of the parasite (see section 2.5.1). A T25 flask containing $1x10^5$ Vero cells per flask with 5% FCS IMDM was prepared 24 hours in advance of sub-culturing and incubated at 37°C, 5% CO^2.

Previously infected flasks were monitored for tachyzoite growth, when approximately 60 -70% of Vero cells were infected, cells were detached from the flask using a cell scraper. Tachyzoites were counted using a Neubauer haemocytometer and $2x10^5$ sub-cultured into the new T25 flask containing $1x10^5$ Vero cells per flask, ensuring media was replaced with 5ml 2% FCS IMDM prior to the addition of tachyzoites. Greater numbers of tachyzoites were produced in an

identical manor, however, a T75 tissue culture flask containing $3x10^5$ Vero cells with addition of $1x10^6$ tachyzoites were used.

2.6 Oocyst production

2.6.1 Oral infection of cats

Two male cats (see section 2.1.1) were used as *T. gondii* donors by infecting each animal with approximately 10^3 tissue cysts (half a *T. gondii* infected mouse brain, refer to section 2.7 for details), mixed with cat food. Faeces were collected daily from 3 days post infection until 20 days post infection and examined for oocysts (see section 2.6.2 and Appendix 8.3)

2.6.2 Identification, extraction and purification of oocysts using salt floatation and differential centrifugation.

2.6.2.1 **Identification**

To identify oocysts from cat faeces the Saturated Salt Floatation Technique was used, as described in Buxton *et al* (1988). Briefly, approximately 1g of faeces was suspended in 10mls sterile dH_2O and centrifuged for 5 minutes at 1000g. The supernatant was discarded and the pellet resuspended in 10mls saturated sodium chloride (NaCl) solution (see Appendix 8.1.8), and centrifugation repeated as previously. The meniscus was transferred to a McMaster slide and visualised under the microscope for the presence of oocysts. The oocysts were identified by their spherical structure and measured approximately 11 to 13µm. If oocysts were present the remainder of the sample was processed as described in section 2.6.2.2

2.6.2.2 **Extraction and purification**

Cat faeces were diluted 1:10 with water and homogenised in a Waring laboratory blender (Fisher Scientific, Loughborough, UK) to form a suspension, then filtered

through 1mm mesh to remove hair and lumps of debris. Fifty milliliter aliquots of the suspension were centrifuged for ten minutes at 1000g and the pellet resuspended in 50mls saturated NaCl solution, followed by further centrifugation at 1000g for 20 minutes. The resulting meniscus (approximately 5-10mls) was carefully removed, diluted 1:10 with dH_2O and centrifuged for 20 minutes at 1000g. Supernatant was removed and the resulting pellet resuspended in 20mls 50% saturated NaCl solution and centrifuged for 20 minutes at 1000g, again the meniscus removed and further diluted 1:10 with dH_2O followed by centrifugation at 500g for 20 minutes. The final pellets were resuspended in 2% sulphuric acid (H_2SO_4) and an aliquot diluted 1:50 with dH_2O for counting using a Neubauer haemocytometer. For processing larger quantities of faeces the methodology was up-scaled using 200ml of faeces suspension and an appropriate increase in reagent volumes. Although some oocysts may have already sporulated, a maximum number of sporulated oocysts is desirable and should therefore undergo further sporulation (see section 2.6.3).

2.6.3 Sporulation of oocysts

Only sporulated oocysts are infective and therefore for infection studies a maximum number should be sporulated. Purified oocyst (see section 2.6.2.2), were transferred to a T25 or T75 tissue culture flask (size dependant on final volume) which contained a hole within the lid which allowed a small piece of rubber tubing to be inserted. The flask was then connected to an air pump which was set to slowly release air bubbles into the oocyst H_2SO_4 suspension. The flask was then incubated at 23^{o}C for 4 days before being examined for signs of sporulation by light microscopy. Once approximately 90% had sporulated, using a differential oocyst count (number of sporulated vs. number of non-sporulated oocysts), oocysts were stored at 4^{o}C and used for infection studies within 2 months.

2.6.4 Preparation of oocyst inoculum

Sporulated oocysts (see section 2.6.3) were washed in sterile 1x PBS (see Appendix 8.1.6) and centrifuged at 1000g for ten minutes. The pellet was resuspended in a

suitable volume of PBS for counting and the number of sporulated oocysts counted. The volume of the PBS/oocysts suspension was adjusted depending on the number of infective oocysts required.

2.7 Tissue cyst production

At Moredun Research Institute Porton mice (see section 2.1.3) were routinely used for the production of tissue cysts. Mice were orally infected with $1x10^4$ M4 tachyzoites (see sections 2.4.1 and 2.5) and if tissue cysts were required an appropriate number of mice culled at least six weeks post infection. Remaining mice were maintained for use until individual animals began to show clinical signs of *T. gondii* infection, at which point the affected mouse was culled (as described in section 2.1.3.1).

2.7.1 Homogenisation of infected mouse brains

Following mouse post mortem (see section 2.1.3.2) an entire mouse brain was homogenised by passing through an increasing gradient of fine gauge needles. Briefly, one millilitre of PBS was added to each individual mouse brain, an 18G needle was attached to a one ml plastic syringe and the mouse brain passed through the needle at least five times to form a homogenate. The brain homogenate was then passed through a 21G needle followed by a 25G needle, ensuring that the brain material could pass through each needle five times.

2.7.2 Determining numbers of tissue cysts by light microscopy and preparation of tissue cyst inoculum

To determine the number of tissue cysts in mouse brain homogenate (see section 2.7.1), 10µl was placed onto a McMaster slide and the number of tissue cysts counted across the entire field. Once the number of tissue cysts present in 10µl of homogenate was determined, the inoculum was prepared according to the number of tissue cysts required using sterile PBS.

2.8 Mouse Bioassay

Porton mice (see section 2.1.3) which are known to be particularly sensitive to *T. gondii* infection were used for all mouse bioassays.

2.8.1 Pepsin/Acid digest of porcine tissues for mouse bioassay

All porcine tissues, used for mouse bioassay, were digested with pepsin/acid as described by (Dubey, 1998a). Briefly, connective tissue, fat and epithelium from muscle tissue (50g) was removed and cut into 1-2cm pieces. Cut tissues were homogenised at low speed for 15 seconds in a robust household blender, and then at high speed with the addition of 100mls sterile saline for 30 seconds. The homogenate was transferred into a 500ml glass Duran bottle and the blender rinsed with 100mls sterile saline to ensure removal of all homogenate. Two hundred and fifty millilitres of pre-warmed (37^{o}C) acid/pepsin solution (see Appendix 8.1.1) was added to the homogenate and incubated for 60 minutes at 37^{o}C, with frequent mixing during incubation. The homogenate was then filtered through two layers of gauze into a 500ml jug and poured into two 250ml polycarbonate centrifuge bottles, which were centrifuged for 10 minutes at 1200g. The supernatant was gently poured off and the sediment, from both 250ml tubes, were combined with 20mls PBS in a 50ml tube. To neutralize the solution 15mls of 1.2% sodium carbonate (see Appendix 8.1.9) was added by gently mixing (mixture will effervesce and lid should be unscrewed after mixing to release air). The neutralised solution was centrifuged for 10 minutes at 1200g, supernatant was poured off gently and pellet resuspended in 3mls sterile saline containing 400µg/ml penicillin 400units/ml streptomycin.

2.9 Antigen preparation from tachyzoites

2.9.1 RH tachyzoite culture for antigen preparation

Tachyzoites of the RH stain (see section 2.4.1) were grown in pre-cultured Vero cells (ATCC No. CCL-81) in IMDM with 2% FCS (see section 2.5) at a ratio of 1:5 (Vero cells : *T. gondii* tachyzoites). The tachyzoites were cultured until all Vero cells were lysed and free tachyzoites were visible in the medium. The medium containing tachyzoites was pooled from each flask and antigen prepared as described in 2.9.2.

2.9.2 Antigen preparation

Culture medium containing free RH strain tachyzoites (see 2.9.1) was centrifuged for 10 minutes at 675g and supernatant discarded. The pellet was washed twice with 50mls PBS (675g for 10 minutes), and the final pellet resuspended in 25mls of PBS. Parasite number was calculated using a Neubauer haemocytometer and adjusted to a final concentration of 10^9 tachyzoites / ml in antigen extraction buffer (see Appendix 8.1.2), with saponin and octylglucosideare (Sigma-Aldrich Company Ltd., Dorset, UK) added at a final concentration of 0.5% each. The suspension was incubated for 16 hours at 4^{o}C on a blood tube rotator, followed by centrifugation for 30 minutes at 50,000g at 4^{o}C. The supernatant was collected and antigen concentration measured using a Pierce BCA Protein Assay Kit (ThermoFisher, Northumberland, UK). Aliquots of supernatant were prepared and stored at -80^{o}C until use.

2.10 Enzyme linked immunosorbent assay (ELISA)

2.10.1 *T. gondii* human IgG ELISA

An in house human IgG ELISA was adapted from the methodology by Opsteegh *et al* (2010) and the final optimisation of the ELISA is described. Microwells of a Greiner Bio-One 96 well medium binding plates were coated with 100µl of solubilized RH antigen (see section 2.9,) at a concentration of 6µg/ml in carbonate

buffer pH9.6 (see 8.1.3) and incubated at 4^{o}C overnight. Following incubation plates were washed 3 times with 300µl PBS (pH7.2) containing 0.05% Tween 20 (PBST – see Appendix 8.1.7). Control sera and test sera were diluted 1/100 in PBST and 100µl of the dilution added to the appropriate microwell, in duplicate. Plates were incubated at room temperature (22 – 25^{o}C) for 1 hour. Plates were washed three times with PBST as previously, followed by addition of 100µl conjugate (anti-human IgG alkaline phosphotase – Sigma Aldrich, Dorset, UK) to each microwell, diluted 1/1000 in PBST. Plates were incubated for 1 hour at room temperature. Following incubation each well was washed 3 times in PBST as previously and 100µl of 1-step PNPP (Thermo Scientific Pierce, Cramlington, Northumberland, UK) was added to each well. The plate was incubated in the dark at room temperature for 30 minutes. Optical density (OD) was measured at 405nm using a Dynex-MRXII microplate reader (Dynex Technologies Limited, Worthing, West Sussex, UK) installed with Revelation software. Cut off values were determined as described in section 4.2.7.3.

2.10.2 Commercial *T. gondii* IgG ELISA (ID.vet)

The ID Screen Toxoplasmosis Indirect Multi-species ELISA kit (ID.vet, Montpellier, France) was used for porcine and mouse serum samples. The supplied manufactures instructions were followed. Briefly, 10µl of test serum was diluted in 90µl of Dilution Buffer 2 and added to the supplied microwells, which are pre coated with recombinant *T. gondii* tachyzoite surface protein (SAG1) as the antigen, and incubated for 45 minutes at room temperature. Diluted serum was then removed and wells washed three times with 300µl of Wash Solution, followed by the addition of 100µl Conjugate 1X. Microwells were incubated for 30 minutes at room temperature, conjugate removed and wells washed three times with 300µl Wash Solution. 100µl of the Substrate Solution was added to each well and incubated for 15 minutes at room temperature, the reaction was then stopped with 100µl Stop Solution and read at an O.D of 450nm.

According to the manufacturer's instructions an ELISA was valid if the mean value of the positive control O.D. (OD_{pc}) was greater than 0.035 ($OD_{pc} > 0.035$), and if the

ration of the mean O.D. values for the positive and negative controls (OD_{pc} and OD_{nc}), were greater than 3.5 ($OD_{pc}/OD_{nc} > 3.5$).

The interpretation of the result was classed as percent seropositivity (SP) value which was calculated using the following formula:

$$SP = \frac{OD_{sample} - OD_{nc}}{OD_{pc} - OD_{nc}} \times 100$$

A sample with an SP value of 50% or higher was positive, a negative result was an SP of 40% or less, and the result classed as doubtful if the SP was between 40% - 50%.

2.11 DNA Extraction

2.11.1 Precellys method

DNA was extracted from approximately 1g of homogenised tissue. Briefly, tissue was placed into a CK28 precellys tube (tubes prefilled with ceramic beads with a diameter of 2.8 mm - Peqlab, Sarisbury Green, Hampshire, UK), containing 1000µl Nuclei Lysis Solution (Promega, UK), and the tissue homogenised using a Precellys® 24 homogeniser (Peqlab) using two cycles of 50 seconds at 380g (6500rpm). 400µl of homogenate was further processes with 900µl Nuclei Lysis Solution (Promega), and incubated overnight in a water bath at 55^{o}C (the remaining unused tissue homogenate was stored at -20^{o}C for future use). Once removed the lysate was allowed to cool to room temperature, 300µl Protein Precipitation Solution (Promega) was added and mixed for 20 seconds using a vortex, then incubated on ice for 5 minutes. Following incubation the mixture was centrifuged at 13,000g for 5 minutes and the resulting supernatant transferred to a 2ml Eppendorf tube, containing 900µl isopropanol, mixed by inversion and incubated at -20^{o}C overnight. The DNA was pelleted by centrifugation at 13,000g for 5 minutes, supernatant removed and DNA pellet washed with 600µl 70% ethanol. Centrifugation was repeated for 2

minutes at13,000g, supernatant removed and the DNA pellet allowed to briefly air dry. The final pellet was re-suspended in 200µl distilled H_2O. The DNA was stored at 4^oC for immediate use or at -20^oC for longer term storage.

2.11.2 GentleMacs method

For larger quantities of DNA, approximately 5 - 8g of tissue was place into a gentleMACS M tube (Miltenyi Biotec, Woking, Surrey, UK) containing 6mls of Nuclei Lysis Solution (Promega) and samples homogenised at using a GentleMacs tissue dissociator (Miltenyi Biotec). All the homogenate was transferred into a 15ml centrifuge tube and incubated overnight at 55^oC. Following incubation samples were allowed to cool to room temperature and 1.5ml Protein Precipitation Solution (Promega) was added and mixed by inversion several times, then incubated on ice for 5 minutes. Samples were centrifuged at 1200g for 30 minutes and the supernatant transferred into a 15ml centrifuge tube containing 7.5ml isopropanol. After gentle mixing by inversion, precipitated DNA was centrifuged for 10 minutes at 1000g and the pellet washed with 2ml 70% ethanol, supernatant removed and pellet briefly air dried and resuspended in 700µl distilled H_2O. The DNA was stored at 4^oC for immediate use or at -20^oC for longer term storage.

2.12 Polymerase chain reaction

2.12.1 Standard PCR reaction

Standard 20µl PCR reactions contained 2µl 10x custom PCR mix – SM0005 (45mM Tris-HCl. 11mM $(NH_4)_2SO_4$, 4.5mM $MgCl_2$, 0.113mg/ml BSA, 4.4µM EDTA and 1.0mM dATP, dATC, dGTP, dTTP – ABgene, Epsom, Surrey, UK), 0.75 units BioTaq (Bioline, London, UK), and 2µl DNA. Positive control DNA was included in all PCR runs and consisted of *T. gondii* DNA derived from tachyzoites grown in tissue culture, negative controls were also included and consisted of sterile dH_2O or DNA extraction controls. All reactions were made to a final volume of 20µl with

sterile dH_2O. Primers, their concentration, and cycling conditions varied depending on the specific reaction (see sections 2.12.2 and 2.12.4).

2.12.2 Diagnostic nested ITS1 PCR

The nested PCR targets the multicopy 18S-5.8S rRNA internal transcribed spacer (ITS1) region of the parasite, and is routinely used at Moredun Research Institute for diagnostic purposes for detection of *T. gondii* infection within DNA from host tissue samples. Primers and methodology were adapted from Buxton *et al* (2001), and Hurtado et al (2001). To improve the sensitivity of the technique, each reaction was carried out in triplicate.

First round reactions were conducted as described for the standard PCR reaction (see section 2.12.1), using 5µM of primers NN1-ext-F and NN1-ext-R ((Buxton *et al.*, 2001) (see Table 2-5). Cycling conditions were 5 min at 95°C, followed by 35 cycles of 1 min at 95°C, 1 min at 55°C, 1 min at 72°C, and a final extension period of 5 min at 72°C. To reduce unused primers from the primary PCR, first round PCR products were diluted 1:5 with dH_2O. The conditions for each second round reaction were identical to the first, except 2µl of diluted first round product was used instead of DNA, and 5µM of primers Toxo_NP-1_F and Toxo_NP-2_R ((Hurtado *et al.*, 2001), (see Table 2-5) were used. All PCR products were visualised following electrophoresis in 2% agarose gels incorporating Biotum Gel Red™ (Cambridge Bioscience Ltd, Cambridge, UK).

Table 2-5: Primers used for diagnostic PCR.

Primers 5' - 3' (external forward, external reverse internal forward, internal reverse')		Nested PCR Product Size (bp)
NN1_ext_F	tca acc ttt gaa tcc caa	227
NN2_ext_R	cga gcc aag aca tcc att	
Toxo_NP-1_F	gtg ata gta tcg aaa ggt at	
Toxo_NP-1_F	act ctc tct caa atg ttc ct	

2.12.3 Host actin and inhibition control PCR

To ensure DNA was present for each animal and to ensure that any negative result was not due to PCR inhibition, a PCR reaction which amplified host actin was carried out from the diluted ITS1 first round products. The reaction used the same standard PCR as previously (see section 2.12.1) but contained 2µl diluted first round product, with 4µM of primers Uni_Actin_F3 and Uni_Actin_R3 (see Table 2-6). Cycling conditions were as previously described for the diagnostic nested ITS1 PCR (see section 2.12.2). All PCR products were visualised following electrophoresis in 2% agarose gels incorporating Biotum Gel Red™ (Cambridge Bioscience Ltd, UK).

Table 2-6: Primers used for actin PCR

Primers 5' - 3' (forward and reverse)		Product Size (bp)
Uni_Actin_F3	cat cct gcg tct gga cct	132
Uni_Actin_R3	tag cag agc ttc tcc ttg atg tc	

2.12.4 Genotyping Mn-PCR-RFLP

This method was adapted from work previously described by (Prestrud *et al.*, 2008) and (Su *et al.*, 2010) and the methodology summarised in Figure 2-2. Briefly, each 20µl multilocus PCR reaction was set up as described in section 2.12.1, but contained 0.1µM of forward and reverse external primers for the following markers – 5'SAG2, 3'SAG2, SAG3, GRA6 and BTUB (see Table 2-7), 0.75 units BioTaq (Bioline, London, UK), 5.85µl dH_2O and 2µl DNA. To improve the sensitivity of the technique each reaction was carried out in quadruplicate. First round cycling conditions were 4 min at 95^oC, followed by 25 cycles of 30 seconds at 94^oC, 1 min at 53.7^oC, and 2 min at 72^oC. First round PCR products were diluted 1:1 with dH_2O. The nested second round reaction used separate internal primers for each marker and used the diluted first round PCR products instead of DNA. Each individual 20µl second round reaction contained 2µl 10x custom PCR mix (SM0005), 0.3µM primers (individual forward and reverse internal markers for 5'SAG2, 3'SAG2,

SAG3, GRA6 and BTUB – see Table 2-7) 0.75 units BioTaq, 13.85µl dH_2O, and 2µl of diluted first round PCR product. Cycling conditions were 4 min at 95^{o}C, followed by 40 cycles of 30 seconds at 94^{o}C, 1 min at 60^{o}C, and 1.5 min at 72^{o}C. RFLP typing was achieved by preparing an enzyme digest which contained 2µl of the second round product, 2µl of 10x NEB Buffer 4 (New England Biolabs, Hitchin, Herts, UK), 4 units of the appropriate restriction enzyme (New England Biolabs – see Table 2-7) made up to a final volume of 20µl in dH_2O. Each digest was incubated for 1hr at the appropriate temperature depending on the restriction enzyme used (see Table 2-7). Digested products were visualised following electrophoresis using a 3% Metasieve Agarose gel incorporating Biotum Gel Red™ (Cambridge Bioscience Ltd, Cambridge, UK).

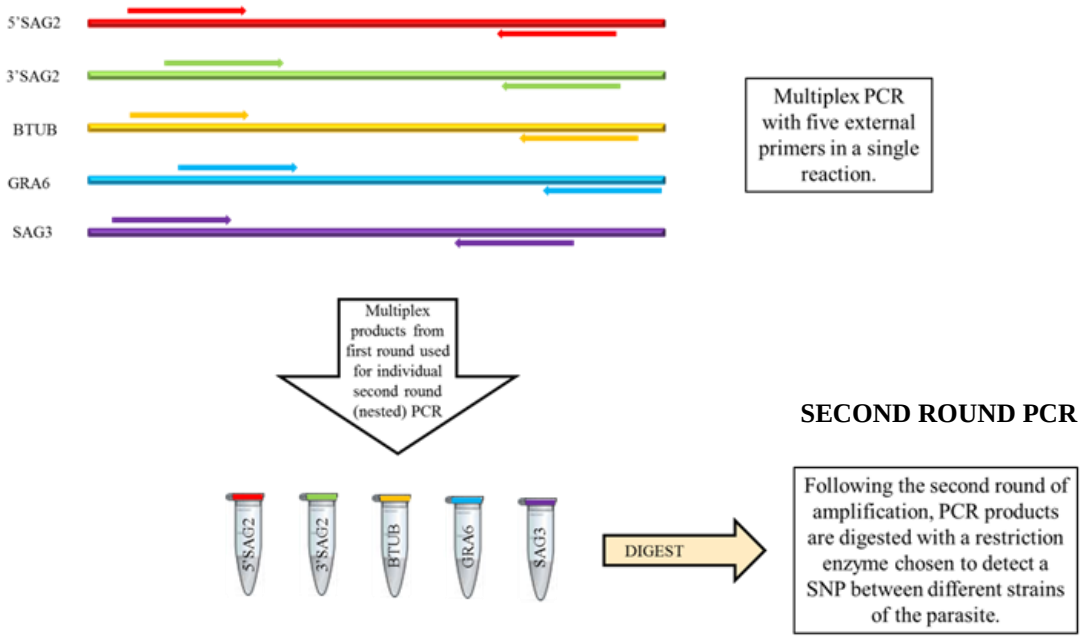

Figure 2-2: PCR-RFLP Genotyping methodology. An initial multiplex reaction followed by nested PCR. Products generated are then digested by a restriction enzyme chosen to detect a SNP which differs between parasite strains. Digested products are resolved on a 3% Metasieve agarose gel .

Table 2-7: Primers used for Mn-PCR-RFLP

Marker	Primers 5' - 3' (external forward, external reverse internal forward, internal reverse)		Nested PCR Product Size (bp)	Restriction Enzyme Digest	Reference
5'SAG2	5'SAG2_ext_F	gtc acc tgc aac agg aac ac	242	MboI 1hr @ 37°C	Howe *et al.* 1997; Su *et al.* 2006
	5'SAG2_ext_R	gca tca aca gtc ttc gtt gc			
	5'SAG2_int_F	gaa atg ttt cag gtt gct gc			
	5'SAG2_int_R	gca aga gcg aac ttg aac ac			
3'SAG2	3'SAG2_ext_F	tct gtt ctc cga agt gac tcc	222	HhaI 1hr @ 37°C	Howe *et al.* 1997
	3'SAG2_ext_R	tca aag cgt gca tta tcg c			
	3'SAG2_int_F	att ctc atg cct ccg ctt c			
	3'SAG2_int_R	aac gtt tca cga agg cac ac			
SAG3	SAG3_ext_F	caa ctc tca cca ttc cac cc	225	NciI1hr @ 37°C	Grigg *et al.* 2001
	SAG3_ext_R	gcg cgt tgt tag aca aga ca			
	SAG3_int_F	tct tgt cgg gtg ttc act ca			
	SAG3_int_R	cac aag gag acc gag aag ga			
GRA6	GRA6_ext_F	att tgt gtt tcc gag cag gt	344	MseI 1hr @ 37°C	Khan *et al.* 2005a; Su *et al.* 2006
	GRA6_ext_R	gca cct tcg ctt gtg gtt			
	GRA6_int_F	ttt ccg aga agg tga cct			
	GRA6_int_R	tcg ccg aag agt tga cat ag			
BTUB	BTUB_ext_F	tcc aaa atg aga gaa atc gt	411	BsiEI+TaqI 1hr @ 60°C	Khan *et al.* 2005a; Su *et al.* 2006
	BTUB_ext_R	aaa ttg aaa tga cgg aag aa			
	BTUB_int_F	gag gtc atc tcg gac gaa ca			
	BTUB_int_R	ttg tag gaa cac ccg gac gc			
SAG1	SAG1_ext_F	gtt cta acc acg cac ctt gag	390	n/d	Grigg *et al.* 2001
	SAG1_ext_R	aag agt ggc agg ctc agt ga			
	SAG1_int_F	caa tgt gca cct gta gga agc			
	SAG1_int_R	gtg gtt ctc cgt cgg tgt gag			
alt. SAG2	alt.SAG2_ext_F	gga acc cga aca atg agt tt	546	n/d	Khan *et al.* 2005a; Su *et al.* 2006
	alt.SAG2_ext_R	gca ctg ttg tcc agg gtt tt			
	alt.SAG2_int_F	acc cat ctg cga aga aaa cg			
	alt.SAG2_int_R	att tcg acc agc ggg agc ac			
C22-8	C22-8_ext_F	tga tgc atc cat gcg ttt at	521	n/d	Khan *et al.* 2005a; Su *et al.* 2006
	C22-8_ext_R	cct cca ctt ctt cgg tct ca			
	C22-8_int_F	tct ctc tac gtg gac gcc			
	C22-8_int_R	agg tgc ttg gat att cgc			
C29-2	C29-2_ext_F	acc cac tga gcg aaa aga aa	446	n/d	Khan *et al.* 2005a; Su *et al.* 2006
	C29-2_ext_R	agg gtc tct tgc gca tac at			
	C29-2_int_F	agt tct gca gag tgt cgc			
	C29-2_int_R	tgt cta gga aag agg cgc			
L358	L358_ext_F	tct ctc gac ttc gcc tct tc	418	n/d	Khan *et al.* 2005a; Su *et al.* 2006
	L358_ext_R	gca att tcc tcg aag aca gg			
	L358_int_F	agg agg cgt agc gca agt			
	L358_int_R	ccc tct ggc tgc agt gct			
PK1	PK1_ext_F	gaa agc tgt cca ccc tga aa	903	n/d	Khan *et al.* 2005a; Su *et al.* 2006
	PK1_ext_R	aga aag ctc cgt gca gtg at			
	PK1_int_F	cgc aaa ggg aga caa tca gt			
	PK1_int_R	tca tcg ctg aat ctc att gc			
Apico	Apico_ext_F	tgg ttt taa ccc tag att gtg g	640	n/d	Su *et al.* 2006
	Apico_ext_R	aaa cgg aat taa tga gat ttg aa			
	Apico_int_F	gca aat tct tga att ctc agt t			
	Apico_int_R	ggg att cga acc ctt gat a			

2.13 Sequencing and analysis

2.13.1 Purification of PCR products

All PCR products sent for sequencing were excised from agarose gel and purified using the Promega Wizard SV gel and PCR clean up system (Promega, UK) in accordance with the manufacturer's instructions. Briefly, a band was cut from an agarose gel, weighed and transferred into a sterile 1.5ml eppendorf tube to which 10µl of Membrane Binding solution per 10mg was added and mixed thoroughly. The sample was incubated at 60^{o}C until the gel slice had completely dissolved. The dissolved mixture was transferred into a SV Minicolumn and Collection Tube and incubated at room temperature for 1 minute, followed by centrifugation at 16,000g for 1 minute. The Minicoulmn, which contained the bound DNA, was washed twice with Membrane Wash Solution at 16,000g for 5 minutes followed by centrifugation for 5 minutes at 16,000g to remove any residual ethanol for the Wash Solution. DNA was eluted from the Minicolumn by incubating with 35µl of sterile dH_2O at room temperature for 1 minute, followed by centrifugation at 16,00g for 1 minute. DNA was quantified (see section 2.13.2) and stored at -20^{o}C until further use.

2.13.2 DNA quantification

DNA concentration and purity were determined using a Nanodrop ND1000 spectrophotometer (Labtech International Ltd, Uckfield, East Sussex, UK). DNA was measured at an absorbance of 260nm, whilst purity was measured using the ratio between readings at 260nm and 280nm.

2.13.3 Sequencing

Purified PCR products were sent to GATC (GATC, Konstanz, Germany) using their "Lightrun" sequencing service, where sequencing was performed using an Applied Biosystems 3730xl DNA Analyser. 5µl of purified PCR product was sent at a

concentration of between 20 and 80ng/µl containing 5µl of the appropriate forward or reverse primer at a concentration of 5pmol/µl.

2.13.4 Sequence analysis

Sequences were analysed using BioEdit software (biological sequence alignment editor for windows 95/98/NT/2000/XP). A free download is available at http://www.mbio.ncsu.edu/bioedit/bioedit.html

2.13.5 Sequence databases and search engines

Sequence databases used throughout included NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and ToxoBD (http://toxodb.org/toxo/).

2.13.6 Submission of sequences to GenBank

Sequences which were submitted for GenBank accession numbers were completed in accordance with BankIt (http://www.ncbi.nlm.nih.gov/BankIt/help.html).

Chapter 3: Prevalence and genetic diversity of *T. gondii* from wild mammalian carnivores in Great Britain[1]

Aims:

- Determine the prevalence of *T. gondii* in wild mammalian carnivores by molecular detection of parasite DNA.

- Identify the most sensitive and informative PCR-RFLP markers to enable genotyping of *T. gondii* positive wildlife samples.

- Determine the *T. gondii* lineages present within carnivorous wildlife in Great Britain and whether mixed or atypical genotypes exist in this study population.

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[1]The work described herein forms the basis of a peer-reviewed publication (see Appendix 8.5):

Burrells A, Bartley P. M, .Zimmer I. A., Roy S, Kitchener A. C., Meredith A., Wright S. E., Innes E. A., Katzer F. (2013) Evidence of the three main clonal *Toxoplasma gondii* lineages from wild mammalian carnivores in the UK. *Parasitology* **140**, 1768-1776

3.1 Introduction

Wildlife are known intermediate hosts for *T. gondii*, infected animals harbour tissue cysts containing hundreds (even thousands) of infective bradyzoites (Dubey *et al.*, 1998a). Studying wildlife provides an indication of the prevalence of *T. gondii* and the strains present in the environment. Studying carnivorous wildlife is particularly important as these animals become infected not only via *T. gondii* oocysts in the environment, but also via carnivory, consuming other infected host species. It is this feature which makes wild mammalian carnivores a useful sentinel species, providing an indication to not only the prevalence but also the strains present within the environment.

Molecular characterisation of the parasite using multilocus nested PCR-RFLP can identify the predominant lineages, as well as recombinant or mixed infections, which contain a combination of alleles from type I, II and III (Su *et al.*, 2006). This technique has been used to identify the alleles present in a variety of species worldwide, including wildlife, livestock and humans (Canon-Franco *et al.*, 2013; Halova *et al.*, 2013; Wang *et al.*, 2013), and has also been used to identify atypical strains which are comprised of unique genotypes (Pena *et al.*, 2013). Although research of *T. gondii* in wildlife has been carried out worldwide on a range of species, including grey wolves (*Canis lupus*), bobcats (*Lynx rufus*) and white-tailed deer (*Odocoileus virginianus*) in the US (Dubey *et al.*, 2013b; Yu *et al.*, 2013) and red foxes (*Vulpes vulpes*), rodents and roe deer (*Capreolus capreolus*) in Europe (Aubert *et al.*, 2010; Herrmann *et al.*, 2012), recent figures regarding the prevalence of *T. gondii* in British wildlife is limited and is predominantly based on serological detection other than molecular detection. The added bonus of *T. gondii* detection using molecular technologies is the ability to complete strain genotyping using PCR-RFLP, which cannot be achieved using serology based methods.

Serological data from the United Kingdom has found a *T. gondii* seroprevalence in red foxes of 20% (Hamilton *et al.*, 2005), whilst in Eurasian badgers (*Meles meles*), the prevalence was found to vary between 39.0% - 77% (Anwar *et al.*, 2006). Another study examined the prevalence of the parasite in wild brown rats (*Rattus*

norvegicus), and found a mean seroprevalence of 35.0% (Webster, 1994). In addition, although not considered a wildlife species, the prevalence within 125 sheep flocks (which included a representative sample of the entire Scottish flock), described an overall *T. gondii* seroprevalence of 56.6% (Katzer *et al.*, 2011). This indicates a high level of environmental contamination from *T. gondii* oocysts. Unfortunately none of the work previously described allowed parasite strain genotyping, which can have epidemiological importance, particularly when comparing genotypes to those found in livestock and human infections. One study which identified the different *T. gondii* alleles present in woodmice (*Apodemus sylvaticus*) within North Yorkshire, UK, found evidence of alleles for type I, II and III using PCR-RFLP (Thomasson *et al.*, 2011). However this study only used a single RFLP marker (SAG3), meaning mixed infections or atypical strains would not be identified. More recently a study investigating the seroprevalence of *T. gondii* in Eurasian otters (*Lutra lutra*) from England and Wales found a high incidence of the parasite, where 39.5% of animals were seropositive (Chadwick *et al.*, 2013). Whilst in Ireland research by Halova *et al* (2013) examined both the seroprevalence and molecular detection of *T. gondii* in food producing animals (sheep, pigs, chicken and deer). These results show a greater number of animals to be positive by serological detection (seropositivity ranged from 36% in sheep, 18% in chickens, 4.7% in pigs, and 6.6% in deer) when compared to molecular detection of the parasite from diaphragm samples (*T. gondii* DNA was detected in 13% of pigs, 4.2% of deer and 3.6% of sheep). However, the study also used meat digestion liquids to detect *T. gondii* DNA from pigs previously used in a *Trichinella* spp survey, using this methodology an increase in the molecular detection of the parasite was observed with 50% of animals reported as *T. gondii* positive.

Within this study host DNA from wild mammalian carnivores was used to not only detect the presence of parasite DNA, but also genotype the parasite using multilocus nested PCR-RFLP across five markers (SAG3, GRA6, 5'SAG2, 3'SAG2 and BTUB). However, initial testing of twelve markers (additional markers included SAG1, altSAG2, C22-8, C29-2, L358, PK1 and Apico) was also carried out and five were selected as the most informative and sensitive to be used for this study. Using

five markers will not only identify alleles for the predominant *T. gondii* lineages but will also enable identification of mixed or atypical genotypes.

Therefore the aim of this study was to determine the prevalence of *T. gondii* in wild mammalian carnivores by PCR, and to genetically characterise the genotype present using five of the most sensitive and informative PCR-RFLP markers.

3.2 Materials and methods

3.2.1 Optimisation and sensitivity of nested PCR-RFLP markers

Initially twelve PCR-RFLP markers (see
Table 2-5) were used to test the sensitivity of each marker and to enable optimisation of the PCR conditions. Murine host DNA which had been spiked with tachyzoite DNA derived from the M4 strain of *T. gondii* (see 2.4.1), was prepared at the following dilutions 10^{2}, 10^{1}, 10^{-1},10^{-2}, 10^{-3} tachyzoites / ml. Methodology for the genotyping Mn-PCR-RFLP was followed (see 2.12.4) using all twelve primers described in Table 2-7. However, during thermal cycling (see 2.12.4) the annealing temperature for each first round PCR was assessed on a temperature gradient which ranged from 53.1^{o}C – 61.4^{o}C.

3.2.2 DNA from wildlife samples

Tissue samples were collected from Eurasian badger, red fox, American mink, European polecat, and stoat, as described in section 2.3 and also further detailed in Table 2-4. DNA extractions were completed using 1g of tissue as described in section 2.11.1

3.2.3 Detection of *T. gondii* by ITS1 PCR

All DNA samples were amplified using the diagnostic nested ITS1 PCR (see 2.12.1) and electrophoresed in 2% agarose gels. A sample was regarded as positive if an amplicon of 227bp was present in one or more of the three reactions per sample. Multiple negative controls (dH_2O) as well as extraction controls were included in each PCR run, and results only accepted if these reactions were negative.

3.2.4 Detection of host DNA

DNA samples were amplified using an actin PCR to ensure host DNA was present and that PCR inhibitors were absent (see section 2.12.3). This was carried out to ensure that any negative result observed was a true negative and not due to PCR inhibition.

3.2.5 Genetic characterisation of *T. gondii* by multiplex nested PCR-RFLP

Genotyping was applied to all *T. gondii* positive samples (as described in section 2.12.4) using the five markers identified as the most sensitive and informative (SAG3, GRA6, 5'SAG2, 3'SAG2 and BTUB).

3.2.6 DNA sequencing and sequence analysis

Twenty five SAG3 PCR products were directly sequenced using both forward and reverse SAG3 internal primers (see Table 2-7). Products were purified (see section 2.13.1) and sequencing completed at GATC Biotech (section 2.13.3), and results analysed using BioEdit sequence analysis software (section 2.13.4), and consensus sequences generated. To enable identification of polymorphisms, a 166bp fragment of the published sequences representing the three clonal archetypal *T. gondii* lineages were used as reference sequences, which included RH – type I, Pru – type II and CEP – type III (GenBank accession numbers AF340227, AF340228, AF340229 respectively). Direct sequencing using forward and reverse internal primers was also completed to obtain representative sequences for 5'SAG2, 3'SAG2, GRA6, and BTUB.

3.3 Results

3.3.1 Five most informative and sensitive PCR-RFLP markers identified

Following adjustment of the first round PCR annealing temperature it was found that 53.7^{o}C was the optimum annealing temperature (data not shown) to enable detection of parasite DNA over twelve markers (as listed in Table 3-1). Markers 5'SAG2 and 3'SAG2 were the most sensitive and reliably detected *T. gondii* DNA in dilutions as low as 10^{-1} per ml (see Table 3-1). In addition, when these markers are used in combination with each other, they can distinguish between the three main archetypal lineages. An additional seven markers (SAG3, GRA6, BTUB, SAG1, C22-8, L358 and Apico) could detect parasite DNA at a dilution which contained approximately one tachyzoite per ml. The PK1 marker was the least sensitive and was only able to detect parasite DNA at a concentration which contained approximately 100 tachyzoites per ml, as described in Table 3-1.

The markers SAG3 and GRA6 are two of the most informative markers, as a single restriction digest (NciI and MseI respectively – see Table 2-7) of PCR products generated from these markers can distinguish between the tree archetypal lineages (I, II and III) (Khan *et al.*, 2005a; Su *et al.*, 2006). Also PCR products generated from the BTUB marker can be used to distinguish between the tree archetypal lineages using a double restriction enzyme digest (BsiEI and TaqI – see Table 2-7).

From these results the five most sensitive and informative markers to be used in this study were 5'SAG2, 3'SAG2, SAG3, GRA6 and BTUB. These markers represent four distinct loci within the *T. gondii* genome (as further described in section 1.4).

Table 3-1: Sensitivity of detection of *T. gondii* by PCR with 12 different markers using an annealing temperature of 53.7°C. Key: "+" = positive result, "-" = negative result.

Marker	Dilution *T. gondii* / ml diluted in host DNA					
	10^{2}	10	1	10^{-1}	10^{-2}	10^{-3}
5'SAG2	+	+	+	+	-	-
3'SAG2	+	+	+	+	-	-
SAG3	+	+	+	n/d	n/d	n/d
GRA6	+	+	+	-	-	-
BTUB	+	+	+	-	-	-
SAG1	+	+	+	-	-	-
alt. SAG2	+	+	-	-	-	-
C22-8	+	+	+	-	-	-
C29-2	+	+	-	-	-	-
L358	+	+	+	-	-	-
PK1	+	-	-	-	-	-
Apico	+	+	+	-	-	-

3.3.2 Prevalence off *T. gondii* varies between host species and tissues tested

Overall 22.8% (89/390) of the animals tested were positive for *T. gondii*. The prevalence varied depending on the host species (see Table 3-2), 31.4% (22/70) of polecats tested positive, whilst only 6.0% (5/83) of foxes were positive. Mink, badger and ferret had a prevalence of 29.2% (19/65), 25.0% (16/64) and 23.2% (23/99) respectively. Stoats showed a higher prevalence with 44.4% (4/9) of animals tested PCR positive, however it should be noted that this group had a lower representative sample size and was collected from a geographically smaller area than the other species tested.

Multiple tissues were used to test for the presence of *T. gondii* in mink, badger and stoat. Mink brain tissue accounted for 20.0% (13/65) of positive samples within this species, followed by tongue and heart tissue (10.8% and 1.5% respectively), whilst in badgers, brain tissue accounted for 5.0% (3/60) of positive samples, while tongue and heart tissue had a positivity of 6.3% (2/32) and 7.7% (4/54) respectively (see Table 3-3). Multiple tissues from the same animal were found to be positive for mink, and badger (see Figure 3-1), with an individual badger having up to three tissues positive for *T. gondii*.

All DNA samples tested were positive for the detection of host DNA. Therefore any negative result observed in the ITS1 PCR (or negative result from the same DNA when using the genotyping PCR-RFLP primers), was not due to degraded DNA or the presence of PCR inhibitors.

Table 3-2: Samples collected for detection of *T. gondii* and parasite prevalence by diagnostic nested ITS1 PCR

							95% CI	
Species	Provider	Locations of samples	Tissue samples collected	No. of animals tested	No. of animals positive	Mean % Positive	Lower	Upper
Ferret (*Mustela furo)*	National Museum of Scotland	Scotland	brain	99	23	23.2	15.3	32.8
Red Fox (*Vulpes vulpes*)	University of Edinburgh & Moredun Research Institute	Scotland	brain	83	5	6.0	1.9	13.5
European Polecat (*Mustela putorius*)	National Museum of Scotland	England, Scotland, Wales	brain	70	22	31.4	20.9	43.6
American Mink (*Neovison vison*)	The Food and Environment Research Agency	Scotland	brain, tongue, neck muscle	65	19	29.2	18.6	41.8
Eurasian Badger (*Meles meles*)	Moredun Research Institute	Scotland	brain, tongue, heart, lung, liver, kidney, spleen, spinal cord, clotted blood, submandibular lymph node, neck muscle, hind leg muscle	64	16	25.0	15.0	37.4
Stoat (*Mustela erminea*)	Moredun Research Institute	Scotland	brain, tongue, heart, lung, liver, kidney, spleen, clotted blood, neck muscle, hind leg muscle	9	4	44.4	13.7	78.8

Table 3-3: Individual tissues collected and *T. gondii* positivity.

Species	Number of tissues positive / Number of tissues collected (%)										
	Br	Tn	He	Ne	Lu	Kid	Sp	Bl	Hlm	Sln	Sc
Ferret	23/99 (23.2)	-	-	-	-	-	-	-	-	-	-
Red Fox	5/83 (6.0)	-	-	-	-	-	-	-	-	-	-
European Polecat	22/70 (31.4)	-	-	-	-	-	-	-	-	-	-
American Mink	13/65 (20.0)	7/64 (10.8)	1/65 (1.5)	-	-	-	-	-	-	-	-
Eurasian Badger	3/60 (5.0)	2/32 (6.3)	4/54 (7.7)	3/54 (5.6)	1/52 (1.9)	1/52 (1.9)	3/52 (5.8)	1/52 (1.9)	2/52 (3.8)	2/37 (5.4)	2/12 (16.6)
Stoat	2/9 (22.2)	0/2 (0.0)	0/1 (0.0)	2/7 (28.6)	0/1 (0.0)	0/1 (0.0)	0/1 (0.0)	0/1 (0.0)	0/2 (0.0)	-	-

Br = Brain, Tn = Tongue, He = Heart, Ne = Neck Muscle, Lu = Lung, Kid = Kidney, Sp = Spleen, Bl = Clotted Blood, Hlm = Hind leg muscle, Sln = Submandibular lymph node, Sc = Spinal Cord

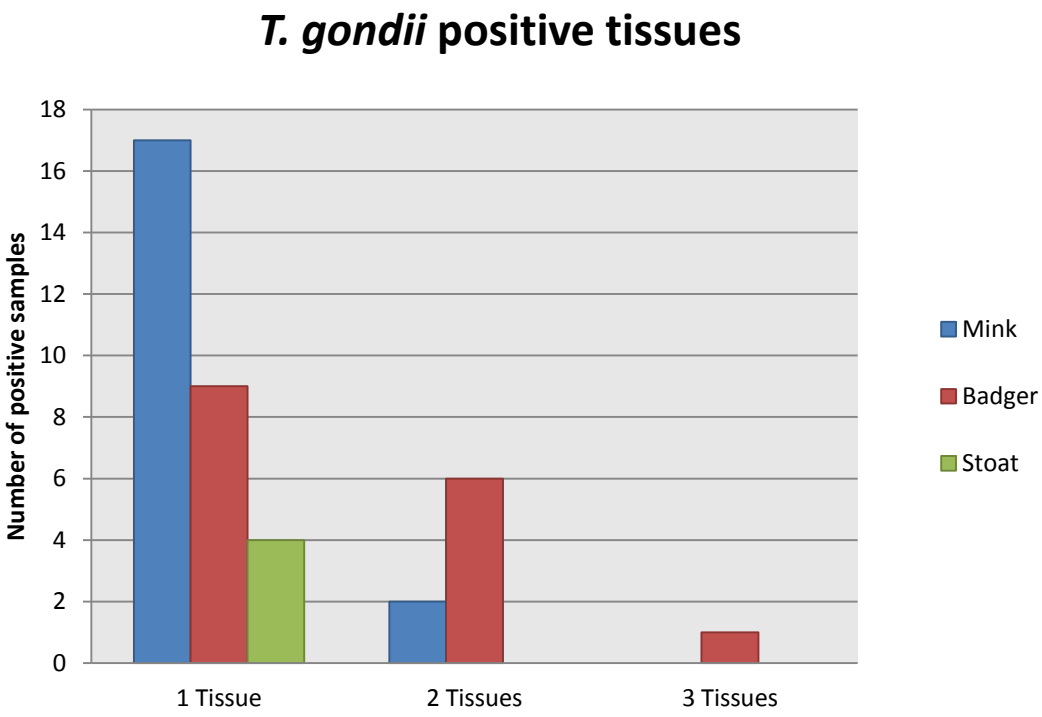

Figure 3-1: Samples which were positive for more than one tissue by ITS1 PCR. Badger showing the greatest number of *T. gondii* positive tissues (n=3).

3.3.3 PCR-RFLP genotyping reveals evidence of alleles for all three main clonal lineages

Of the 89 animals that tested positive for *T. gondii* by ITS1 PCR (see section 3.3.2), 30 were successfully amplified with PCR-RFLP primers for three or more markers across three loci (33.7%). These included nine polecats, twelve ferrets, six mink, two badgers and one fox (see Table 3-4). Two polecats (Pc07 and Pc26) only amplified at a single locus (SAG3), which identified a type I allele in both animals. Attempts to amplify products at additional loci in these animals failed.

A type III lineage was identified across all five markers (four loci) in one polecat (Pc39), however, the predominant lineage across all species genotyped was type II (90.6%). PCR-RFLP genotyping across all markers (four loci) for the type II lineage was achieved in 23 animals which included five mink, 11 ferrets, and seven polecats. Partial typing of the type II lineage was seen in six animals; two badgers, one fox, one mink, one ferret and one polecat.

Within this group of animals, multi locus genotyping highlighted the presence of alleles for the three main clonal lineages of *T. gondii*, with no evidence of recombinant genotypes or atypical strains.

As previously stated, all DNA samples tested positive for the detection of host actin DNA, therefore any samples which were negative for PCR-RFLP genotyping were not due to PCR inhibition or degraded DNA.

Table 3-4: *T. gondii* PCR-RFLP genotyping from thirty two infected animals across five markers.

Reference/ Species	Animal ID	PCR-RFLP Marker					Genotype
		5'SAG2	3'SAG2	SAG3	GRA6	BTUB	
RH - Type I	Reference	I or II	I or III	I	I	I	I
M4 - Type II	Reference	I or II	II	II	II	II	II
NED - Type III	Reference	III	I or III	III	III	III	III
Polecat	Pc39	III	I or III	III	III	II or III	III
Polecat	Pc07, Pc26	na	na	I	na	na	I*
Polecat	Pc10, Pc21, Pc27, Pc30, Pc34, Pc43, Pc53	I or II	II	II	II	II	II
Polecat	Pc63	na	na	II	II	II	II
Ferret	Ft04	na	na	II	II	II	II
Ferret	Ft02, Ft05, Ft15, Ft39, Ft42, Ft49, Ft50, Ft55, Ft64, Ft82, Ft83	I or II	II	II	II	II	II
Mink	Mk39	na	II	II	II	II	II
	Mk02, Mk12, Mk38, Mk54, Mk08	I or II	II	II	II	II	II
Fox	Fx31	I or II	II	na	II	na	II
Badger	Ba50, Ba62	I or II	na	II	II	II	II

na, no amplification. *based on a single allele identified at the SAG3 locus. I = type I; II = type II; III = type III; I or II = type I or II; I or III = type I or III; II or III = type II or III.

3.3.4 Sequencing identified nucleotide differences at the SAG3 locus

Direct sequencing of 25 SAG3 PCR products (11 ferrets, 10 polecats, three mink and one badger) which had previously been genotyped by PCR-RFLP is highlighted in Figure 3-2). The results confirm that the type I *T. gondii* alleles identified in two polecats by PCR-RFLP (Pc07 and Pc26) were identical to a 166bp region of the archetypal type I lineage RH at the SAG3 locus (GenBank accession no. AF340227).

The *T. gondii* strain identified in a polecat (Pc39), which was classified as type III by PCR-RFLP, showed 100% sequence identity to the archetypal type III lineage: CEP (GenBank accession no. AF340229) as shown in Figure 2-2. In twenty of the animals the *T. gondii* strain classified as type II by PCR-RFLP also had a sequence identity to the archetypal type II lineage: Pru (GenBank accession no. AF340228).

Finally, the *T. gondii* strain identified in two polecats (Pc10 and Pc34) showed slight variation compared to the type II archetypal lineage Pru, with sequences from both animals showing a single nucleotide substitution (Pc10 at 134bp and Pc34 at 147bp, GenBank accession numbers KC928252 and KC928253 respectively), both sequences were blasted against the NCBI and ToxoDB databases but did not show 100% identity to any other sequence. To ensure PCR error was not responsible for the observed single nucleotide polymorphisms, both PCR's were repeated and products re-sequenced which confirmed the results.

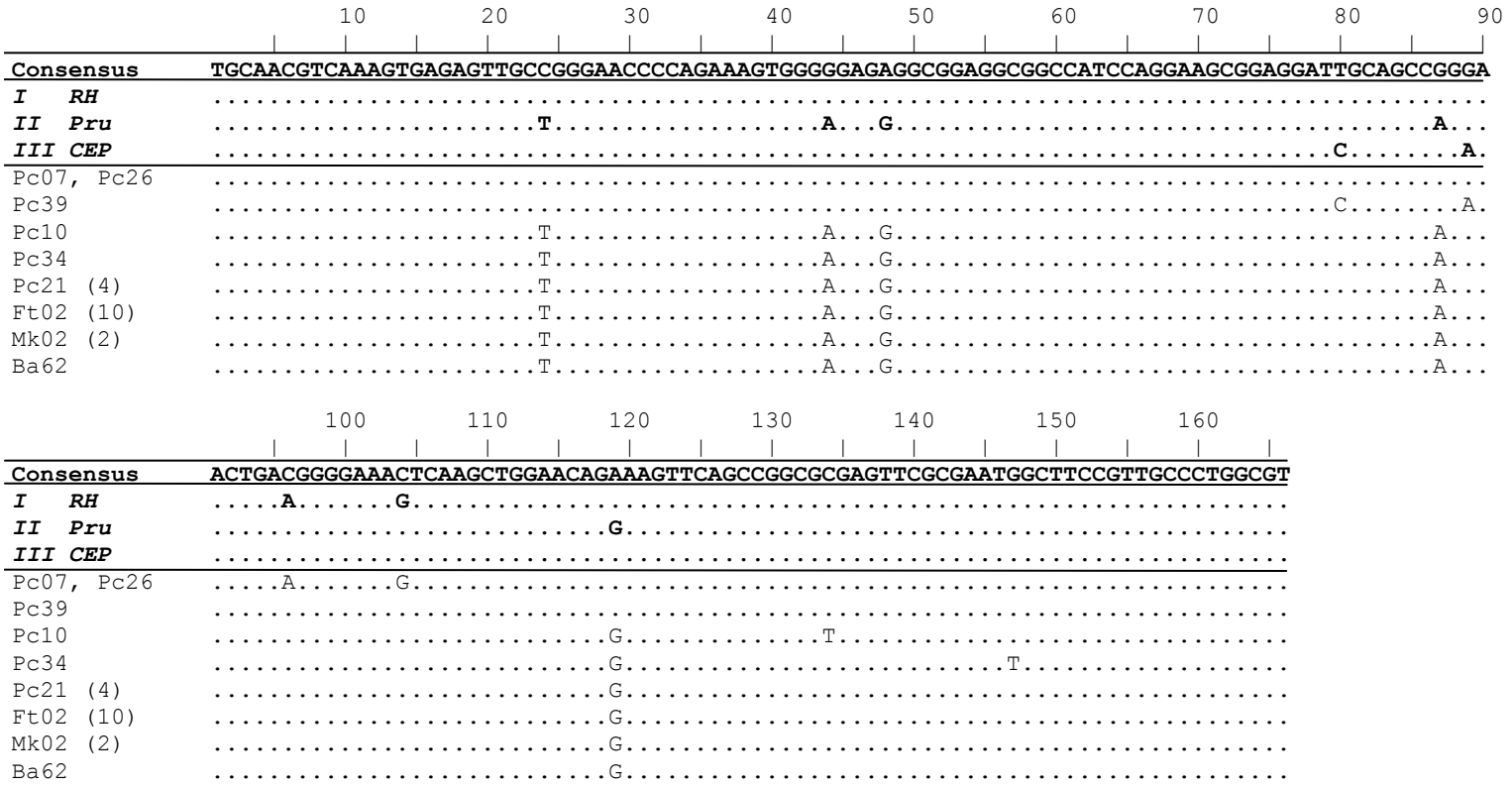

Figure 3-2: Sequence alignment at the SAG3 locus. Consensus sequence indicates the nucleotides shared by a minimum of two of the three archetypal clonal lineages RH (I), Pru (II) and CEP (III). The sequence alignment shown for Pc21 (4) also represents identical sequencing for four other polecat samples (Pc27, Pc30, Pc43 and Pc53), whilst Ft02 (10) represents identical sequencing for 10 other infected ferrets (Ft05, Ft15, Ft39, Ft42, Ft49, Ft50, Ft55, Ft64, Ft82, and Ft83), and Mk02 (2) represents identical sequencing for two other mink samples (Mk08 and Mk54).

It should be noted that direct sequencing was also carried out for several other amplicons generated from 5'SAG2, 3'SAG2, GRA6 and BTUB. These sequences correlated with the results obtained from PCR-RFLP (see Table 3-4). Sequencing using SAG3 proved to be more informative, providing additional information about the genotype than PCR-RFLP alone.

Representative sequences were submitted to GenBank (http://www.ncbi.nlm.nih.gov/genbank) and are available online using the following accession numbers; KC928254 – representative of twenty isolates (SAG3 Type II allele) KC928250 – Pc07 (SAG3 Type I allele), KC928251 – Pc39 (SAG3 Type III allele), KC928252 – Pc10 (SAG3 Type II allele, single base pair substitution), KC928253 - Pc34 (SAG3 Type II allele, single base pair substitution), KC928255 (GRA6 Type II), KC928256 (GRA6 Type III), KC928257 (BTUB Type II), KC928258 (5'SAG2 Type II), KC928259 (3'SAG2 Type II).

3.4 Discussion

Data describing the prevalence of *T .gondi* in wildlife within Great Britain is limited, even less information is known about the genotypes, which may be circulating within the host populations and subsequently the wider environment. However research carried out in Europe has shown not only further information on the prevalence of the parasite, but also on the genotypes within these host species. The results from this chapter help to complete the knowledge regarding the prevalence of *T. gondii* and the genotypes present in the environment within Great Britain. The results show that the prevalence of *T. gondii* in British wild mammalian carnivores varies between 6.0% - 44.4% depending on the host species (see Table 3-2). Analysis of identical tissues for the detection of *Neospora caninum* was recently published (Bartley *et al.*, 2013), and through personal communication with the author of this manuscript, a comparison between this research and the current study has identified seven animals (1.8%) infected with both with *T. gondii* and *N. caninum*. This includes one badger, one fox and five polecats.

The overall prevalence of *T. gondii* may be affected by the number and type of tissues available for testing within each species (Table 3-3). Stoats have the highest prevalence (44.4%), however only nine animals were tested by PCR for the presence of *T. gondii* DNA, whereas for the other species examined the sample number was greater (n= 64 - 99), as described in Table 3-2. The availability of only nine stoats means that this group does not constitute a representative sample for the species. To fully evaluate the prevalence of *T. gondii* within stoats a greater number of animals should be sampled, however on this occasion we were limited to this small number. The lowest prevalence was observed in foxes (6.0%), however a previous serum based study in red foxes from the UK found a *T. gondii* seroprevalence of 20.0% (Hamilton *et al.*, 2005). Other studies within Europe have highlighted a higher seroprevalence, including 73.7% (14/19) in France (Aubert *et al.*, 2010), 56.0% (115/206) in Ireland (Murphy *et al.*, 2007), and 74.5% (152/204) to 84.7% (149/176) in Germany depending on the region (Herrmann *et al.*, 2012). Although it should be noted that lower figures are observed for the molecular detection of the parasite. In Belgium 18.8% (57/304) of foxes tested positive for *T. gondii* by real time PCR (De

Craeye *et al.*, 2011) and in Germany 13.4% (20/149) to 18.4% (28/152) of foxes were identified as positive by PCR, which differed substantially from the serological results of 74.5% to 84.7% (Herrmann *et al.*, 2012). The work carried out by Hermann *et al* (2012) showed that molecular detection resulted in a lower prevalence when compared to serological detection of the parasite. Unfortunately the tissues available for our study did not allow a seroprevalence study to be undertaken, however in the future this is something that should be considered, and if serum is not available perhaps using meat juice in an ELISA, taken directly from the tissues would be something to consider for future experiments (Berger-Schoch *et al.*, 2011; Glor *et al.*, 2013) . Our prevalence rate is lower than many European seroprevalence studies, as serum based detection appears to result in an increased *T. gondii* infection detection rate. Lower values for the molecular detection of the parasite are likely to be due to a combination of the inhomogeneous distribution of *T. gondii* tissue cysts in the host and the small sample size used for DNA isolation (approximately 1g of tissue). Testing a greater number of tissue samples from each species by PCR could increase the overall detection rates, as it is possible that *T. gondii* infection was not detected in some animals as a particular tissue was not available for testing. An example of this is highlighted within the current study, where a selection of badger tissues were positive for *T. gondii* while corresponding brain samples tested negative (see Table 3-3). It should also be noted that where multiple tissues were collected from individual animals (mink, badger and stoat), that more than one tissue could be positive for *T. gondii* by ITS1 PCR. This was particularly noted in a badger, where *T. gondii* was detected in three individual tissues from this animal (see Figure 3-1). Sampling different tissues from the same animal will increase the detection level, for example, in badgers, based on the presence of *T. gondii* DNA in brain alone the prevalence rate is only 5% (see Table 3-3), however, by testing multiple tissues from each animal the overall prevalence increased to 25% The inconsistency in the detection of the parasite between different tissues from the same animal could reflect the infection burden, which may provide an indication of host resistance or susceptibility to *T. gondii*.

Molecular detection of parasite DNA does enable strain genotyping by PCR-RFLP, which cannot be achieved by serology based methods. Genotypic analysis of the parasite is important as it can identify recombinant genotypes or atypical strains. Different strains of the parasite can be more virulent in different hosts, or when a species has not been exposed to the parasite during their evolution (Innes, 1997). Examples of this have been observed in Australian marsupials (Parameswaran *et al.*, 2010), Californian sea otters (Miller *et al.*, 2004); (Sundar *et al.*, 2008) and even humans (Carme *et al.*, 2009b; Ferreira *et al.*, 2011). Our research shows that the strain most prevalent throughout Europe and North America (type II), is the main lineage identified within the wildlife species tested (see Table 3-4). However a *T. gondii* type III lineage was identified in one polecat and an allele for type I was identified within two different polecats (see Table 3-4), indicating that alleles for the three main clonal lineages are present in the British environment. A recent publication, which incorporated genotyping for only one marker (SAG3) in woodmice (*Apodemus sylvaticus*) from an area in North Yorkshire, also described the presence of all three alleles within the study population. A total of 22 samples from *T. gondii* infected mice were genotyped: 12 were identified to contain type I, nine as type II and one as type III (Thomasson *et al.*, 2011). Although information relating to strains found in wildlife in Britain is still lacking there are reports of parasite genotypes belonging to type III and I lineages in European wildlife and livestock (de Sousa *et al.*, 2006; Waap *et al.*, 2008; Berger-Schoch *et al.*, 2011; De Craeye *et al.*, 2011), and also evidence of atypical strains (Berger-Schoch *et al.*, 2011). Our current study using five RFLP markers did not identify any atypical strains or recombinant genotypes within the species tested, however, it should be noted that application of additional markers at other loci may identify more novel *T. gondii* genotypes from these samples. Although we identified type II as the predominant linage by PCR-RFLP (see Table 3-4), sequence analysis at the SAG3 locus provided additional information across the amplicon. Two isolates identified as type II by PCR-RFLP differed by single base pair substitutions (see Figure 3-2), which was confirmed by further repeating PCR and sequencing of the original samples. The two sequences (represented by GenBank accession numbers KC928252 and KC928254) are unique as they did not show 100% identity to any

other sequence within the ToxoDB or NCBI databases. Additional sequencing of these PCR products at the GRA6 locus showed no further base pair polymorphisms (data not shown). Additional nucleotide changes, within the type II lineage, have also been reported from *T. gondii* infected sheep in the Netherlands, where sequencing of the GRA6 gene identified a G to A substitution (Opsteegh *et al.*, 2010a). This indicates that there is a degree of variability within the type II lineage, which is not identifiable by PCR-RFLP analysis alone.

Not all DNA samples, which tested positive by ITS1 PCR, were successfully amplified for genotyping; only 35.6% of positive samples were successfully genotyped at three or more loci. Primers used for the detection of parasite DNA were designed around the multicopy ITS1 region (Hurtado *et al.*, 2001), this PCR is therefore more sensitive than the genotyping PCR-RFLP, which is based on single copy genes, and is merely the result of copy number of the DNA target. Similarly, previous studies found only 22 out of 84 DNA samples from *T. gondii* positive mice could be genotyped at the SAG3 locus (Thomasson *et al.*, 2011), 12 out of 20 *T. gondii* infected fox samples from the Saxony-Anhalt federal state in Germany could be genotyped across nine markers (Herrmann *et al.*, 2012), and 31 out of 167 fox samples, which were seropositive, could be genotyped across 10 markers (Prestrud et al., 2008). Interestingly, amplification of *T. gondii* DNA from Pc07 and Pc26 using markers 5'SAG2, 3'SAG2, GRA6 and BTUB was unsuccessful despite numerous attempts, which included increased concentrations of target DNA. For these samples genotyping was only possible at the SAG3 locus, which identified an allele for type I by both PCR-RFLP (see Table 3-4) and direct sequencing (see Figure 3-2). One possible explanation for this is that SAG3 has been reported to be the most sensitive genotyping marker (Khan et al., 2005), which suggests that parasite DNA was not present at a high enough concentration in the sample to enable detection by potentially less sensitive PCR-RFLP markers.

This study highlights not only the prevalence of *T. gondii* across a variety of wild British mammalian carnivores, but also provides novel data regarding the strains present within the environment that could potentially be transmitted to cats, livestock and even humans within Great Britain.

Chapter 4: Prevalence of *T. gondii* from human hosts in Scotland

Aims

- Examine the prevalence of *T. gondii* infection from two different study groups; Scottish blood donors (seroprevalence) and human brain samples (molecular detection).

- Discover the rate of seroconversion (or reversion) within Scottish blood donors and whether any recent infections were a result of oocyst or tissue cysts infection.

- Determine whether age, gender, geographical location, or cause of death may be linked to the risk of *T. gondii* infection.

- Research the genotypes present from *T. gondii* positive human brain samples.

4.1 Introduction

One third of the human population are predicted to be infected with *T. gondii* (Tenter *et al.*, 2000). However, current *T. gondii* prevalence figures for the UK are limited and even less information is available specifically for Scotland. Infection is generally asymptomatic, however those who are immunocompromised or women who acquire the infection for the first time during pregnancy are particularly at risk (Hohlfeld *et al.*, 1989). Therefore, for women of childbearing age individuals who test seronegative for *T. gondii* are more at risk of miscarriage and future complications with the unborn child, if they become infected with the parasite for the first time during pregnancy. The majority of seroprevalence studies have focused on pregnant women (Flatt & Shetty, 2012; Fonseca *et al.*, 2012; Gebremedhin *et al.*, 2013), and are not representative of the population as a whole.

Research has suggested that *T. gondii* infection rates for those who live in a rural location or work outdoors (such as farmers) are higher, compared to those who live and work in more urban/city locations (Nash *et al.*, 2005; Stanford *et al.*, 1990). It is thought this difference is due to an increased risk of exposure from environmental oocysts in rural areas. Determining exactly how a seropositive individual became infected (whether via oocysts or tissue cysts) has recently become possible, using a sporozoite specific antigen (*T. gondii* embryogenesis-related protein [TgERP]) which will enable the identification of individuals infected via oocysts (Hill *et al.*, 2011). However this method is only suitable for detecting recent infections (approximately six to eight months after initial oocyst infection).

More recently links have been made between *T. gondii* infection and various psychological problems, such as schizophrenia, suicide and increased risk taking behaviour (Flegr *et al.*, 2002b; Lester, 2010; Nascimento *et al.*, 2012). Currently there are no reports in the literature from individuals within the UK regarding these psychological conditions and their potential link with *T. gondii* infection.

Within this study two different groups of individuals were studied with an objective to obtain a current estimate for the prevalence of *T. gondii* infection in people in Scotland. These two groups included serum samples from a cohort of blood donors

(n = 1403) located in Glasgow and Dundee, and human brain tissue from individuals located in the Edinburgh area obtained due to sudden death (n= 151).

Serum samples were obtained from blood donors taken at four different collection periods, which spanned the years 2006 – 2009. Each individual was assigned a specific ID number to enable tracking throughout each collection period and which also enable the data to be anonymised. Information regarding age, gender and location of residence was also made available from a database generated by Health Protection Scotland, who previously selected some of the samples for a longitudinal cryptosporidiosis study (Pollock *et al.,* 2013). Tracking of each individual over time also allows for possible detection of *T. gondii* IgG seroconversion or reversion, as without repeated exposure or parasite recrudescence due to immunosuppression, IgG levels may fall below detectable levels. In addition collaboration with D. Hill (USDA) allowed samples, which had recently seroconverted, to be tested using the newly identified sporozoite specific antigen (TgERP) (Hill *et al.*, 2011). In addition, the collection of samples from two different locations (Glasgow = urban environment and Dundee = more rural environment), allow for any significant differences to be identified between the two regions.

Human brain tissue was used for the molecular detection of the parasite, and was collected over a five year period (2008 – 2012). This allows generation of additional up to date information on the prevalence of the parasite, to determine if there are any specific relationships between cause of death and detection of *T. gondii*, and also to identify any trends between age and gender and parasite prevalence. Strain genotyping using PCR-RFLP is also possible on any *T. gondii* positive samples. Although limited information exists on strains that infect immunocompromised individuals (such as HIV and organ transplant patients) (Ajzenberg *et al.*, 2002), very little data about which strains effect a "healthy" population with no known underlying cause of illness is not known. Information from these individuals would also be more representative of the general population.

The main aims of this study were to obtain up to date figures for the prevalence of *T. gondii* in the Scottish population, and determine any link between age, gender, location or cause of death. The research will also identify whether seroconversion (or indeed reversion) of *T. gondii* IgG occurs, and finally whether recent infections were due to the tissue cyst or oocyst stage of the parasite. The molecular detection of the parasite from human brains allows strain genotyping to be completed, therefore, another aim of this research was to identify the genotypes present within the study population.

4.2 Materials and Methods

4.2.1 Human reference sera

Test reference / control sera was obtained from the Scottish *Toxoplasma* Reference Laboratory (Scottish *Toxoplasma* Reference Laboratory, Inverness, UK), who routinely test human samples for the presence of *T. gondii* IgG. The control sera supplied had previously been dye tested (see section 1.9.1 for details of this technique) and calibrated as international units per milliliter (iu/ml) as defined by the World Health Organisation (see Table 4-1). These controls not only enabled the standardisation of an in house *T. gondii* IgG ELISA, but also acted as reference sera for testing other human sera.

Table 4-1: Human reference sera supplied by the Scottish *Toxoplasma* Reference Laboratory. Sera representing a range of possible infection results.

Dye test result (IU/ml)	125	30	8	<2
T. gondii infection status	Strong positive	Positive	Borderline	Negative

4.2.2 Human test sera

Human serum test samples (n = 3274) were obtained in conjunction with Health Protection Scotland and the Scottish Parasite Diagnostic Laboratory from individual blood donors (n = 1403) from two cohorts, Glasgow (n = 1102) and Dundee (n = 301), as described in further detail in section 2.2.1.

4.2.3 Human brain DNA samples

Tissue samples from human brains (n = 151) were obtained from the MRC Sudden Death Brain and Tissue Bank over a five year collection period (2008 – 2012) as described in section 2.2.2. Pooled brain tissue consisting of approximately 1g of medulla, cerebellum and pons (regions of the hind brain, see Figure 2-1) from each individual were used for DNA extraction using the GentleMacs method (see section 2.11.2).

4.2.4 Human anti *T. gondii* IgG ELISA

In order to test the human sera samples, an in house human anti-*T. gondii* IgG ELISA was optimised during this study. Control sera, individual test sera and serum blank controls were tested in duplicate. Details of the final methodology used for all test sera are described in section 2.10.1. Miss A. Woodall should be acknowledged for her assistance in testing the serum samples using this ELISA. OD values were corrected for blank measurements and plate to plate variation (see section 4.2.7.1).

4.2.5 Detection and genotyping of *T. gondii* DNA

Following the extraction of DNA from human brain tissue (see section 4.2.3), samples were used to detect the presence of *T. gondii* DNA using the diagnostic ITS1 PCR described in section 2.12.2. To determine the genotype of *T. gondii* positive DNA samples genotyping was completed using two of the most sensitive and informative molecular markers (SAG3 and GRA6), using the methodology described in section 2.12.4.

4.2.6 Donor / case information

For the serology study (blood donors), information was recorded for every blood donor, which included details regarding their age, gender and location, this enabled a longitudinal serological profile of each individual donor. For the human brain

samples, basic case data was available for each individual which included information about age, gender and cause of death. To ensure individuals could not be identified, details for all samples for both the serological detection of *T. gondii* (blood donors), or molecular detection of the parasite (human brain samples) were anonymised.

4.2.7 Data analysis

4.2.7.1 Corrected OD values and plate to plate variation

To analyse any variation between each plate when testing serum samples using the human IgG ELSA (see section 4.2.4), positive and negative control samples (see Table 4-1) and serum blank controls (both tested in duplicate), were added to each plate. Once all the OD readings were measured, the average OD readings for the blank controls on each plate were subtracted from the OD values of the sera on that specific plate. Then, these blank corrected OD values for the serum control samples (n = 8 – duplicates of each positive and negative control sample) were plotted against their appropriate average blank corrected OD value over all the plates tested (a regression line). Using the appropriate regression line the standardised blank corrected OD values for the test sera on that specific plate was calculated (their ODc value). Plates were excluded if; 1) any of the duplicates for more than one of the control samples had a coefficient of variation (CV = standard deviation of replicates / mean of replicates) above 20%, or 2) if the R^2 value was less than 0.95. In addition individual sera were excluded from analysis if the CV value was above 20%. Any sera which had an OD value below 0.1 any CV value was accepted (although the OD value of both samples from this duplicate had to be below 0.1). Finally, as some of the ODc values were negative a blank value of 0.05 was added for all sera, with all values $\log^{10}$ transformed. When a new batch of positive and negative control samples were used, their average OD across all plates tested was plotted against the average OD of the control samples used on all plates using the previous batch. Plates containing a new batch of positive and negative controls were only accepted if the R^2

was less than 0.95. Acknowledgements should be made to Dr M. Opsteegh for completion of and assistance with the linear regression plots and plate to plate variation (Opsteegh *et al.*, 2010b).

4.2.7.2 Seroprevalence in Scottish blood donors using a binormal mixture model

When a population can be presumed as seropositive or seronegative, the distribution can be assumed to follow normal distribution. As antibody titers are usually lognormally distributed (Thrusfield, 2005) the ODc values were log transformed. This allows for analysis between the two variables (seropositive or seronegative), both of which are presumed to be normally distributed but with a different standard deviation and mean. A binormal mixture model takes these factors into account, and estimates two standard deviations, two means and the mixing parameter. The estimated prevalence is determined by the mixing parameter, whereby the area under the curve of the positive distribution divided by the total area under both curves. The binomial mixture model was performed in Mathematica (v7.0, Wolfram Research, Illinois, USA), as described in Opsteegh *et al*, 2010. Acknowledgements should be made to Dr M. Opsteegh and Dr A. Swart for completion of and assistance with the binormal mixture model (see Figure 4-1).

4.2.7.3 Determining cut off values to asses seropositivity and analysis between different groups

Identifying an individual as either positive or negative for *T. gondii* enables further analysis to be completed, such as difference between seropositivity and gender, age, location or identification of seroconversion. This was carried out using a cut off value which allowed each individual to be scored as either seropositive or seronegative. The figure used as the cut off value was obtained using a receiver operating characteristic (ROC) curve which incorporated the two distributions from a binornal mixture model where the sum of sensitivity and specificity were both maximized.

4.2.7.4 Identification of sporozoite specific antigen in serum samples from individuals that showed signs of seroconversion.

Using the cut-off value obtained from section 4.2.7.3, 50µl of serum from individuals who had been identified as seronegative at one collection period, but who later became seropositive in a subsequent collection period (seroconversion), were sent to D. Hill (United States Department of Agriculture, Beltsville, USA) for the identification of antibodies to the sporozoite specific antigen *T. gondii* embryogenesis-related protein (TgERP). This protein elicits an antibody response in humans (and also mice and pigs), who have previously been exposed to sporulated oocysts, therefore identifying individuals that have been infected with this stage of the parasite. Only serum samples from individuals where the ELISA result identified recent signs of seroconversion were sent for analysis as the TgERP antibody response is only detectable for 6 to 8 months following initial infection (Hill *et al.*, 2011). The methodology for the ELISA is described by Hill *et al* (2011), briefly, 96 well plates were coated with 2µg/ml TgERP in 0.1M carbonate buffer, pH 9.6 and the ELISA completed as described by Gamble *et al* (2005). Reference sera for positive and negative controls were also incorporated into the ELISA, with all plates read using a wavelength of 405ηm. A positive cut off value was determined using the mean OD of the negative reference samples plus three times the standard deviation. Acknowledgements should be made to Dr D. Hill for testing the samples from this study.

4.3 Serological Results

4.3.1 Seroprevalence, and determination of cut- off value using a binormal mixture model

All results from collection period 1 (n = 947 individuals) were used to formulate a binormal mixture model (see Figure 4-1). Collection period 1 was used to produce this model, as once repeat donors had been identified this group contained the greatest number of individuals over all collection periods. Using the statistical methodology described in section 4.2.7.2, fitted distributions were plotted and the binormal mixture model generated.

The model describes the overall *T. gondii* seroprevalence for this donation period, which using the binormal mixture model is estimated at 11.0% (104/947) (see Table 4-2). As described in section 4.2.7.3 the determination of a cut off value using ROC curve analysis could be performed using the data derived from the binormal mixture model. The cut off value was estimated at logODc -0.48, where specificity and sensitivity was estimated at 99.0% and specificity at 99.4%. This cut off value was used for further analysis of all donations; determining seropositivity with age, gender and location, across all donation periods. Using the cut off value, *T. gondii* seroprevalence for the first donation period was estimated at 11.9% (see Table 4-2).

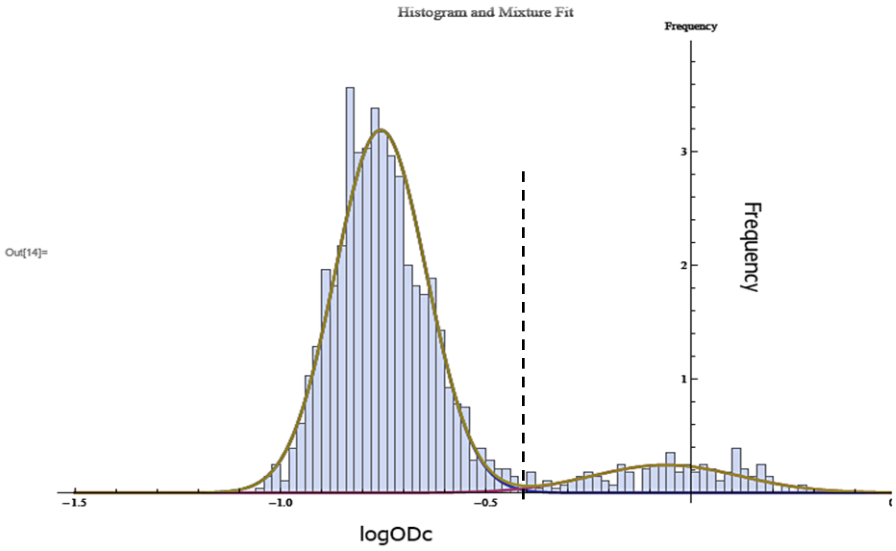

Figure 4-1: Human serology *T. gondii* binormal mixture model. Frequency distribution of logODc values generated from Scottish blood donors (n = 947) using the human anti-IgG *T. gondii* ELISA. Bars represent individual blood donors, and the curved line shows the normal distributions. The black dashed line indicates the cut off value (log ODc -0.48) used for subsequent analysis.

Table 4-2: Estimation of *T. gondii* seroprevalence of blood donors from Glasgow and Dundee. Prevalence was determined using the binormal mixture model and then the cut off value (logODc -0.48) extrapolated from the model. The cut off value can be used to further anyalyse the data. *accounts for repeat donations, each individual is counted only once. Collection period 1 = 24/04/2006 – 26/07/2006, collection period 1 – 4 = 24/04/2006 – 09/02/2009.

Seropositivity determined by:	Percentage positivity (%)	Total number of individuals (n)	Total number of individuals *T. gondii* positive (n)	Collection Period
Binormal Mixture Model	11.0%	947	104	1
Cut off value (logODc -0.48)	11.9%	947	113	1
Cut off value (logODc -0.48)	13.0%	1403	183	1-4*

4.3.2 Overall seroprevalence determined by cut off value and seroprevalence within each collection period.

Using the cut off value (logODc -0.48) determined in section 4.3.1, the overall prevalence of *T. gondii* during the 4 year collection period (2006 – 2009) was estimated at 13.0% (183/1403), as described in Table 4-2 and Table 4-3. This figure took into account repeat donations and included each individual only once throughout the entire collection period. The cut off value (see 4.3.1) was also used to determine seroprevalence within each collection period (see Table 4-3). Again repeat donations were common from blood donors throughout an individual collection period, and the results ensure that each individual was identified and counted only once. In addition, repeat donors which were seropositive were counted once as positive, which was based on the first observation of seropositivity.

Table 4-3: Collection period and *T. gondii* seropositivity. Seroprevalence takes into account repeat donations from each collection period. *repeat donations taken across all collection periods where each individual is counted only once over the four year period (2006-2009)

Period	Collection Period	No. Samples - includes repeat donations (n)	No. Individuals - no repeat donations (n)	No. *T. gondii* positive - no repeat donations (n)	*T. gondii* seropositivity - no repeat donations (mean % positive)	95% CI (lower and upper values)
1	24/04/2006 - 24/07/2006	947	947	113	11.9	9.9 – 14.2
2	26/07/2006 - 14/08/2007	811	811	105	12.9	10.7 – 15.5
3	04/04/2008 - 29/08/2008	766	697	84	12.1	9.7 – 14.7
4	01/09/2008 - 09/02/2009	749	606	76	12.5	10.0 – 15.4
1-4	24/04/2006 - 09/02/2009	3273	3061	378	12.3	11.2 – 13.6
All*	24/04/2006 - 09/02/2009	1403	1403	183	13.0	11.3 – 14.9

The results show little variation between collection periods and varies from 11.9% (113/947) in collection period 1, to 12.9% (105/811) in collection period 2 as highlighted in Figure 4-2 and Table 4-3.

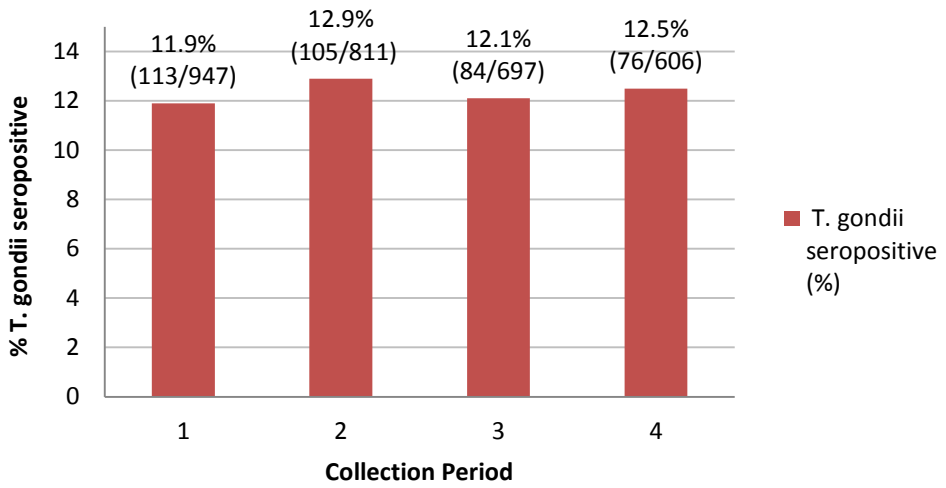

Figure 4-2: *T. gondii* seroprevalence within Scottish blood donors by collection period. Details of each collection period are described in Table 4-3.

4.3.3 Evidence of seroconversion over a four year period (2006 – 2009)

Longitudinal analysis over this four year period allowed individuals to be identified and monitored for their *T. gondii* IgG status (seroconversion or reversion). Using a positive cut-off value of OD 0.41 (D. Hill personal communication) clear seroconversion (*T. gondii* negative to positive) was observed in six individuals (see Table 4-4, donor ID's 404, 414, 342, 963, 1246, and 1312. One individual became *T. gondii* seropositive at the beginning of collection period four but then reverted back to seronegative four months later (see Table 4-4, donor ID 816). There were also three individuals who appeared to become seropositive, however, previous results from collection periods prior to this showed that they were not clearly negative

(potentially borderline positive), and therefore have not be classified as a "true" seroconversion (see Table 4-4 donor ID, 215, 721 and 698).

Table 4-4: Evidence of *T. gondii* IgG seroconversion or reversion and TgERP antibody response in Scottish blood donors (2006-2009).

Donor ID	pos	logODc	Odc	Town	Collection period	Date	Status	TgERP ELISA Result
404	N	-0.89	0.13	DUN	1	09/05/2006		0.39
404	Y	0.25	1.76	DUN	3	13/05/2008		1.38
404	Y	0.13	1.34	DUN	4	09/09/2008		0.38
404	Y	0.03	1.06	DUN	4	09/12/2008	SEROCONVERTED	0.27
414	N	-0.63	0.23	DUN	1	09/05/2006		0.22
414	N	-0.77	0.17	DUN	2	15/11/2006		0.17
414	N	-0.99	0.10	DUN	3	20/08/2008		0.12
414	Y	0.00	1.00	DUN	4	17/12/2008	SEROCONVERTED	0.15
215	N	-0.50	0.31	GLA	1	08/05/2006		0.20
215	Y	-0.27	0.54	GLA	2	04/04/2007		0.29
215	Y	-0.28	0.52	GLA	3	21/07/2008	SEROCONVERTED	0.19
342	N	-0.87	0.13	GLA	1	17/05/2006		0.20
342	N	-0.81	0.16	GLA	2	23/08/2006		0.21
342	N	-1.01	0.10	GLA	4	03/09/2008		0.21
342	Y	-0.02	0.96	GLA	4	03/01/2009	SEROCONVERTED	0.22
721	N	-0.58	0.26	GLA	1	07/06/2006		0.18
721	N	-0.52	0.30	GLA	2	04/09/2006		0.22
721	Y	-0.43	0.37	GLA	3	22/04/2008		0.20
721	N	-0.55	0.28	GLA	3	29/07/2008		0.23
721	Y	-0.22	0.60	GLA	4	25/11/2008	SEROCONVERTED	0.32

Table 4-4 continued:

Donor ID	pos	logODc[a]	Odc	Town	Collection period	Date	Status	TgERP ELISA Result
698	N	-0.51	0.31	DUN	1	22/05/2006		0.17
698	Y	0.12	1.30	DUN	2	23/01/2007	SEROCONVERTED	0.16
963	N	-0.66	0.22	GLA	1	10/07/2006		0.25
963	Y	-0.29	0.52	GLA	4	19/02/2009	SEROCONVERTED	0.25
1,246	N	-0.79	0.16	GLA	3	30/07/2008		0.14
1,246	Y	-0.36	0.44	GLA	4	18/12/2008	SEROCONVERTED	0.14
1,312	N	-0.79	0.16	GLA	3	06/08/2008		0.23
1,312	Y	-0.08	0.84	GLA	4	06/12/2008	SEROCONVERTED	0.14
816	N	-0.80	0.16	GLA	1	21/06/2006		0.15
816	N	-0.74	0.18	GLA	2	25/10/2006		0.24
816	N	-0.74	0.18	GLA	3	16/06/2008		0.28
816	Y	-0.37	0.43	GLA	4	08/11/2008	SEROCONVERTED	0.23
816	N	-0.93	0.12	GLA	4	05/02/2009	REVERSION	0.25

Cut off values: a) logODc cut off value for *T. gondii* IgG ELISA = -0.48 b) OD cut off value for TgERP analysis = 0.41.

Key: Y = positive IgG result, N = negative IgG result

4.3.4 Distribution of seroprevalence between two locations, Glasgow and Dundee determined by cut off value

When using the data from all individuals over the four year period, and the cut off value described in section 4.3.1, no difference was observed between donations from Glasgow and Dundee, where prevalence between the two regions was determined at 12.9% (142/1102) and 13.6% (41/301) respectively (see Figure 4-3). However, more individuals who donated blood were from Glasgow (n = 1102) than Dundee (n = 301).

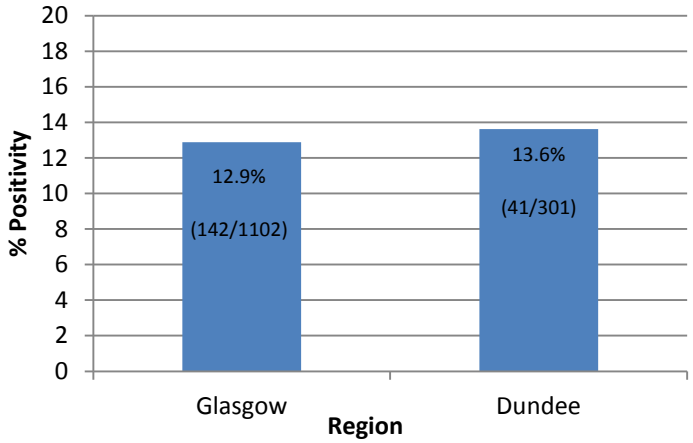

Figure 4-3: *T. gondii* prevalence from blood donors located in Glasgow and Dundee. Prevalence over a four year period (2006 – 2009).

4.3.5 Seroprevalence of *T. gondii* from blood donors in relation to age and gender determined by cut off value

Over the four year period, donations from individuals were not biased towards either gender, with approximately and equal number females (45.9%, 759/1403) and males (54.1%, 644/1403). The majority of donations (40.7%, 571/1403) were obtained from the age group 31-48, whilst only 3.64% (51/1403) contributed for donations from the 63-69 age group (see Figure 4-4).

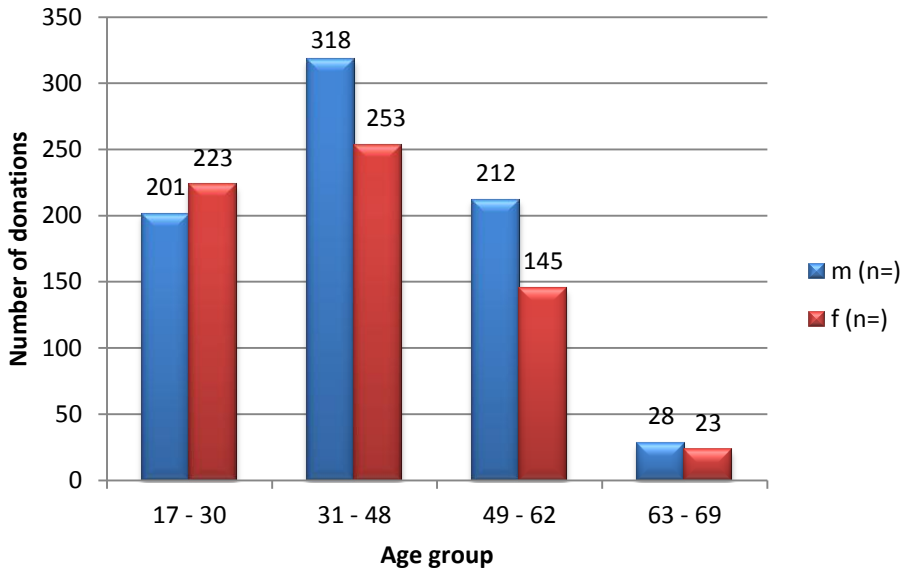

Figure 4-4: Total number and age of blood donors over a four year period (2006 -2009). m = male, f = female.

When observing *T. gondii* seropositivity in relation to age, there was a clear linear relationship between increasing age and an increasing *T. gondii* seropositivity. However the 63-69 age group had the highest prevalence at 23.5% (12/51), whilst the lowest prevalence of 6.37% (27/424) was observed in the 17-30 age group (see Figure 4-5).

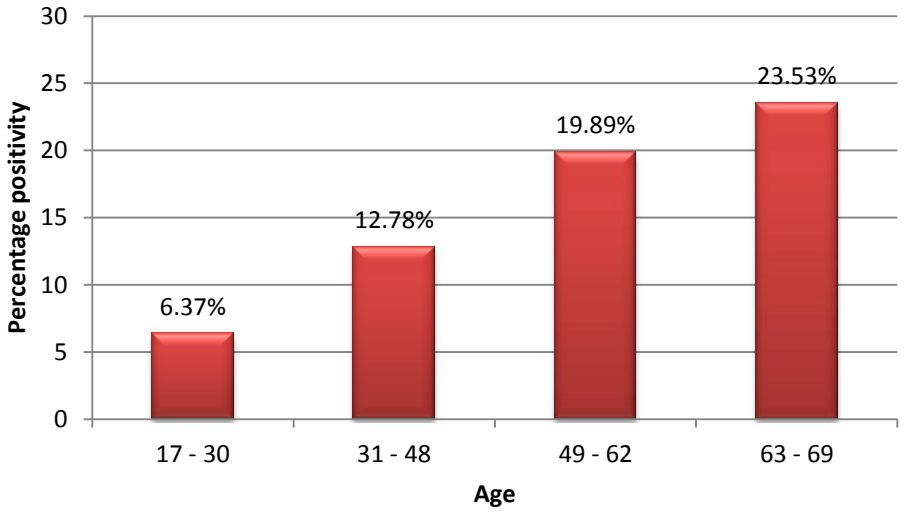

Figure 4-5: *T. gondii* seropositivity of Scottish blood donors in relation to age. Indviduals from both Glasgow and Dundee (2006 – 2009).

When gender is also taken into account, again seropositivity increases with age but there is no significant difference between *T. gondii* infection and gender (see Figure 4-6). This is particularly highlighted in the 63-69 age group, where the percent prevalence between males and females is identical, 11.8% (males n = 6, females n = 6)

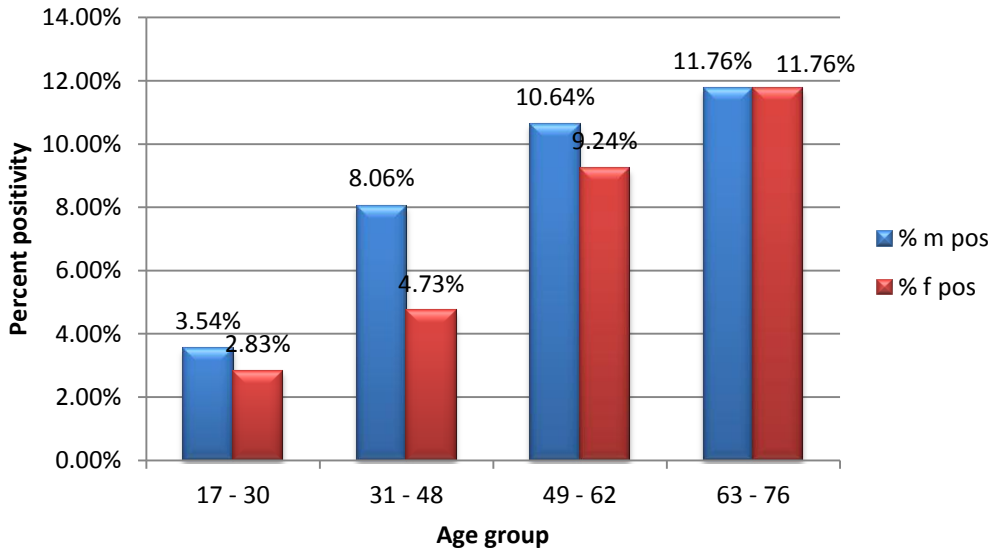

Figure 4-6: *T. gondii* seropositivity of Scottish blood donors in relation to age and gender (2006 – 2009).

4.4 Presence of *T. gondii* DNA in human brain tissue

4.4.1 Prevalence of *T. gondii* DNA in human brain tissue over a five year period (2008 – 2012)

ITS1 PCR was carried out on 151 brain samples from three pooled regions of brain (pons, medulla and cerebellum) per individual. Molecular detection of parasite DNA from all samples (irrespective of age, gender or cause of death) over a five year period (2008 – 2012 inclusive), has shown an overall detection rate of 17.9% (27/151), where prevalence varied throughout the five year period (see Table 4-5). In 2008 parasite DNA was detected in 22.6% (8/35) of individuals, in 2009 and 2010 detection levels were lower 5.4% (2/37) and 15.8% (6/38) respectively. This level then increased in 2011 to 26.1% (6/23) and then peaked in 2012 with a detection rate of 27.8% (5/15).

Table 4-5: Molecular detection of *T. gondii* from human brains by year (2008 - 2012).

Year	No. samples (n)	*T. gondii* positive (n)	% *T. gondii* positive	95% CI (lower and upper values)
2008	35	8	22.9	10.4 – 40.1
2009	37	2	5.4	0.6 – 18.2
2010	38	6	15.8	6.0 – 31.3
2011	23	6	26.1	10.2 – 48.4
2012	18	5	27.8	9.7 – 53.5
2008 - 2012	151	27	17.9	12.1 – 24.9

4.4.2 Effect of age and gender on parasite prevalence in humans

Overall, a greater number of samples were obtained from males, which consisted of 79% (120/151) of all individuals. This gender bias towards males was evident across all ages, except in the 77 – 91 age group (see Figure 4-7). Interestingly, most brain samples were obtained from the 49-62 age group, which accounted for 35.1% (53/151) of all samples.

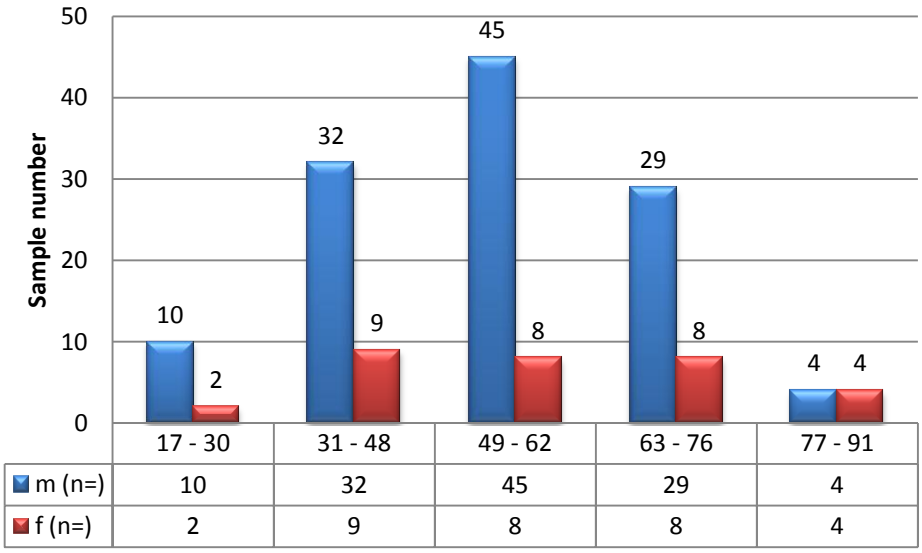

Figure 4-7: Number of individuals across age and gender (2008 - 2012).

Over the five year period, the prevalence of *T. gondii* detected in human brains increased with age (see Figure 4-8) , however, detection levels dropped within the 77-91 age group, but this may be due to a reduced samples size (n = 8, as highlighted in Figure 4-7) compared to the other age groups.

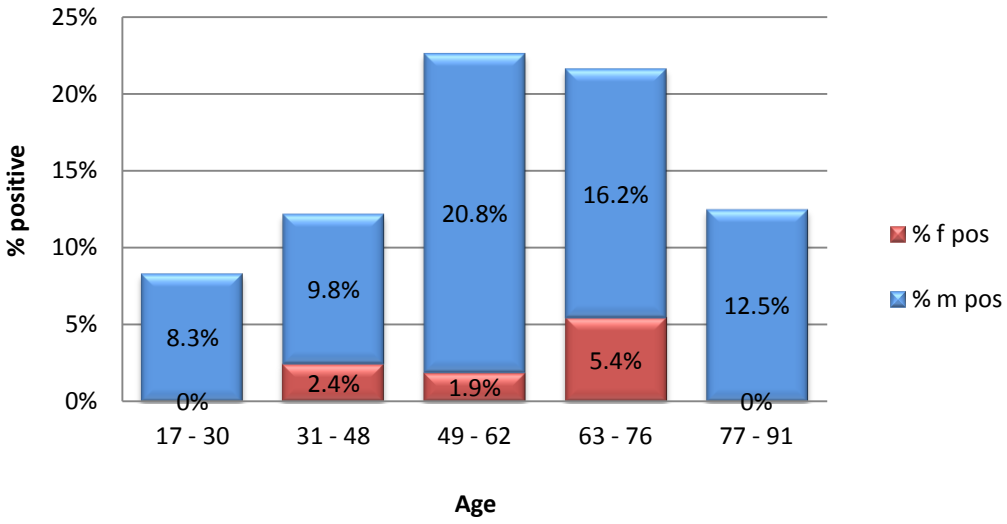

Figure 4-8: Detection of parasite DNA from human brains in relation to age and gender (2008 - 2012).

4.4.3 Relationship between cause of death and parasite detection

Anonymised information from the MRC Sudden Death Brain and Tissue Bank also provided details about the cause of death. As previously described, the overall prevalence of *T. gondii* DNA detected within these brain samples was estimated at 17.9% (see section 4.4.1), but when analysing what each individual had died from the results can be further split, as shown in Table 4-6. This table highlights that the predominant cause of death for all samples obtained was due to heart disease/heart

attack, which accounted for 64.9% (98/151) of all cases. The second most common cause of death was due to suicide (6.0%, 9/151), followed by those who died due to drug overdose (5.3%, 8/151).

Table 4-6: Cause of death, age and prevalence of *T. gondii* from human brains (2008 - 2012).

Cause of death - COD	No. of COD (n)	% COD	No. of all *T. gondii* cases (n)	% *T. gondii* positive	17 - 30	31 - 48	49 - 62	63 - 76	77 - 91	Totals
Heart attack/disease	98	64.9	18	66.7	1	21	43	29	4	98
Brain haemorrhage	6	4.0	2	7.4	0	1	2	1	2	6
Bronchopneumonia	1	0.7	1	3.7	0	0	1	0	0	1
Drug over dose	8	5.3	1	3.7	3	3	1	1	0	8
Familial amyloid polyneropathy	1	0.7	1	3.7	0	0	0	1	0	1
Motor neurone disease	3	2.0	1	3.7	0	0	1	2	0	3
Suicide	9	6.0	1	3.7	3	5	1	0	0	9
Unascertained death	4	2.7	1	3.7	1	1	1	1	0	4
Abdominal aneurysm	1	0.7	1	3.7	0	0	0	1	0	1
Asthma	1	0.7	0	0.0	0	1	0	0	0	1
Breast cancer	1	0.7	0	0.0	0	0	1	0	0	1
Bronchopneumonia & coronary artery atherosclerosis	1	0.7	0	0.0	0	1	0	0	0	1
Hypertensive haematoma	1	0.7	0	0.0	0	0	0	1	0	1
Intracerebral haematoma	1	0.7	0	0.0	0	0	0	0	1	1
Insulin overdose	1	0.7	0	0.0	0	1	0	0	0	1
Marfans Syndrome	2	1.3	0	0.0	0	2	0	0	0	2
Multiple injuries	1	0.7	0	0.0	0	1	0	0	0	1
Pancreatic carcinoma	1	0.7	0	0.0	0	0	0	0	1	1
Peritonitis	1	0.7	0	0.0	0	0	1	0	0	1
RTA	5	3.3	0	0.0	4	0	1	0	0	5
Small vessel disease (brain)	1	0.7	0	0.0	0	1	0	0	0	1
Stroke	1	0.7	0	0.0	0	1	0	0	0	1
Sudden adult death	2	1.3	0	0.0	0	2	0	0	0	2
TOTAL	151	**100.0**	**27**	**100.0**	12	41	53	37	8	**151**

Further analysis of cause of death and the molecular detection of *T. gondii* DNA in the brain of each individual, has highlighted that 66.7% (18/27) of individuals who died due to heart disease/heart attack, were also positive for *T. gondii* (see Table 4-6). *T. gondii* was detected less frequently in individuals who died from brain haemorrhage, bronchopneumonia, drug overdose, familial amyloid disease, motor neuron disease, suicide, abdominal aneurysm, and unascertained death, as highlighted in Figure 4-9 and Table 4-6.

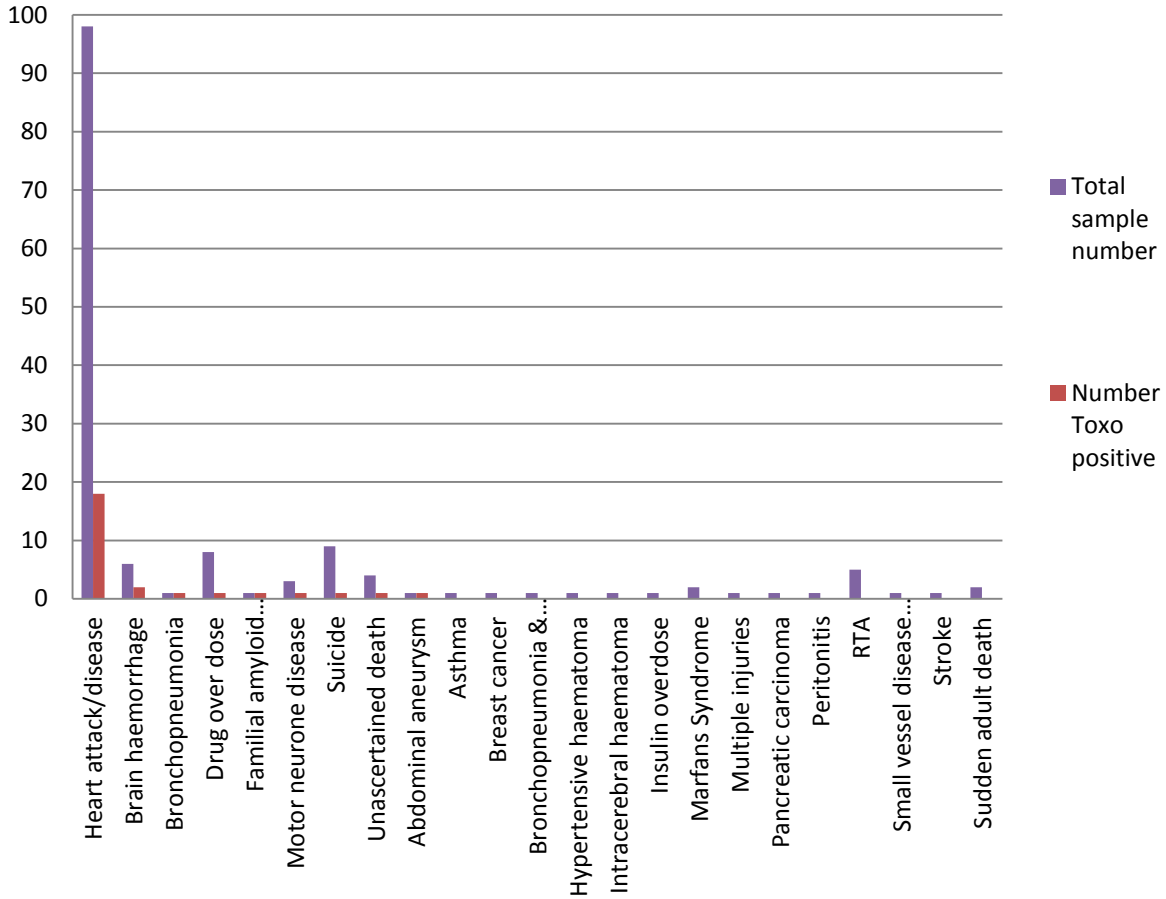

Figure 4-9: Cause of death and detection of *T. gondii* in human brains (2008 - 2012).

4.4.4 Strain genotyping using SAG3 and GRA6

Although multiple attempts were made to amplify DNA from individuals, which were identified as positive for *T. gondii* by ITS1 PCR, only three samples were able to be genotyped by PCR-RFLP using the SAG3 primer and one using the GRA6 primer (see Table 4-7). These results identified two type III and two type I alleles from four different individuals. In addition, *T. gondii* DNA from one individual was successfully sequenced at the GRA6 locus (see Figure 4-10), and also at the SAG3 locus from another individual (see Figure 4-11) further confirming the identification of type I and type III genotypes by PCR-RFLP.

Table 4-7: *T. gondii* PCR-RFLP genotyping across two markers from four infected humans.

				PCR-RFLP genotyping		
Sample ID	**Gender**	**Age**	**Cause of death**	**SAG3**	**GRA6**	**Genotype**
019/08	M	58	Heart attack/disease	na	I*	I
004/10	M	50	Heart attack/disease	III	na	III
021/10	M	27	Drug overdose	I[#]	na	I
013/11	M	58	Heart attack/disease	III*	na	III

[#] = weak band; * = sequencing completed, na = no amplification
I = type I; III = type III. M = Male

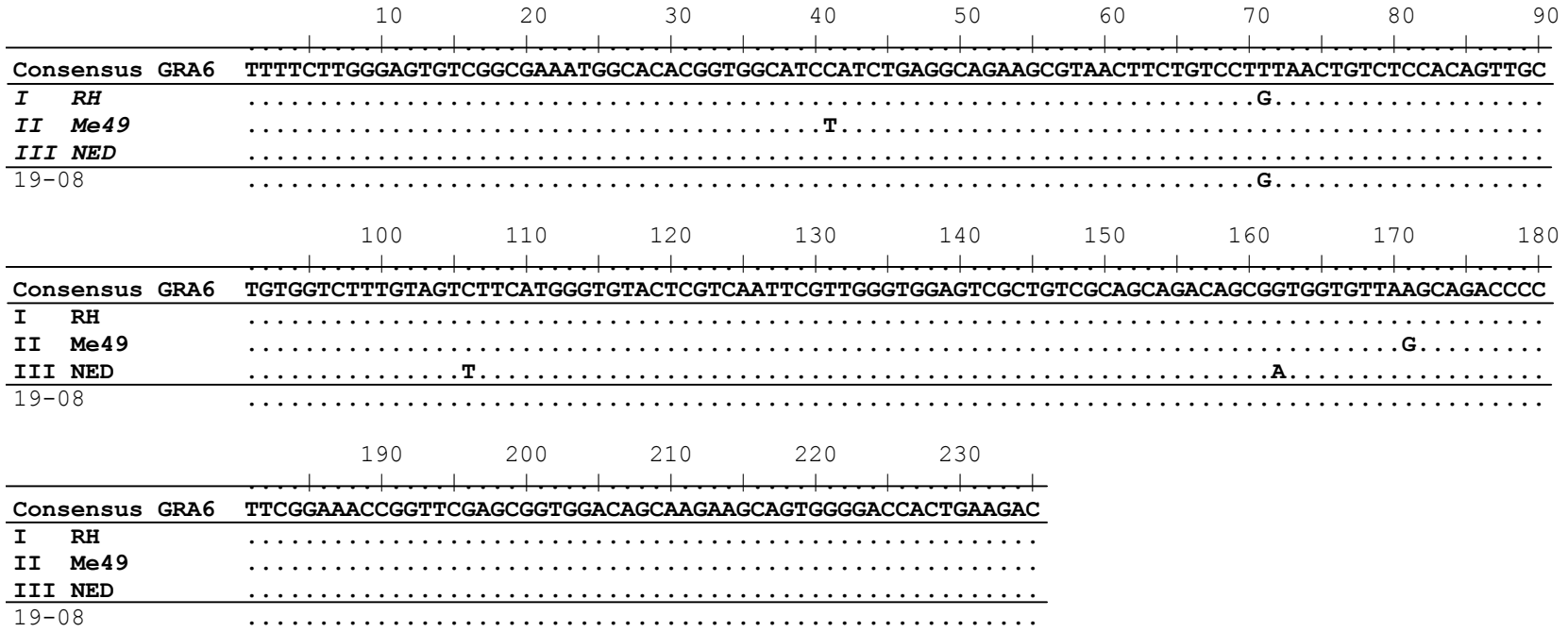

Figure 4-10: GRA6 sequencing of *T. gondii* detected from a human brain. Consensus sequence indicates the nucleotides shared by a minimum of two of the three archetypal clonal lineages RH (type I), Me49 (II), and CEP (III), GenBank accession numbers AF239283, AF239285 and AF239287 respectively. Sequencing shows that *T. gondii* type I was identified from a human brain (sample ID 19-08), using GRA6 primers.

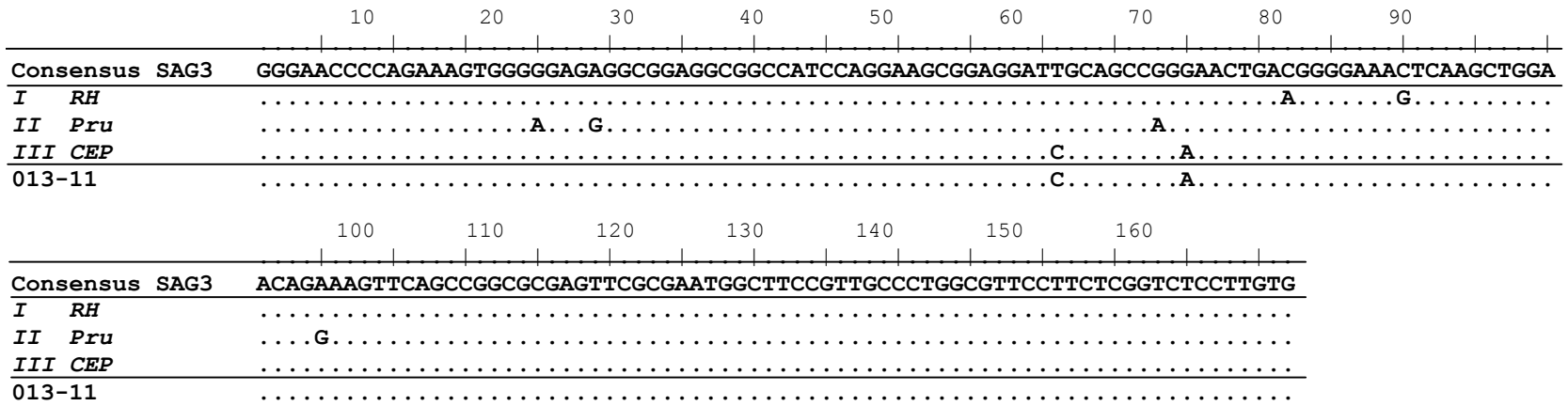

Figure 4-11: SAG3 sequencing of *T. gondii* detected from a human brain. Consensus sequence indicates the nucleotides shared by a minimum of two of the three archetypal clonal lineages RH (type I), Pru (II), and CEP (III), GenBank accession numbers AF340227, AF340228 and AF340229 respectively. Sequencing shows that *T. gondii* type III was identified from a human brain (sample ID 13-11), using SAG3 primers.

4.5 Discussion

The primary aim of this study was to obtain an estimate of *T. gondii* seroprevalence in the Scottish population using two different study groups. Firstly using samples from Scottish blood donors who represent a broad age range of individuals and also includes a similar number of samples from both males and females, (as opposed to the majority of *T. gondii* seroprevalence studies which mainly focus on women of childbearing age). Secondly prevalence was determined by molecular detection of the parasite DNA from brain tissue in individuals who had died due to sudden death, with no known underlying clinical disease.

When examining the seroprevalence of *T. gondii* from Scottish blood donors, the result over a four year period (2006 – 2009), was estimated (using the cut off value) at 13.0% (see Table 4-2 and Table 4-3). This figure is slightly lower than the prevalence determined by molecular detection in human brains over a five year period (2008 – 2013), which was estimated at 17.9% (see Table 4-5). This difference is likely to be due to the variation in the number of samples obtained within each age group, and the number of samples available at each age group. The total number of individuals representing samples for the molecular detection of *T. gondii* were significantly lower than the number of individuals recruited for serological detection of the parasite (n=151 and n=1403 respectively). In addition, within the samples used for the molecular detection of the parasite, there was not a representative sample size for each age group or gender, as highlighted in Figure 4-7. The majority of samples (35.1%), were collected from the 49 – 62 age group whilst only 7.9 % represented the 17 – 30 age group. In contrast the age of individuals in the serology study was more uniform (see Figure 4-4), with the 49 – 62 age group representing 30.2% of the population and 25.4% of the 17 – 30 age group. These factors are likely to be responsible for the higher figure observed for the prevalence of *T. gondii* when using the human brain samples.

When examining each group individually further comparisons can be made. In particular the serology study shows a direct link between seropositivity and age (see Figure 4-5), with increasing age the as detection of *T. gondii* also increases. This

correlation between age and prevalence of the parasite has been described in humans and animals (Allain *et al.*, 1998; Alvarado-Esquivel *et al.*, 2007; Bellali *et al.*, 2013; Katzer *et al.*, 2011), and reflects the importance of the post-natal route of acquisition of infection.

The molecular detection of the parasite from human brains initially follows this pattern (see Figure 4-8), however prevalence begins to fall in the 77 – 91 age group. The observed reduction in prevalence is likely to be due to only eight individuals being tested and does not constitute as a representative sample size. Had there been a greater number of samples available for testing, it would be likely that the trend observed in the current serology study would be repeated.

Another objective of the serology study was to examine if there was any significant difference between the two different locations in which individual donors lived (Glasgow – more samples from an urban location, Dundee – more samples from a rural location). The results did not identify any significance between the two regions, which is in keeping with research carried out by Allain *et al* (1998), who also reported similar findings during a study examining the prevalence of *T. gondii* in pregnant women in east England. Differences between the two locations may not have been observed as individuals could reside in rural locations but commute to urban/city areas on a daily basis for work.

Once the serology data was analysed using the cut-off value (log ODc -0.48), calculated using the binormal mixture model (see Figure 4-1), it was possible to identify individuals who, over time, had become seropositive (or indeed those who had reverted back to seronegative) as shown in Table 4-4. Clear seroconversion was evident in six individuals, an additional three also appeared to seroconvert however in these cases their sera, which tested from previous collection periods, was found to be borderline positive. One donor (donor ID = 816) appeared to clearly seroconvert and then in the following period reverted back to seronegative (see Table 4-4). For each of these ten donors all sera from every collection period were sent to D. Hill (United States Department of Agriculture, Beltsville, USA) for ELISA analysis using the sporozoite specific antigen. The results from this analysis only identified one

positive serum sample (from donor = 404), indicating that this individual had been infected by ingestion of sporulated oocysts other than infection with bradyzoites (tissue cysts). It was also observed that following the positive result from the *T. gondii* IgG ELISA, the ODc value for this sample (collection period 13/05/2008), was greater than any other sample tested (ODc = 1.76) as shown in Table 4-4. Although antibodies to the sporozoite antigen could not be detected in the remaining samples (indicating bradyzoite infection), it should also be noted that the ODc values following the *T. gondii* IgG ELISA were lower when compared to the sample which gave a positive result for the sporozoite antigen which could have affected the sensitivity of the assay. In addition it has been shown that antibodies to TgERP are only detectable in humans (and pigs) for six to eight months after infection, with antibody detection rates lower in persistently infected individuals then those with acute infection (Hill *et al.*, 2011). It is possible to test for acute infection using an ELISA, which can detect *T. gondii* specific IgM antibodies. This antibody response is produced one week after infection and then begins to decrease after approximately one month, with levels of IgM generally undetectable by about six months (Rossi, 1998). Therefore it may be useful to test the sera which were analysed for the presence of antibody to TgERP using an IgM ELISA, this would help to determine whether the negative results were due to a persistent infection or as a direct result of bradyzoite infection from consumption of infected undercooked meat.

When a sample of human brain tissue was collected from the Sudden Death Brain Bank, cause of death for that specific individual was also recorded (in addition to age and gender). This information was particularly useful for potentially comparing *T. gondii* prevalence to schizophrenia, suicide and fatalities from road traffic accidents. The cause of death reported from individuals included those who had died due to suicide or fatal road traffic accidents, but also a broad range of other factors, which caused sudden death, as listed in Table 4-6. Although in the current study information is not available on the mental health status of each individual, it can be presumed that this sample set covers a broad range of individuals. The results from this study show no clear association between suicide and molecular detection of *T. gondii*, which is similar to results by Samojłowicz *et al* (2013) who found no

significant difference between *T. gondii* prevalence and sudden death from victims of road traffic accidents. However, suicide only accounted for 6.0% (9/151) of all cases (see Table 4-5). What does initially appear to be apparent, is the number of cases where heart attack/disease is reported as the cause of death and detection of *T. gondii* from their hind brain. The results show that 66.7% of individuals who were positive for *T. gondii* also died due heart attack/disease (see Table 4-6 and Figure 4-9). However, as can clearly be observed in Figure 4-9, a significantly greater number of brain samples came from cases of sudden death due to heart attack/disease, 64.9% (98/151) of all cases (which is the most frequent cause of death in the UK). Therefore it is unlikely that the prevalence of *T. gondii* has any role or link to those who die due to heart attack/disease. Nevertheless, several publications have reported potential involvement with *T. gondii* and complications with the heart (Chandenier *et al.*, 2000; Chapman *et al.*, 1995; Dixit *et al.*, 2007), and more recently a publication described a possible link to acute myocardial infarction (AMI) and *T. gondii* infection (Hamidinejat *et al.*, 2013), where 66.7% (32/48) of cases of AMI where also associated with the presence of anti-*T. gondii* IgG. However any possible link between the parasite and heart complications will require further investigation, and would have to take into account lifestyle habits.

Results from both the serological and molecular detection of the parasite provide more up to date information on the prevalence of the parasite within the Scottish population. The results from the serology study show that there is very little variation within prevalence and the different collection periods (2006 – 2009), which varies from 11.9% (113/947) in collection period 1 to 12.9% (105/811) in collection period 2 (see Table 4-2). In contrast, prevalence determined by molecular detection of the parasite did vary quite dramatically from year to year, with 5.4% (2/37) being the lowest level recorded in 2009 and 27.8 (5/18) in 2012 (see Table 4-5). The year by year variation observed by molecular detection is likely to be due to both the inhomogeneous distribution of tissue cysts and in the case of year 2012 too small a sample size to be considered representative of that year. When comparing the prevalence from both sets of results (serological detection vs. molecular detection), seropositivity from blood donors across all four collection periods (which excluded

repeat samples) was determined at 13.0% (183/1403) (see Table 4-2), whilst molecular detection was slightly higher at 17.9% (27/151) (see Table 4-5). The higher prevalence rate from molecular detection of *T. gondii* is most likely due to sample collection from individuals who were older, with 64.9% of samples obtained from those aged 49 or over, compared to only 29.1% of samples collected from blood donors aged 49 or over. As previously discussed, the correlation between increasing age and an increase in the prevalence of *T. gondii* has been reported in humans and animals, which provides a likely explanation for the higher prevalence observed within the results for molecular detection of the parasite.

Recently a study in the UK which involved 2610 pregnant women in London reported a seroprevalence of 17.3% (Flatt & Shetty, 2012), which is similar to our reported figure for the molecular detection of the parasite (17.9%). However, in this publication when the figure was adjusted to include only those of UK origin (67.7%), the figure dropped to 11.9%, which is identical to our reported seroprevalence figure for collection period 1 (see Table 4-2). Previous seroprevalence studies from the UK where positivity was also measured using *T. gondii* IgG, levels have varied from 7.7% (1031/13328) from pregnant women in east England in 1998 (Allain *et al.*, 1998), and 9.1% from pregnant women in Kent, England (Nash *et al.*, 2005). The results reported within this body of work are from both genders, across a broad age range (17 – 91 years), and are therefore more representative of the general population than studies carried out on pregnant women. Both in this study and others the prevalence of *T. gondii* is low when compared to other European counties, where prevalence can be as high as 55.4% in France, 23.6% in Spain and 26% in the Netherlands (Bellali *et al.*, 2013; Hofhuis *et al.*, 2011; López-Fabal & Gómez-Garcés, 2013), therefore there is a greater risk of women in the UK to become infected for the first time during pregnancy, with a greater risk of congenital toxoplasmosis.

Although strain genotyping was not particularly successful (due to a high concentration of host DNA compared to a low concentration of parasite DNA), two different alleles (type I and type III) from four different individuals were identified (see Table 4-7). In addition, direct sequencing of two of these amplified PCR

products, using GRA6 and SAG3 markers, further confirmed the results observed by PCR-RFLP (see Figure 4-10 and Figure 4-11). In order to obtain further genotyping information from *T. gondii* positive samples, which fail to produce any PCR amplicons when using the less sensitive PCR-RFLP primers, future work should incorporate magnetic capture PCR (MC-PCR) to isolate any *T. gondii* DNA that may be present. It is possible that this methodology may increase the sensitivity, therefore resulting in more amplicons for genotyping. MC-PCR has previously been used for strain genotyping of *T. gondii* (specifically targeting the GRA6 gene), whereby parasite DNA is isolated from host DNA using a GRA6 specific biotin labelled capture-oligonucleotide and streptavidin coated magnetic beads (Opsteegh *et al.*, 2010a). Due to the invasive process required for obtaining human tissue samples, information on parasite strains isolated from humans is limited, but it is thought that strains (in particular atypical strains) are likely to cause a difference in the severity of disease and hence the clinical symptoms observed (Boothroyd & Grigg, 2002; De Salvador-Guillouet *et al.*, 2006; Grigg *et al.*, 2001), similar to what is also seen in other species (Fernandez-Aguilar *et al.*, 2013; Howe & Sibley, 1995; Miller *et al.*, 2004).

Chapter 5: Vaccination and challenge of pigs to evaluate *T. gondii* tissue cyst formation

Aims

- Evaluate the effectiveness of a live attenuated strain of *T. gondii* (S48) in its ability to reduce viable tissue cysts in porcine tissues.

- Investigate whether detection of parasite DNA using homogenised porcine tissues, prior to inoculation into mice, will be as sensitive as mouse bioassay.

- Using a mouse bioassay, determine which porcine tissues the parasite preferentially disseminates to following challenge with either oocysts or tissue cysts.

5.1 Introduction

Due to the omnivorous nature of pigs, they can become infected not only via consumption of *T. gondii* oocysts in the environment but also from accidentally consuming bradyzoites (tissue cysts) from infected rodent cadavers (Kijlstra *et al.*, 2008). In addition pigs which are outdoor reared are more likely to become infected with the parasite compared to those reared indoors (Kijlstra *et al.*, 2004; van der Giessen *et al.*, 2007). Although clinical signs following *T. gondii* infection in pigs are rare, and certainly not a large enough problem to warrant a commercial vaccine against the parasite, the formation of parasitic tissue cysts in the muscles of infected animals can pose a significant risk for food safety and is thought to be one of the most important sources of *T. gondii* infection for humans, particularly when pork is eaten undercooked or raw (Dubey *et al.*, 1991). Therefore, a vaccine which can reduce or eliminate the formation of infective tissues cysts in pigs would be beneficial for pork and pork products intended for human consumption. Previous research into the reduction of the formation of tissue cysts in pigs has included work using live and killed vaccines approaches (Dubey *et al.*, 1994; Dubey *et al.*, 1998b; Garcia *et al.*, 2005; Jongert *et al.*, 2008; Kringel *et al.*, 2004) (as further described in section 1.11.2). It is currently not known whether the commercially available vaccine Toxovax®, which is used to protect against ovine abortion caused by *T. gondii*, offers any protection against tissue cyst formation and was highlighted as a "data gap" in a recent report commissioned by the UK Food Standards Agency (AMCSF, 2012). Toxovax® is comprised of tachyzoites from the "incomplete" strain of *T. gondii* S48. S48 was originally isolated from a case of ovine abortion in New Zealand, where after approximately 3000 passages in mice it lost the ability to develop into tissue cysts (bradyzoites) in mice and oocysts in cats (Buxton & Innes, 1995).

Currently, the gold standard for assessing the viability of *T. gondii* tissues cysts is by mouse bioassay, however, not only does this method require experimental animals with *in vivo* mouse studies, but it is also time consuming, expensive and hazardous due to the preparation of potentially infective tissue cysts (Esteban-Redondo *et al.*, 1999). An attempt to move away from this methodology would also be beneficial in

reducing the amount of animals used for the bioassay. Molecular technologies have become more sensitive for the detection of parasite DNA, for example by use of nested PCR, qPCR and magnetic capture qPCR (Calderaro *et al.*, 2006; Hurtado *et al.*, 2001; Opsteegh *et al.*, 2010a), and these alternative options should be explored.

It is known that infection by both oocysts and bradyzoites will result in the formation of tissue cysts within the meat of an infected animal. Experiments in the 1980's using mouse bioassays highlight these findings (Dubey, 1988; Dubey *et al.*, 1986), but as previously mentioned, molecular technologies have developed over the past twenty years, therefore re-assessing the distribution of parasite tissue cysts within specific tissues from infected animals should be re-addressed. This information would be useful to researchers for determining which particular tissues would be most useful for detection of parasite DNA, in for example, animal experiments or abattoir studies. Also knowing whether the bradyzoite (tissue cyst) stage of the M4 strain of *T. gondii* was as effective at establishing itself in the host as the oocysts stage, would be beneficial for future animal challenge experiments. If bradyzoite challenge is as effective as oocyst challenge in causing infection (both in terms of the resulting host immunological response, and establishment of tissue cysts), then future experiments using the M4 strain in pigs would not require the preparation of oocysts, which involves yet another animal model, the cat.

It has been shown that low numbers of oocysts, (even one single oocyst), are sufficient to cause infection and subsequent development of viable tissue cysts in pigs (Dubey *et al.*, 1996). In the current study the experimental infection with either 1000 M4 *T. gondii* oocysts or tissue cysts was designed to reflect a challenge, which may be similar to that obtained in the environment, but also a dose which would avoid severe clinical signs such as anorexia, severe prostration, and even death as observed in a previous studies where pigs were challenged with more than 10^4 oocysts (Dubey *et al.*, 1998b; Garcia *et al.*, 2008)

The objective of this study was threefold; evaluate the effectiveness of a live attenuated strain of *T. gondii* (S48) in its ability to reduce viable parasitic tissue cysts in porcine tissues; to investigate whether detection of parasite DNA using

homogenised porcine tissues prior to inoculation into mice would be as sensitive as mouse bioassay; and to compare between the two infective stages of the parasite and their ability to develop into infective tissues cysts.

5.2 Experimental design

An overview of the timescale of the experiment can be seen in Appendix 8.2.

5.2.1 Pig vaccination and challenge

A total of 23 pigs were used in the experiment which were separated into five experimental groups (G) (see Table 5-1 and Figure 5-1); G1 (n = 5) un-vaccinated and tissue cyst challenged animals, G2 (n = 5) un-vaccinated and oocyst challenged animals, G3 (n = 5) vaccinated and oocyst challenged animals, G4 (n = 5) vaccinated non-challenged animals, G5 (n = 3) un-vaccinated and non-challenged animals. The five animals in G3 and G4 were vaccinated SC with $1.2x10^5$ S48 tachyzoites (see section 2.4.1) 4 weeks prior to experimental challenge -28dpi). Four weeks following vaccination (0 dpi) animals in G2 and G3 were orally challenged with 1000 *T. gondii* M4 oocysts (see section 2.6 for oocyst preparation), whilst animals in G1 were orally challenged with 1000 *T. gondii* M4 tissue cysts (see section 2.6). All 23 animals were culled six weeks post challenge (42 dpi) and porcine tissues collected at post mortem (see Table 2-2 for list of tissues collected at PM)

Table 5-1: Animal groupings for pig vaccination and *T. gondii* challenge.

Group	Vaccination and / or challenge		Number of animals
	-28 dpi	0 dpi	
G1	n/a	1000 M4 tissue cysts	n = 5
G2	n/a	1000 M4 oocysts	n = 5
G3	$1.2x10^5$ tachyzoites	1000 M4 oocysts	n = 5
G4	$1.2x10^5$ tachyzoites	n/a	n = 5
G5	n/a	n/a	n = 3

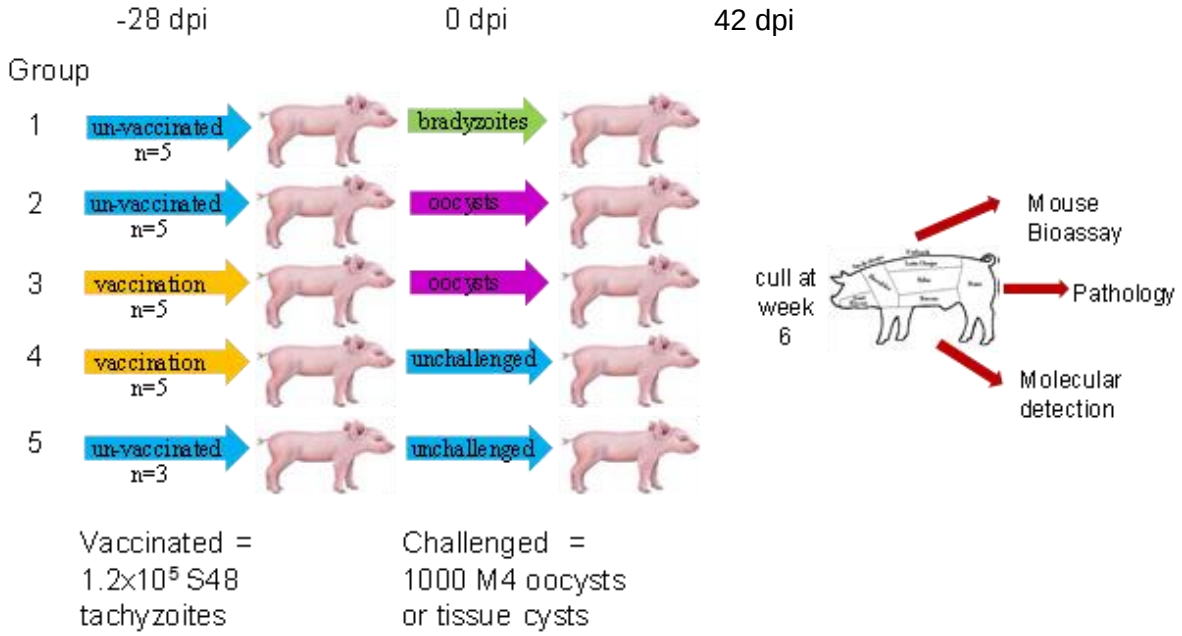

Figure 5-1: Overview of experimental design for vaccination and *T. gondii* challenge of pigs.

5.2.2 Mouse bioassay to assess the viability of *T. gondii* tissue cysts

Mouse bioassay is the gold standard for determining the viability of *T. gondii* tissue cysts (as further descried in section 1.10.2). In this study specific porcine tissues were removed at post mortem (see Table 2-2 for a complete list of tissues removed at post mortem) and a selection of these tissues from pigs in groups 1, 2 and 3 (tissue cyst challenged, oocyst challenged and vaccinated and oocyst challenged pigs respectively) were used in a mouse bioassay. The tissues used for the bioassay were separated into three different 50g groups depending on the tissue type (as described in Table 5-2), and included the following groups; "Brain" (50g of brain), "Food" (12.5g each of chop, loin, left tricep and left semitendinosus) and "Other" (12.5g each of diaphragm, heart, tongue and masseter).

By using specific porcine tissues from experimental groups 1, 2 and 3, not only could the effect of vaccination on parasite tissue cysts viability be assessed, but also the

viability of tissue cysts from the two infective stages of the parasite, oocyst and bradyzoites.

Table 5-2: Porcine tissues used for mouse bioassay.

Mouse bioassay tissue group	Porcine tissue
Brain	Brain*
Food	Chop, loin, left tricep, left semitendinosus
Other	Diaphragm, heart, tongue, masseter

***A total of 50g of brain was used. For the "food" and "other" groups 12.5g of the appropriate tissue was collected and used to make a total of 50g to process for mouse bioassay.**

Tissues from pigs in groups 4 and 5 (vaccinated non-challenged and the negative control group animals) were not included in the bioassay. However, in these two groups, the same tissues which were processed for mouse bioassay were also tested for the presence of *T. gondii* DNA by ITS1 PCR (see section 2.12.2).

5.3 Materials and Methods

5.3.1 Temperature monitoring

Rectal temperatures of all pigs were monitored 5 days prior to vaccination (-33 dpi) and up to 14 days following vaccination (-14 dpi) as described in section 2.1.2.1 Due to the increase in the size of the animals, temperatures were recorded using a Thermochip® (see section 2.1.2.1) -2 dpi until 14 dpi.

5.3.2 Clinical observations

5.3.2.1 **Pigs**

All pigs were observed daily throughout the duration of the experiment and their health status evaluated as described in section 2.1.2.1.

5.3.2.2 **Mice**

Mice were monitored twice daily (up to three times daily for the first 14 days after challenge with the bioassay inocula) and their health status evaluated as described in section 2.1.3.1. Any animal which obtained a score of 3 or above was euthanized prior to the end of the experiment, with signs of infection including hunched appearance, tottering gait or reluctance to move.

5.3.3 Preparation of bioassay inocula

An acid/pepsin digest was carried out on a total of 50g of different porcine tissues as described in section 2.8.1 and by Dubey *et al* (1998). The specific tissues used for bioassay are described in section 5.2.2 (see Table 5-2). The resulting digested material was re-suspended in 3mls of 4x penicillin / streptomycin solution and 400µl of each inocula was injected into three mice by IP route.

5.3.4 Molecular detection of *T. gondii*

DNA was extracted from porcine tissues, mouse brains and bioassay inocula as described in section 2.8.1. An ITS1 PCR (see section 2.12.2) specific for *T. gondii* was used to identify the presence of parasite DNA from these samples, PCR was carried out in triplicate for each sample and included a positive control and multiple negative controls (see section 2.12.1).

5.3.5 Serology

Sera was collected from all pigs from 5 days prior to vaccination (-33 dpi) and then on a weekly basis until the end of the experiment (42 dpi). Sera from the mice used in the bioassay was obtained at the end of the experiment (week 6), or when animals were euthanised due to clinical signs of *T. gondii* infection. The commercially available ID Vet ELISA kit was used to detect IgG antibodies raised against the *T. gondii* SAG1 antigen (see section 2.10.2).

5.3.6 Statistical analysis

5.3.6.1 Comparison between *T. gondii* IgG levels from pigs within different challenge groups using the Student *t*-test.

To analyse the statistical significance between the different porcine challenge groups following the *T. gondii* IgG ELISA were calculated using the Student *t*-test. Levels of significance are quoted as "Probability" (*p*) where a p-value ≤ 0.05 indicated 95.0% probability; ≤ 0.01 indicated a probability of 99.0% and ≤ 0.001 of indicates a probability of 99.9%.

5.3.6.2 Mouse bioassay survival

A Kaplan-Meir survival curve was plotted to compare the mean proportions of mice survivability for different groups. To compare the mean survival proportions of mice between different groups (tissue cyst challenged, oocyst challenged and vaccinated and oocyst challenged), a Fisher's exact test was conducted within each organ (brain, food and other) as well as pooling the data across all organs. This analysis was completed by Mintu Nath from Biomathematics and Statistics Scotland (BioSS, The Kings Buildings, Edinburgh, UK).

5.4 Pig Challenge Results

5.4.1 Clinical responses in pigs following vaccination and challenge were minimal.

5.4.1.1 Temperature

Rectal temperatures of all animals were recorded from days -5 to 14 days post vaccination (-33 dpi to -14 dpi respectively) as described in section 5.3.1. The mean animal temperature within each group did not appear to fluctuate within this time frame as shown in Figure 5-2. It can also be seen that there was no obvious difference between the mean temperature of the animals which were vaccinated (groups 3 and 4) compared to those which had not been vaccinated, (groups 1, 2 and 5). Due to the increasing size of the animals, temperature monitoring following challenge (-2 dpi to 14 dpi) was not as accurate, as the technique used for recording temperature was an implanted Thermochip®. Temperatures using this method fluctuated greatly due to the location of the chip resulting in vast variations of temperature readings, which were not reliable or informative and could not be used in the analysis of the experiment.

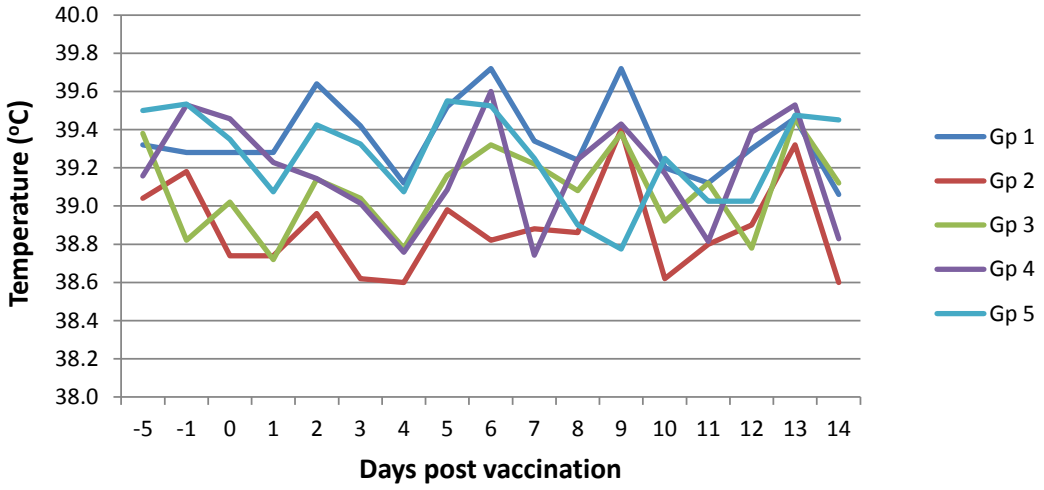

Figure 5-2: Average porcine temperature per experimental group following vaccination with S48 tachyzoites.

5.4.1.2 Clinical observations

All animals were monitored daily throughout the experiment and their health status was scored as described in 2.1.2.1. Throughout the experiment all animals scored 0 for demeanour, except on 7 dpi, where five animals in groups 1 and 2 had a score of 1. These animals appeared subdued/depressed for approximately 24 hours, although they were interested in food and movement was unaffected.

5.4.2 Vaccinated and challenged pigs seropositive by d21 dpi, with no significant difference in % seropositivity between oocyst and tissue cyst challenged animals.

Using the ID Vet ELISA described in section 2.10.2, all pigs tested negative for the presence of *T. gondii* IgG, at the beginning of the experiment (see Table 5-3).

Table 5-3: *T. gondii IgG* levels in pigs prior to vaccination. All animals were negative for *T. gondii* IgG prior to the start of the experiment (-33 dpi)

Group	% SP at -33 dpi
1 (Tissue Cysts)	0.7
2 (Oocysts)	0.4
3 (Vacc + Oocysts)	0.3
4 (Vacc)	0.2
5 (Neg CTL)	0.6

SP = % seropositivity as determined by the commercially available ID vet *T. gondii* IgG ELISA.

At 21 dpi all animals, apart from those in group 5 (negative control animals), tested IgG positive and the average % seropositivity per group was calculated (as described in section 2.10.2). The average % seropositivity for animals in group 1 was 130%, whilst the group 2 average was 128%, group 3 had the highest average at 156% and group 4 an average of 137% (see Figure 5-3). The average % seropositivity for those animals in group 5 was 4%, indicating that they had remained seronegative for *T. gondii* IgG. These results not only show that the animals in groups 1 and 2 had successfully mounted an immune response to challenge from either tissue cysts or oocysts, but there was no significant difference between the two results (p = 0.787, see Table 5-4). Those animals in group 4 had produced an immune response to vaccination with the live attenuated strain of the parasite (S48) which was not significantly different to those animals in groups 1 ($p \leq 0.05$) and 2 ($p \leq 0.05$) as shown in Table 5-4. Group 3 animals displayed the highest % seropositivity (156%) which was significantly higher ($p \leq 0.01$) than animals in groups 1, 2 and 4 (see Table 5-4 and Figure 5-3).

Table 5-4: Statistical analysis of *T. gondii* IgG levels from pigs within different challenge groups at 21 dpi.

Comparing Group	With Group				p-value / Probability
	2	3	4	5	
1	0.787	**0.011**	0.434	**0.0000**	**p-value**
	n/a	**95.00%**	n/a	**>99.9%**	**Probability**
2		**0.024**	0.396	**0.0000**	**p-value**
		95.0%	n/a	**>99.9%**	**Probability**
3			**0.040**	**0.0000**	**p-value**
			95.0%	**>99.9%**	**Probability**
4				**0.0000**	**p-value**
				>99.9%	**Probability**

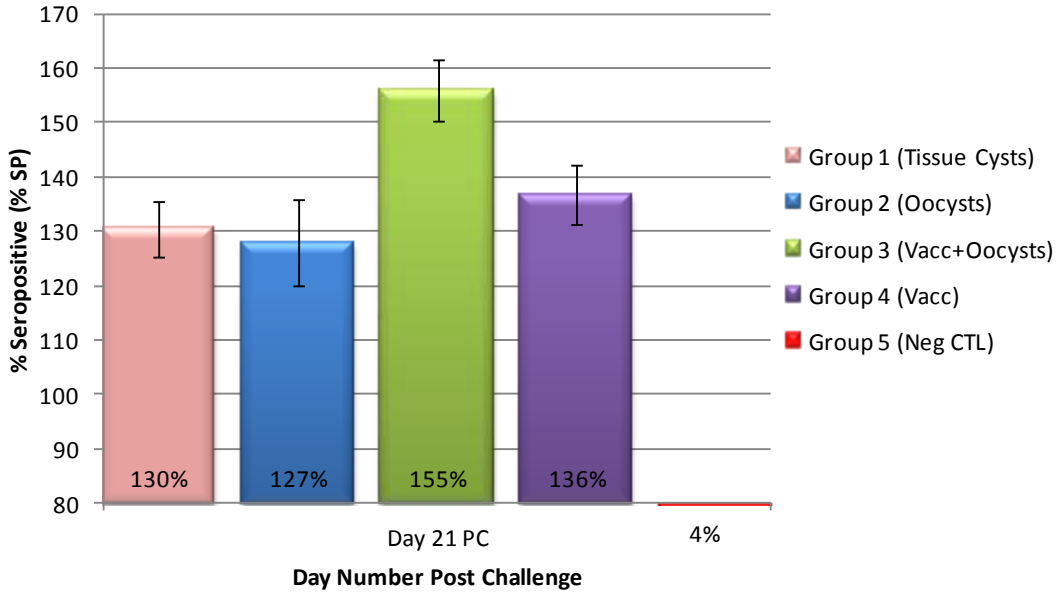

Figure 5-3: Average *T. gondii* IgG levels in pigs 21 days after challenge. Error bars plotted using the Standard Error of the Mean (SEM).

In addition, animals from group 1 and 2 were also tested for the presence of *T. gondii* IgG at 7 and 14 days PC (Figure 5-4). At 7 days PC both groups were seronegative, however, at 14 days PC the average percent seropositivity had increased within both groups (group 1 SP = 89%, group 2 SP = 72%) indicating a *T. gondii* IgG positive result. At day 7 post challenge there was no significant difference observed in percentage seropositivity between group 1 and group 2.

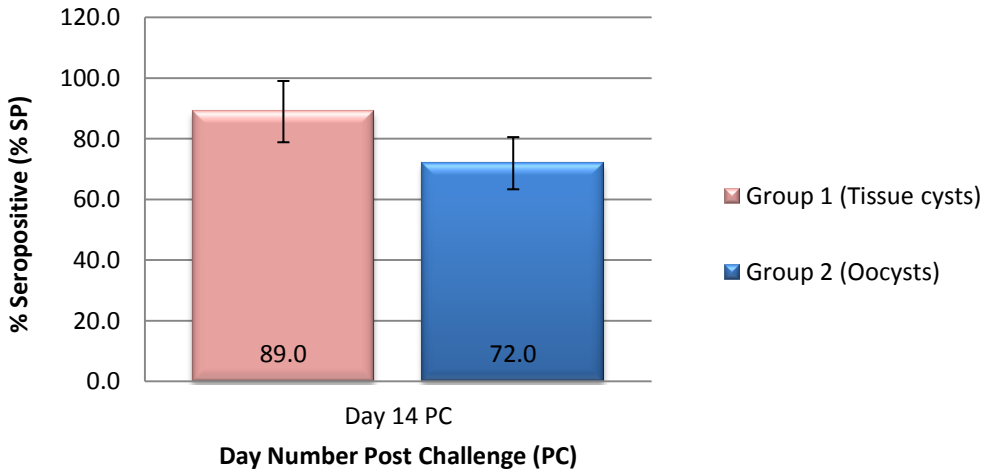

Figure 5-4: Average *T. gondii* IgG levels in pigs 14 days after challenge. Error bars plotted using the Standard Error of the Mean (SEM).

5.4.3 *T. gondii* DNA detected in a limited number of pig tissues.

As highlighted in the experimental design (see section 5.2) all animals were culled six weeks post challenge. At post mortem thirty five different tissue samples (see 2.1.3.2) were collected from each animal, of which 16 were used for bioassay and molecular detection of *T. gondii* directly from pig tissue (see Table 2-2). *T. gondii* was only detectable by ITS1 PCR in five tissues from two different animals from group 1, which had been challenged with *T. gondii* tissues cysts alone (see Table 5-5). The results show that parasite DNA was detected in four different tissues (brain – CNS1, heart, chop and masseter) from animal 830, and also from one tissue (heart) from animal 832, both pigs belonged to group 1 (tissue cyst infected animals). *T. gondii* DNA was not detected in any of the other tissues from pigs in group 1, therefore 6.25% (5/80) of all tissues from group 1 were positive for *T. gondii*. In addition, parasite DNA could not be detected by ITS1 PCR from any tissues from pigs within experimental groups 2, 3, 4 and 5.

Table 5-5: Detection of parasite DNA from porcine tissues. A total of five tissues from two different animals were positive by ITS1 PCR, all other porcine tissues from the remaining experimental groups were ITS1 negative.

Group No.	Pig No.	Tissue Type	Tissue Number	No. PCR pos (triplicate)
1	830	Brain (CNS1)	1A	1/3
1	830	Heart	4A	3/3
1	830	Chop	4B	3/3
1	830	Masseter	4K	1/3
1	832	Heart	4A	3/3

5.5 Mouse Bioassay Results

5.5.1 Mouse survival rate as an indication of viable *T. gondii* tissue cysts from pig tissues - mouse bioassay

5.5.1.1 Overall Survival rates

The mouse bioassay has been described as the gold standard in determining the viability of *T. gondii* tissue cysts (see section 1.10.2). Porcine tissues were collected at post mortem, and sixteen tissues were processed for mouse bioassay as described in Table 2-2. In total 68.9% (93/135) of mice survived until the end of the six week experiment (see Figure 5-5), although 42 mice were culled prior to the end of the six week bioassay (2 mice on day 10, 19 on day 11, 19 on day 12 and 2 on day 18) as their health status score (see section 2.1.3.1) was recorded as 3 or above due to signs of *T. gondii* infection.

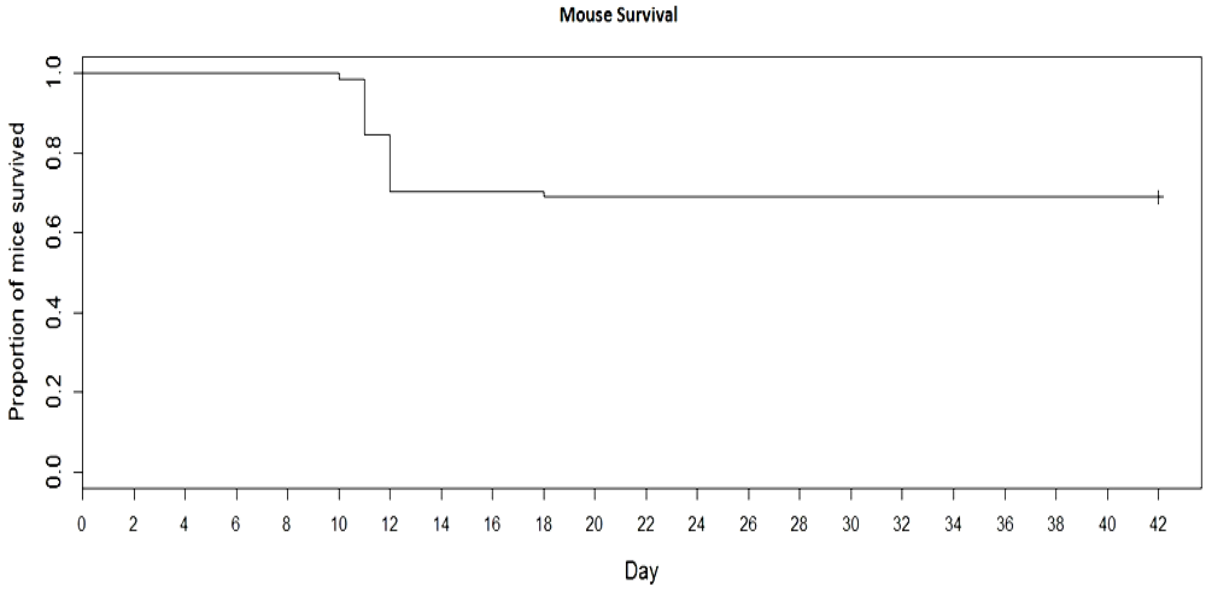

Figure 5-5: Overall mouse survival.

5.5.1.2 Mouse survival is greater in mice inoculated with tissues from vaccinated and challenged pigs, with no significant difference in mortality between mice inoculated with tissues from oocyst or bradyzoite challenged pigs.

When further analysing the results in relation to mouse survival with each individual challenge inocula (Table 5-2), the results show that at the end of the 6 week bioassay 100% (45/45) of group 3 mice (animals which had been inoculated with tissues from pigs which had been vaccinated with S48 and then challenged with oocysts of the M4 strain of *T. gondii*) had survived (see Figure 5-6 and Figure 5-7). However, mice in both groups 1 and 2 (animals had received homogenised tissues from pigs infected with M4 *T. gondii* tissue cysts or oocysts) began to die within 10 days post challenge (see Figure 5-6). The mouse survival rate at the end of the 6 week bioassay within groups 1 and 2 were 55.6% (25/45) and 51.1% (23/45) respectively (see Figure 5-6 and Figure 5-7). By week 6 there is a significant difference (p = 1.545e-08) between those mice which received porcine tissues from animals in group 3 (vaccinated and oocyst challenged pigs) compared to mice which received tissues from animals infected by the same stage of the parasite (oocysts) which had not been vaccinated (group 2), as shown in Figure 5-6. What can also be observed from this figure is that there is no significant differnce (p = 0.833) between the mouse survival rates from animals in group 1 and 2, representing the two infective stages of *T. gondii* (tissue cyst or oocyst infection).

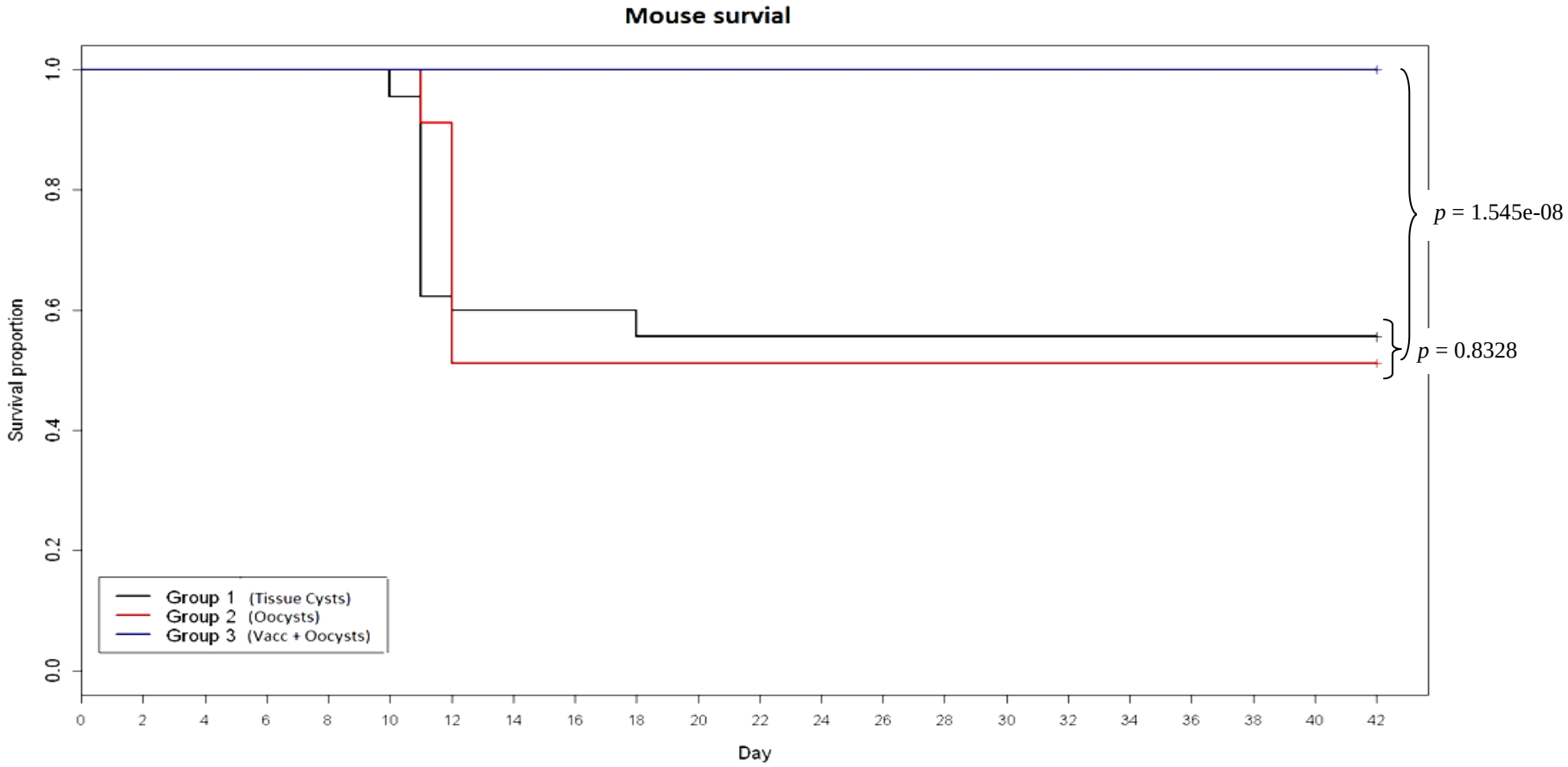

Figure 5-6: Mouse survival rates in relation to challenge inocula. There is no significant difference between groups 1 and 2, whilst there is a significant difference between groups 2 and 3.

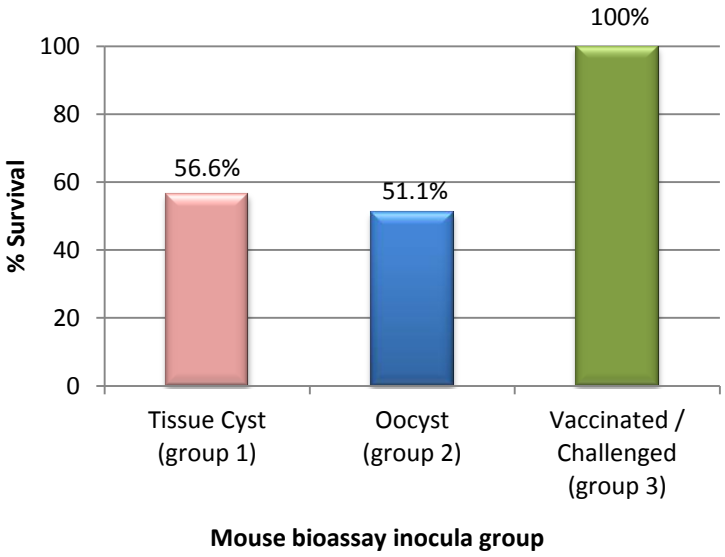

Figure 5-7: Mouse survival rates at 6 weeks PI in relation to challenge inocula.

When further comparing mouse survival and the two infective stages of *T. gondii* (tissue cysts and oocysts) and also taking into account the different tissue groups used to inoculate mice (brain, food and other – see Table 5-2), the "other" and "brain" tissue groups appear to cause a significantly higher number of deaths than the food group, as shown in Figure 5-8. The mouse survival curves in Figure 5-9 for group 1 (tissue cysts), and Figure 5-10 for group 2 (oocyts) further highlight this result.

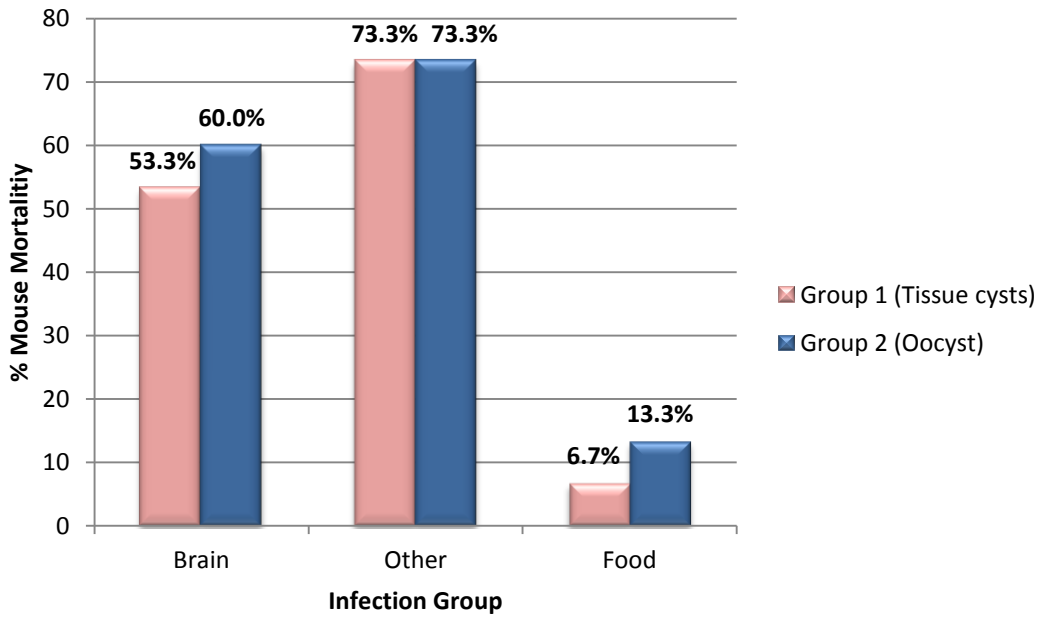

Figure 5-8: Percentage of mouse mortalities related to tissue group and challenge type.

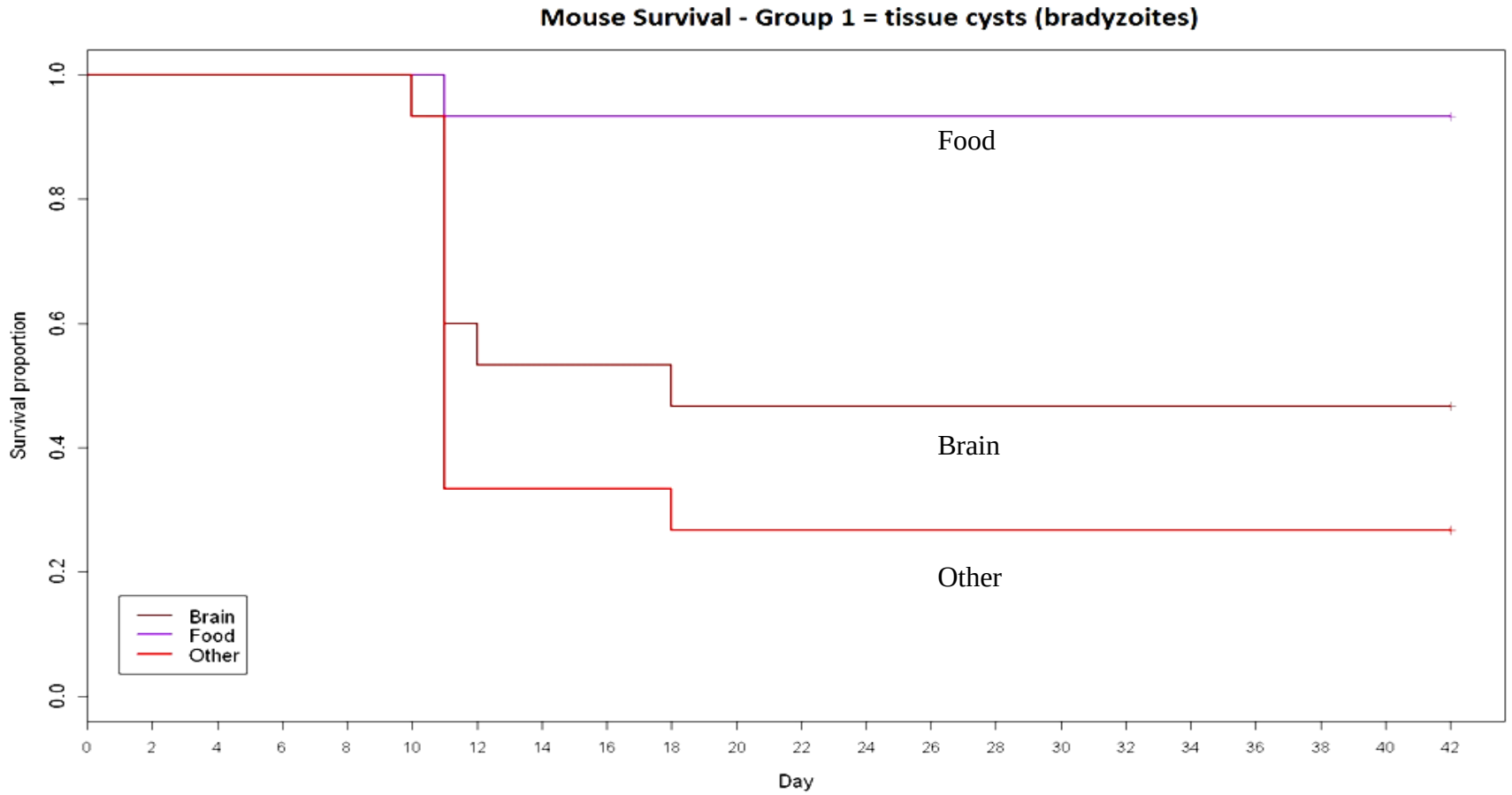

Figure 5-9: Mouse survival rates from animals in group 1 in relation to specific porcine tissues. "Brain" and "Other" group causing the greatest number of mouse mortalities.

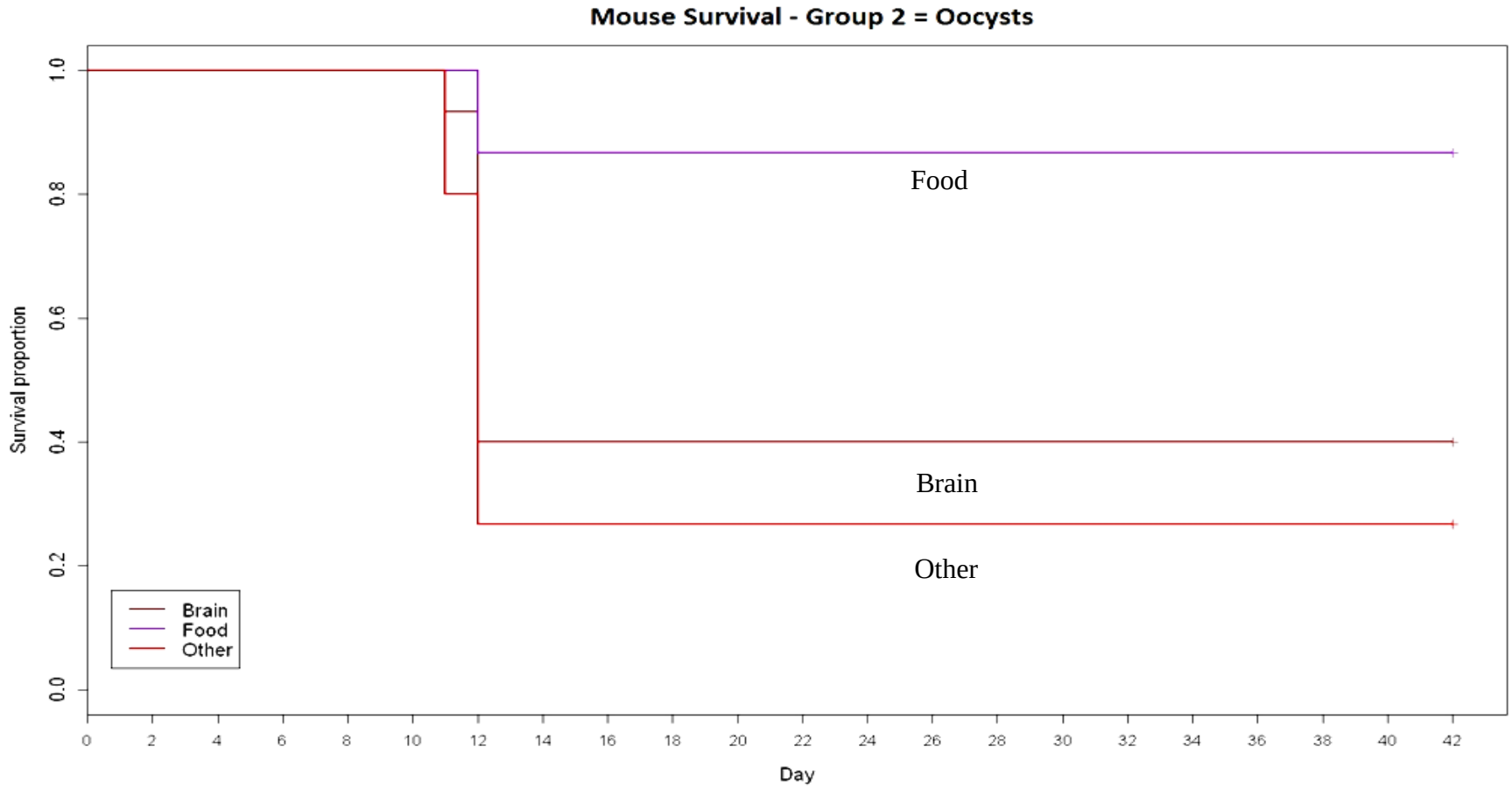

Figure 5-10: Mouse survival rates from animals in group 2 in relation to specific porcine tissues. "Brain" and "Other" group causing the greatest number of mouse mortalities.

5.5.2 Molecular detection of *T. gondii* from mouse brain is more sensitive than DNA extracted from bioassay inocula.

From the 42 mice which were culled prior to the end of the six week mouse bioassay (see section 5.5.1.1), *T. gondii* DNA was detected in 100% (42/42) of these mice by ITS1 PCR, using DNA which had been extracted from each individual mouse brain as summarised in Table 5-6 and in more detail showing each individual mouse result in Table 5-7. In should also be noted that three mice, which survived until the end of the experiment, were also ITS1 positive. These mice had been inoculated with porcine tissues from animals in either group 1 or group 2 (tissue cyst challenged or oocyst challenged pigs respectively). When mouse brain DNA was extracted from the remaining 90 (66.7%) mice, which had survived until week 6 PI, all brains were negative for *T. gondii* DNA (see Table 5-7).

Table 5-6: Summary of *T. gondii* DNA detected by ITS1 PCR in mouse brains following bioassay with porcine tissues.

Mouse Group	ITS1 positive mice (n)	% ITS1 positive	ITS1 negative mice (n)	% ITS1 negative
1	21[a]	46.7	24	53.3
2	24[b]	53.3	21	46.7
3	0	0	45	100
TOTAL	45	33.3	90	66.7

[a] = Including one mouse (C5-4) which survived until Wk6 PI.

[b] = including two mice (C11-1 and C11-2) which survived until Wk6 PI.

Table 5-7: Detection of *T. gondii* DNA by ITS1 PCR following bioassay from individual mouse brains – individual mouse data.

Cage	Mouse No.					Inocula group
1	1^{b}d	2^{b}d	3^{b}d	4^{f}	5^{f}	**Group 1** (Tissue Cysts)
2	1^{f}	2^{o}d	3^{o}d	4^{o}d		
3	1^{b}	2^{b}	3^{b}	4^{f}	5^{f}	
4	1^{f}	2^{o}	3^{o}	4^{o}		
5	1^{b}d	2^{b}	3^{b}d	4^{f}	5^{f}	
6	1^{f}	2^{o}d	3^{o}d	4^{o}d		
7	1^{b}d	2^{b}d	3^{b}d	4^{f}	5^{f}	
8	1^{f}d	2^{o}d	3^{o}d	4^{o}d		
9	1^{b}	2^{b}	3^{b}	4^{f}	5^{f}	
10	1^{f}	2^{o}d	3^{o}	4^{o}d		
11	1^{b}	2^{b}	3^{b}d	4^{f}	5^{f}	**Group 2** (Oocysts)
12	1^{f}	2^{o}	3^{o}	4^{o}		
13	1^{b}d	2^{b}d	3^{b}d	4^{f}	5^{f}	
14	1^{f}	2^{o}d	3^{o}d	4^{o}d		
15	1^{b}	2^{b}d	3^{b}d	4^{f}	5^{f}	
16	1^{f}	2^{o}d	3^{o}d	4^{o}		
17	1^{b}d	2^{b}d	3^{b}d	4^{f}	5^{f}	
18	1^{f}	2^{o}d	3^{o}d	4^{o}d		
19	1^{b}	2^{b}	3^{b}	4^{f}d	5^{f}d	
20	1^{f}	2^{o}d	3^{o}d	4^{o}d		
21	1^{b}	2^{b}	3^{b}	4^{f}	5^{f}	**Group 3** (Vacc+oocysts)
22	1^{f}	2^{o}	3^{o}	4^{o}		
23	1^{b}	2^{b}	3^{b}	4^{f}	5^{f}	
24	1^{f}	2^{o}	3^{o}	4^{o}		
25	1^{b}	2^{b}	3^{b}	4^{f}	5^{f}	
26	1^{f}	2^{o}	3^{o}	4^{o}		
27	1^{b}	2^{b}	3^{b}	4^{f}	5^{f}	
28	1^{f}	2^{o}	3^{o}	4^{o}		
29	1^{b}	2^{b}	3^{b}	4^{f}	5^{f}	
30	1^{f}	2^{o}	3^{o}	4^{o}		

ITS1 Positive ITS1 Negative

d = culled due to signs of Toxo; * = survived until Wk6 but ITS1 positive; tc = tissue cysts observed by light microscopy.
[b] = mice inoculated with homogenised tissues from the porcine "brain" group.
[f] = mice inoculated with homogenised tissues from the porcine "food" group.
[o] = mice inoculated with homogenised tissues from the porcine "other" group.

DNA which had been extracted from the inocula used to challenge the mice was also tested for the presence of *T. gondii* DNA by ITS1 PCR. All inocula (n = 45) were tested (15 inocula per experimental group). The results from this show that the inocula comprised of porcine tissues from pigs in group 1 (tissue cyst challenged pigs), 33.3% (5/15) inocula were positive for *T. gondii* DNA, whilst the inocula generated from group 2 pigs (oocyst challenged pigs), 46.7% (7/15) of inocula were positive. However, *T. gondii* DNA could not be detected in any of the inocula (0/15) comprised from tissues of the group 3 pigs (vaccinated and challenged pigs) as highlighted in Table 5-8).

Table 5-8: Comparison between detection of *T. gondii* DNA by ITS1 PCR from bioassay challenge inocula and in the brains of mice challenged with the same material.

Inocula generated from pigs - group no.	Inocula ITS1 positive (%)	Mouse brain identified as ITS1 positve[a] (%)	Infections undetected by PCR of inocula alone (%)
1 (Tissue cysts)	33.3 (5/15)	53.3 (8/15)	20 (3/15)
2 (Oocyst)	46.7 (7/15)	60.0 (9/15)	13.3 (2/15)
3 (Vacc+oocyst)	0 (0/15)	0 (0/15)	0 (0/15)
Total	26.7 (12/45)	37.8 (17/45)	11.1 (5/45)

[a] = to enable the comparison one ITS1 positive mouse indicates a positive result for using the same challenge inocula.

A comparison between parasite DNA detected directly from the brains of experimental mice in the bioassay and the presence or absence of DNA detected from exactly same the material used to challenge mice can be seen in Table 5-8. This comparison shows that although parasite DNA could be detected in the inocula from both pigs in group 1 and 2, mouse bioassay with the inocula and subsequent detection of *T. gondii* DNA in the brain is more sensitive than testing for the

presence of parasite DNA in the inocula alone, as 20% of infections were undetected from group 1 and 13.3% from group 2.

5.5.3 Limited serological detection of *T. gondii* in mice following bioassay.

Serum samples from all mice (n=135) were collected at post mortem and tested for the presence *T. gondii* IgG antibodies using the commercially available ID vet ELISA (see section 2.10.2). One mouse (mouse number C11-2), which was culled on day 42, tested IgG positive with 189% seropositivity (*T. gondii* DNA was also detected from the brain of this mouse (see Table 5-7). Another mouse (mouse number C5-3) which was culled on day 18 gave a borderline result of 44% seropositivity (*T. gondii* DNA was also detected from the brain of this animal, and upon microscopic examination of a brain smear, a small tissue cyst was also observed from this mouse (see Table 5-7). Mouse C11-1 was classified as seronegative however a value of 23% seropositivity was greater than all the remaining mice where seropositivity was below 3%, again this animal was euthanized due to signs of *T. gondii* infection on d18 PI (see Table 5-9) and tested positive by PCR (see Table 5-7)

Table 5-9: Detection of *T. gondii* IgG in mice following inoculation with porcine tissues (bioassay).

Mouse no.	Group no.	IgG seropositivity (%)	Result	Day PI mouse euthanised
C5-3	1	44	Borderline	d18
C11-1	2	23	Negative	d18
C11-2	2	189	Positive	d42

All remaining mice (n=132) were IgG seronegative (below 3% SP)

5.6 Discussion

Mouse bioassay to assess the success of vaccination:

The results from the mouse bioassay clearly show that vaccination with a live attenuated strain of the parasite (S48) and subsequent challenge with *T. gondii* oocysts (M4) in pigs is particularly successful in preventing infective tissue cysts from establishing within porcine tissues. Mouse survival rates from the bioassay at 6 weeks PI show a clear difference between those mice inoculated with porcine tissues from pigs in group 2 (oocyst challenged pigs), compared to mice inoculated with porcine tissues from pigs in group 3 (vaccinated and oocyst challenged pigs) (see Figure 5-6 and Figure 5-7). 100% of mice inoculated with tissues from group 3 pigs survived until the end of the experiment, whilst only 51.1% of mice inoculated with tissues from pigs in group 2 survived, with the first four mice culled on day 11 PI and a total of 22 mice culled by day 18 (see Figure 5-6). As is has been observed that the M4 strain (type II) of *T. gondii* is particularly virulent in Porton mice (L. Gibbard, Moredun Bioservices – personal communication), any viable tissue cysts present in the inocula (homogenised porcine tissue) will cause clinical symptoms with the resulting euthanasia of mice. Therefore in this study reporting of mouse survival was important, as it indicated the presence or absence of viable tissue cysts. As little as one tissue cyst (containing possibly thousands of infective bradyzoites) is enough to cause infection (Dubey *et al.*, 1998a).

Other research, which has studied the vaccination of pigs to reduce tissue cyst burden, has mainly focused on microscopic identification of tissue cysts and / or detection of parasite DNA from mice used in the bioassay (Cunha *et al.*, 2011; Dubey *et al.*, 1994; Dubey *et al.*, 1998b; Freire *et al.*, 2003; Garcia *et al.*, 2006; Garcia *et al.*, 2005; Kringel *et al.*, 2004), in these studies there is no detailed information about mouse survival and clinical signs of *T. gondii* during the bioassay itself, perhaps in these experiments euthanasia of mice due to signs of *T. gondii* infection (such as hunched appearance, tottering gait and moribund) was not common and the majority of mice survived until the end of the study. In fact, very few *T. gondii* studies, which incorporate mouse bioassay to assess the viability of tissue cysts, describe mouse survival rates in any detail. Research by Pena *et al* (2006), did report mouse mortality rates following bioassay of cat tissues in relation

to the genotype present, however, the majority of literature which report using this technique (mouse bioassay) do not provide this information.

In the current study, molecular detection of *T. gondii* DNA from mouse brain following bioassay was also completed. Parasite DNA was detected in 0% (0/45) of mice following challenge with inocula comprised of porcine tissues from animals in group 3 (vaccinated and oocysts challenged pigs), whilst in the positive control groups parasite DNA was detected in 46.7% (21/45) of mice following challenge with inocula comprised of porcine tissues from animals in group 1 (tissue cyst challenge pigs), and 53.3% (24/45) of mice following challenge with inocula from pigs in group 2 (oocyst challenged pigs) (see Table 5-6). Other research, investigating vaccination and *T. gondii* challenge of pigs and subsequent mouse bioassay, have focused on the microscopic detection of tissues cysts from mouse brain following bioassay (da Cunha *et al.*, 2012; Dubey *et al.*, 1998b; Dubey *et al.*, 1991; Garcia *et al.*, 2005) other than detection of *T. gondii* DNA from the mouse brain. Therefore it is difficult to draw direct comparisons between these similar vaccination and challenge experiments, however, as 100% of mice from the bioassay survived following inoculation with tissues from pigs vaccinated and oocyst challenge, vaccination with S48 tachyzoites is a promising strategy for reducing tissue cyst burden in pigs. Previous research into vaccination of pigs against *T. gondii*, which have incorporated mouse bioassay, have not shown such a protective response against tissue cyst formation in mouse brains. For example, following immunisation of pigs with crude *T. gondii* rhoptry proteins plus Quil-A, da Cunha *et al* (2012) found only partial protection from formation of tissue cysts in mouse brains following bioassay of porcine tissues, with the parasite detected in 5/11 (45.4%) of mice in the vaccinated and challenged group. In a similar experiment, partial protection was observed by Garcia *et al* (2005), who used *T. gondii* rhoptry proteins containing immuostimulating complexes (ISCOMS) as an adjuvant to vaccinate pigs. Dubey *et al* (1998b) tested a vaccine incorporating a low dose of irradiated *T. gondii* oocysts, and although fewer tissue cysts in mice were observed, cysts were observed in 45/110 (40.9%) of mice which had received porcine tissues from vaccinated pigs. Further work by Dubey *et al* (1991), who used tachyzoites of a non-persistent strain of *T. gondii* (RH) to vaccinate pigs, again when porcine tissues were used in mouse

bioassay only found partial protection with fewer tissues cysts in mice. In summary, none of the literature currently available can describe 100% protection against tissue cyst formation in mice, following bioassay of porcine tissues.

From the current results, it appears that the vaccine (S48) alone does not induce tissue cysts as results from the mouse bioassay, which included porcine tissues from the vaccinated and challenged animals, were all PCR negative for *T. gondii*. If a positive had been obtained from this group, the experiment had been designed to verify whether infection was due to vaccination (S48) or oocyst challenge (M4). Both S48 and M4 have different genotypes (S48 = type I, M4 = type II), and by incorporating PCR-RFLP genotyping, it would be possible to ascertain whether infection was due to vaccination or oocyst challenge. However, a bioassay incorporating porcine tissues from animals that were vaccinated alone (which was not included in this study), could further support this result, although it is unlikely that positives will be identified.

Comparison between *T. gondii* oocyst and bradyzoite infection in pigs and viability of tissue cysts in the mouse bioassay.

When comparing mouse survival rates between animals, which had been challenged with inocula from tissues of pigs in group 1 (tissue cyst challenged pigs) and group 2 (oocyst challenged pigs), no significant difference was observed between mouse mortality rates resulting from the different infective stages of the parasite (see Figure 5-7). Both infective stages appear to cause similar levels of infection, although a slight difference in clinical observations was seen in tissue cyst challenged pigs (group 1). In addition the IgG response in pigs following challenge with these two stages of the parasite did not differ significantly (see Figure 5-3 and Table 5-4). However, what was apparent was the difference in mouse mortality between the different porcine tissue groups (e.g. “brain”, “other” and “food” groups) used to inoculate mice (see Figure 5-8). A greater number of mortalities occurred from mice inoculated with porcine tissues from both the “other” group (which was comprised of 12.5g each of heart, diaphragm, tongue and masseter tissues) and the “brain” group (which was comprised of 50g pig brain). Molecular detection of parasite DNA from

mouse brain confirmed that all observed mouse mortalities were due to *T. gondii* infection (see Table 5-6).

A similar study, which investigated the distribution of parasite tissue cysts within porcine tissues, was carried out in the 1980's (Dubey *et al.*, 1986). The work investigated two oocyst challenged and four naturally infected pigs, and following acid/pepsin digest and mouse bioassay of 50 – 100g of each individual tissue (which included six commercial cuts of meat and six other organs including brain, heart, tongue and diaphragm), showed by microscopic examination of mouse brains, that *T. gondii* tissue cysts were present in a greater number in mice that had received the inocula containing three commercial cuts of meat (Boston butt, spareribs and bacon) and also three organs (brain, tongue and heart). However, in the naturally infected pigs, tissue cysts were present in a greater number of mice from only two commercial cuts of meat (arm picnic and Boston butt). This work was completed prior to today's more commonly used molecular methodologies and depended upon microscopic confirmation of *T. gondii* tissue cysts from impression smears of mouse brain.

These results along with the current results from this research confirm that porcine tissues such as brain or highly active muscles (such as heart, tongue, diaphragm and masseter), are more likely to harbour a greater number of viable tissue cysts. This knowledge is useful for future research, particularly for collection of tissues from either experimental animals or from animals slaughtered at an abattoir.

Molecular and serological detection of *T. gondii* compared to mouse bioassay.

In the current study, *T. gondii* DNA was detected in the brains of all mice which had been euthanised prior to the end of the experiment (see Table 5-7), again highlighting the virulence of the M4 strain in Porton mice. All except three mice, which survived until the end of the experiment, were *T. gondii* negative, which included 1 mouse from group 1 (tissue cyst challenged mice – mouse number C5-4), and 2 mice from group 2 (oocysts challenged mice – mouse numbers C11-1 and C11-2) as shown in

Table 5-7. The remaining mice were all *T. gondii* negative, which included 53.3% (24/45) of mice from group 1 (tissue cyst challenged mice), 46.7% (21/45) of mice from group 2 (oocysts challenged mice) and 100% (45/45) of mice from group 3 (vaccinated and oocyst challenged mice), as described in Table 5-6).

Overall, the mouse bioassay was a more sensitive method for determining the presence of parasite tissue cysts, in comparison to PCR on DNA extracted from the same inocula used to infect mice in the bioassay; parasite DNA could only be detected in 26.7% (12/45) of samples, compared to 37.8% (17/45) from mouse brains following bioassay (see Table 5-8). In this study, testing only the inocula by PCR would have resulted in 11.1% (5/45) of infections being undetected when compared to *T. gondii* DNA detection in mouse brains following the bioassay. Although in this instance PCR was not a suitable replacement for mouse bioassay, incorporation of this methodology may allow for a reduction in the number of animals that would be required for the mouse bioassay. Another method to consider for use on the bioassay inocula, would be magnetic capture qPCR (MC-qPCR). This technique has been described for isolation of *T. gondii* DNA from meat samples, where the ratio of host DNA to parasite DNA is high (Opsteegh *et al.*, 2010a), this method also enables the quantification of parasite DNA, which in turn could be related to parasite burden. However, the mouse bioassay is currently the only technique available to assess the viability of tissue cysts, compared to the detection of parasite DNA using PCR.

An ELISA was carried out on all mouse sera collected at post mortem (n = 135) for the detection of IgG antibodies against the *T. gondii* antigen P30 (SAG3) (see Table 5-9). Only 1 mouse (C11-2) was identified as seropositive by ELISA (% seropositivity = 189), this individual mouse survived until the end of the experiment, and DNA extracted from the brain was also positive by PCR (see Table 5-6). Another mouse (C5-3), which tested borderline positive by ELISA (% seropositivity = 44) was culled on day 18, parasite DNA was also detected from its brain by ITS1 PCR and on microscopic examination of a brain smear a single tissue cyst was also observed (see Table 5-7). Therefore, it is likely that those animals, which were culled between days 10 and 18, which also tested positive for the presence of *T.*

gondii DNA in their brains, had only just started to mount an immune response against the parasite, however, the levels of IgG present were below that detectable by the ELISA.

Detection of *T. gondii* directly from individual porcine tissues.

As previously discussed, different porcine tissues for the mouse bioassay were pooled and grouped (see Table 5-2), however, DNA was extracted from each individual tissue used to produce the inocula to examine for the presence of *T. gondii* DNA. Unfortunately, parasite DNA could only be detected in 2 pigs from group 1 (tissue cyst challenged pigs) from heart, masseter, chop and brain (see Table 5-5). The most likely reasons as to why detection of parasite DNA was lower in the individual pig tissues than that observed from the inocula, could be due to two factors. Firstly, the sample size of tissue used for DNA extraction, as only 1g was used compared to a total of 50g pooled sample used for preparation of the mouse inocula, therefore due to the inhomogeneous nature of the distribution of *T. gondii* tissue cysts, the likelihood of a 50g tissue sample containing a parasite tissue cyst is more probable than from a 1g sample. Secondly, pigs were challenged with either 1000 *T. gondii* oocysts or 1000 tissue cysts, which is a lower dose than animals recently received during similar *T. gondii* challenge experiment in lambs, using the same Moredun M4 stain of *T. gondii* (Katzer *et al.*, 2014 (under review)). In this experiment lambs were challenged with 500,000 M4 oocysts, and detection of parasite DNA by PCR using just 1g of ovine tissue was successful, therefore it is probable that due to the lower dose of either oocysts or tissue cysts (bradyzoites) in pigs, there are a fewer number of established cysts in porcine tissues. One way to address this problem would be to take a larger sample of each individual tissue, carry out an acid/pepsin digest (see section 2.8.1) and to test for the presence of *T. gondii* DNA from this concentrated sample. During the lamb experiment, a serial cull was carried out which provided evidence of a decrease in detection rate of *T. gondii* DNA by 42 dpi. This reduction in detection of parasites may be due to a mounting immune response in the animals and subsequent protection from establishment from the of the parasite in the host. A serial cull was not included in the pig experiment

and all animals were culled at 42 dpi, therefore time may have played an important role for the limited detection of parasite DNA directly from porcine tissues.
In addition the sensitivity could be further increased by magnetic capture of *T. gondii* DNA, as described by Opseegh *et al* (2011) with subsequent ITS1 PCR (as used in this study) or even qPCR to enable quantification of tissue cysts.

Serological detection of *T. gondii* specific antibodies in pigs following vaccination and challenge.

Pigs generally go onto develop efficient protective immunity against *T. gondii* and quickly recover from infection without any obvious clinical signs. A specific humoral immune response can be detected following infection of pigs, with IgM antibodies against *T. gondii* detectable around 8-10 dpi and IgG within 10-17 dpi (Garcia *et al.*, 2006; Lind *et al.*, 1997). Although IgM levels were not monitored within the current study, results for IgG antibody levels against the parasite show that all pigs which were either vaccinated and / or challenged mounted an immune response against the parasite as expected. *T. gondii* IgG levels were evaluated at days 7, 14 and 21dpi. These results show that although an antibody response can be detected at day 14 dpi, seropositivity was greater at day 21 dpi (see Figure 5-3). Analysis of these results also show that at day 21dpi there was no significant difference between % seropositivity in pigs from group 1 and 2, infected with different stages of the parasite (oocysts vs bradyzoites) (see Figure 5-3). However, these results also show that pigs, which had been vaccinated using S48 tachyzoites at -28 dpi and challenged 4 weeks later with 1000 *T. gondii* M4 oocysts (group 3), had significantly higher IgG levels (95% $p < 0.05$) than those animals which were challenged with 1000 M4 oocysts alone (group 2). This result is not surprising as it has been shown that vaccination of sheep with S48 to prevent ovine abortion, initiates a protective cell mediated immune response in the animal, which involves IFNγ, $CD8^+$ and $CD4^+$ T cells (Innes *et al.*, 1995). The effect of this immune response in relation to IgG levels can be seen from the pigs, which were vaccinated alone (group 4), showing a seropositivity of 136% (see Figure 5-3). It would appear as observed in sheep by Innes *et al* (1995), that the immune response in animals from group 3 had been primed by vaccination and the subsequent challenge with *T. gondii*

oocysts 4 weeks post vaccination increased this response. During this study, samples have been collected that will allow future analysis of the cell-mediated and humoral immune response induced by vaccination.

In conclusion, the vaccination of pigs with the S48 strain of *T. gondii*, followed by challenge with oocysts of the M4 strain and subsequent mouse bioassay (to enable the detection of viable tissue cysts), has shown a significant degree of mouse survival when compared to animals which received tissues from oocyst challenged (but non-vaccinated) pigs. However, detection of parasite DNA directly from homogenised porcine tissues used to inoculate mice was not as sensitive as detection of parasite DNA from the brains of the mice used in the bioassay. The bioassay is likely to be a more sensitive method for detection due to the multiplication of the parasite in vivo. In addition, the mouse bioassay can also asses the viability of *T. gondii* tissue cysts, which cannot be achieved by molecular detection alone. The mouse bioassay also showed that there was no significant difference in the mouse mortality rate (or the molecular detection of parasite DNA from these mice) when animals were inoculated with porcine tissues from pigs which had previously been infected with either tissue cysts or oocysts. However, there was a difference in mouse survival (and the detection of parasite DNA) depending on which tissue group the mice had been inoculated with. A greater number of mice survived which had received porcine tissues from the "Food" tissue group (irrespective of whether they had received porcine tissues from tissue cyst or oocyst infected pigs), compared to mice which had received tissues from the "Brain" and "Other" tissue groups, indicating a greater number of viable tissue cysts within tissues from the "Brain" and "Other" groups.

Chapter 6: General discussion

The protozoan parasite *T. gondii* is widespread in both human and animal hosts. Felids (the definitive host) play a pivotal role in the life cycle of the parasite as oocysts are excreted into the environment from their faeces (Hutchison *et al.*, 1969; Hutchison *et al.*, 1971). However, once the parasite infects an intermediate host it remains persistently infected due to the cyst forming nature of the parasite. Tissue cysts (which contain infective bradyzoites) are most likely to form in the brain and muscle tissue (Dubey, 1988) of an infected host and are a source of contamination for both carnivores and omnivores.

Wildlife and livestock are intermediate hosts for *T. gondii*. In Great Britain very little information is known about the prevalence or genotypes present within these populations. In Chapter 3 (Prevalence and genetic diversity of *T. gondii* from wild mammalian carnivores in Great Britain), the overall prevalence of *T. gondii* from six different species of wild carnivores from Great Britain was estimated at 22.8% (89/390), which varied depending on the host species. Genotyping of DNA from *T. gondii* positive animals identified 90.6% (29/32) to be infected with the type II lineage of the parasite (see Table 3-4). This result is not surprising, especially as type II has been reported to be the predominant lineage found in wildlife from Europe and North America (Ajzenberg *et al.*, 2004; Aubert *et al.*, 2010). However, the results from this chapter also identified a type III lineage and two alleles for type I from three different polecats, indicating a degree of genetic diversity within this wildlife species and potentially the environment. Due to urban expansion the ecosystems which wildlife inhabit are changing, with the potential for zoonotic "spillover" from wildlife to livestock and ultimately human populations likely to increase (Thompson, 2013). Therefore, a greater knowledge of the prevalence of *T. gondii* in wildlife and the stains present within these species is becoming increasingly more important.

Future work, which would be complimentary to this research, would be to investigate the prevalence of *T. gondii* within other wildlife species (not necessarily carnivores), which are used as sources of food for human consumption in Great Britain, such as rabbit, deer and pheasant. As described in Chapter 1 (Introduction), the

seroprevalence of *T. gondii* in livestock species (such as sheep in pigs) from other countries has been shown to be relatively high. Therefore, it would also be beneficial to determine the prevalence of the parasite and the genotypes present from these species within Great Britain, particularly as foodborne transmission of *T. gondii* to humans is most likely to occur from consuming undercooked or raw meat from these animals (Tenter *et al.*, 2000)

Until recently it has not been possible to identify whether acquired infection in humans is due tissue cyst or oocyst contamination. But in 2011 the identification of a sporozoite specific antigen (TgERP) enabled researchers to identify whether an individual had been infected via *T. gondii* oocysts (Hill *et al.*, 2011). In Chapter 4 (Prevalence of *T. gondii* from human hosts in Scotland), this methodology was implemented on serum samples from Scottish blood donors which had recently seroconverted from *T. gondii* seronegative to seropositive, as identification of the antigen is only possible for six to eight months after infection. Although only one sample tested positive (see Table 4-4), indicating that infection was predominantly due to consumption of tissue cysts, it was also observed that the ODc values from the ELISA used to detect *T. gondii* IgG were lower when compared to the serum sample which generated the positive TgERP result (see Table 4-4). Therefore it is possible that some of the TgERP negative sera may have tested positive, but it was not possible to detect this due to the low concentration of antibody. This methodology will prove to be important in the future, particularly when studying disease outbreaks where the cause of infection is unknown, or in countries or regions where *T. gondii* prevalence is high, such as Brazil. Such information may be useful for epidemiological studies and will also enable scientist to implement specific strategies to prevent infection based on which stage of the parasite most commonly causes infection.

The results from Chapter 4 also provide up to date information about the prevalence of the parasite in humans, data which is lacking for the UK population (AMCSF, 2012). The prevalence was calculated using two different study groups. Firstly, the prevalence of the parasite was determined using DNA samples from the Sudden Death Brain Bank, collected over a five year period (2008 – 2012), where an overall

prevalence of 17.9% (27/151) was detected, and a general trend towards an increase in prevalence with increase in age was also observed (see Figure 4-8). The second study population, which included serum samples from Scottish blood donors over a four year period (2006 – 2009), found an overall seroprevalence of 13.0% (183/1403) Table 4-2 (when calculated using a specific cut off value derived from a binormal mixture model – see Figure 4-1). Although both study groups contained samples from a broad section of the population (a "healthy" population, not known to be predisposed to illness), there was a 4.9% difference in prevalence between the two groups. The most likely explanation for this difference is because the samples obtained from the Sudden Death Brain Bank were predominantly collected from individuals who were over 48 years of age, whilst the samples collected from blood donors were much more equally spread across all age groups (see Figure 4-7 and Figure 4-4). The prevalence of *T. gondii* infection within both groups is relatively low when compared to that of other countries such as Germany or France, where prevalence has been reported to be as high as 59.0% (n=4854) and 54.0% (n=13459) respectively (Fiedler *et al.*, 1999; Pappas *et al.*, 2009). Therefore, within Great Britain a greater percentage of individuals are at risk of infection, particularly when taking into consideration women of child bearing age and the risk of congenital toxoplasmosis. In both study groups there was a correlation between increasing age and an increase in *T. gondii* prevalence, indicating that acquired infection plays an important role within the British population.

To complement the genotyping results obtained from carnivorous wildlife in Chapter 3, strain genotyping was also completed for human DNA samples in which *T. gondii* was detected. Due to a low concentration of parasite DNA, genotyping was successful for only four human samples, which identified alleles for type I and III lineages of *T. gondii* (see Table 4-7). This result was particularly interesting, not only because type II is the most common genotype in Europe and North America, but also because in Chapter 3 the predominant linage identified from carnivorous wildlife was type II (see Table 3-4). Following this result, further genotyping of human samples would be beneficial in order to assess whether type I and III strains are predominantly found in a "healthy" human population. One way in which this

could be achieved, using the DNA extracted from the human brains obtained from the Sudden Death Brain Bank, would be to use magnetic-capture PCR (MC-PCR) (Opsteegh *et al.*, 2010a) to separate *T. gondii* DNA from host DNA, which would increase the sensitivity of the genotyping PCR.

The results from Chapter 4 describe an increase in seroprevalence in relation to an increase in age (indicating acquired infection), and one of the most likely sources of acquired infection is from consumption of *T. gondii* tissue cysts from raw or under cooked infected meat (Cook *et al.*, 2000). Therefore, vaccination to reduce or prevent tissue cyst formation in food producing animals could help to reduce the infection rate in humans and the disease burden associated with this (which can be greater than that reported for *Campylobacter* or *E. coli* - see Figure 1-8) (Havelaar *et al.*, 2012).

Pork is has been described to be one of the main sources of foodborne transmission of *T. gondii* (see Figure 1-6) (Dubey, 2009; Tenter *et al.*, 2000). The research carried out in Chapter 5 (Vaccination and challenge of pigs to evaluate *T. gondii* tissue cyst formation) used the S48 strain of the parasite (which is used in Toxovax®, the commercial vaccine against ovine toxoplasmosis) to assess whether it was possible to reduce the number of *T. gondii* tissue cysts in porcine tissues, potentially making pork a safer meat for human consumption. Following vaccination and oocyst challenge to determine whether of viable tissue cysts were present, porcine tissues from the experiment were used in a mouse bioassay. Not only did 100% (45/45) of mice which received porcine tissues from vaccinated and challenged pigs, survive, but no parasite DNA was detected in their brains. In contrast, mice which received porcine tissues from un-vaccinated and challenged pigs, resulted in survival of 51.1% (23/45) of mice (see Figure 5-6 and Figure 5-7). These results are promising, as this is the first description of 100% protection against tissue cyst formation in mice following mouse bioassay of porcine tissues from pigs used in a vaccination and challenge experiment. The results also begin to answer the question raised by the Food Standards Agency, as in a recent report published by the Advisory Committee on the Microbiological Safety of Food (ACMSF), the document states that one of the knowledge gaps relevant to a risk assessment in the UK is: "*vaccines based on live*

attenuated strains of tachyzoites are effective in reducing morbidity in sheep but it is not known whether vaccination has any effect on the formation of tissue cysts" (AMCSF, 2012). From the current research it is now clear that S48 does have an effect in reducing the formation of tissue cysts in pigs. The vaccination of other "high risk" livestock species should be addressed, however, it has also recently been demonstrated that the effect of vaccination of sheep with S48 also results in a reduction in the number of ovine tissue samples in which parasite DNA can be detected (Katzer *et al.*, 2014 (under review)). Despite the success of these results, it would be more desirable to have a vaccine that is not based on live tachyzoites, which would be a safer alternative for use in livestock species. Therefore, as discussed in Chapter 1, future research into the development of vaccines for *T. gondii* should focus on DNA or subunit vaccines, which are thought to be safer for animal and human use (Ivory & Chadee, 2004).

To determine whether molecular detection of parasite DNA by PCR could replace mouse bioassay (a reduction in the use of animal numbers is desirable in any area of scientific research), Chapter 5 also analysed the molecular detection of *T. gondii* DNA from bioassay inocula (homogenised porcine tissues which had been subjected to an acid / pepsin digest to concentrate any tissue cysts present) prior to inoculation into mice. When using PCR to detect *T. gondii* DNA directly from the bioassay inocula, parasite DNA was detected in 26.7% (12/45) of samples, compared to detection in 37.8% (17/45) of samples from mouse brains following bioassay (see Table 5-8). In this instance, the mouse bioassay is more sensitive for the detection of *T. gondii* from animal tissues, which is not surprising, as the parasite multiplies within the mouse as infection continues over the length of the experiment (in this case, 6 weeks). However, in the future, molecular detection of *T. gondii* directly from bioassay inocula may allow researchers to reduce the number of animals used in the mouse bioassay. As mouse bioassay is currently the only method available to test the viability of tissue cysts, it is unlikely that any molecular based detection method will replace this technique in the near future.

The data described within this thesis is important for addressing many of the issues raised in the recent report published by the AMCSF, which highlighted a number of

knowledge gaps regarding the prevalence of *T. gondii* in both animal and human hosts from the UK. Results regarding the prevalence of *T. gondii* in humans is particularly useful for health professionals, especially as 87% (1220/1403) of the human serum samples tested in Chapter 4 were seronegative, indicating a large percent of the population at risk from infection, which includes women of child bearing age who are most at risk during pregnancy, with the potential for congenital transmission of the parasite to the foetus (congenital toxoplasmosis). Although the parasite is killed by proper cooking of pork and pork products, there are also traditional dishes which include raw pork, such as the Thai dish Nam Mu Sod (raw pork parcels), and instances where the meat is not thoroughly cooked, such as accidentally undercooking pork sausages on a BBQ. Therefore, from a public health and food safety point of view, the thesis not only provides useful information on the distribution of the parasite within different porcine tissues, including those sold for human consumption, it also provides promising results to the effectiveness of the S48 of *T. gondii* as a vaccine to reduce tissue cyst formation in pigs.

T. gondii is a parasite of significant public health importance and more research should be undertaken to understand the link between infection in food animals and the potential risk of transmission to humans from consuming infected meat.

Future studies

There are many knowledge gaps which should be addressed regarding the prevalence and public health impact of *T. gondii* in the UK. A continuation of this research should involve a serological study examining the seroprevalence of *T. gondii* in pigs from the UK. The study should ensure that pigs from different housing systems are tested (indoor vs. outdoor reared animals), as it is likely that outdoor reared pigs will have a higher seroprevalence compared to indoor reared pigs, as observed in other countries. It would also be interesting to examine a link between serology and tissue cyst burden. This is something that can initially be achieved using the samples collected from the porcine challenge experiment in Chapter 5. By carrying out a qPCR on DNA extracted from different tissues and comparing this to the OD values

from the ELISA, a link between seroprevalence and tissue cyst burden could be determined. A link between serology and tissue cyst burden would be useful for determining the potential risk of infection from porcine tissues, particularly from slaughtered animals, where the meat will be used for human consumption. It would also be beneficial to examine other livestock species for the presence of tissue cysts, especially as meat such as beef can be eaten raw in the UK, in dishes such as steak tartare or beef carpaccio. It is also important to understand which genotypes are present within different UK livestock species (such as pigs, cattle and sheep). Not only will this assist in understanding the transmission of the parasite (particularly when it is known which strains are present in wildlife and humans), but, in humans, clinical symptoms can vary depending on the strain (as observed with atypical strains of *T. gondii*), therefore, from a public health perspective, it is important to know whether these strains are present within livestock species from the UK.

The technique MC-qPCR, which separates *T. gondii* DNA from host DNA, was not used within the current study. Research which compares the sensitivity of this technique with conventional PCR would be beneficial, particularly when the amount of host DNA is expected to be high compared to parasite DNA (for example in the human brain samples described in Chapter 4, or when samples are collected from an abattoir - where the parasite burden is likely to be lower from a naturally infected animal, than one which has been infected experimentally). Finally, to determine which stage of the parasite (oocysts or tissue cysts) is the most common cause of human infection, further research should be completed using the sporozoite specific antigen (TgERP), using human serum samples from the UK. Data generated from such an experiment will help to assess the risk associated with infection from tissues cysts consumed from raw or undercooked meat, or whether oocysts play a more important role in human infection.

Chapter 7: References

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Chapter 8: Appendix

8.1 Solutions and media

8.1.1 Acid/pepsin solution

For the preparation of 500mls acid/pepsin solution

Pepsin*	2.6g
Sodium chloride	5.0g
Hydrochloric acid	7.0mls

*Porcine stomach pepsin, 1:10,000 biological activity.

Made up to 500mls with dH_2O

8.1.2 Antigen extraction buffer

For preparation of 20mls extraction buffer:

Pepstatin	2µg/ml
PMSF	1mM
Sodium phosphate	50mM
EDTA	2mM

Made up to 20mls with the addition of dH_2O. It should be noted that stock reagents pepstatin and PMSF should be dissolved in ethanol.

8.1.3 Carbonate buffer (pH9.6)

For preparation of 1000mls of carbonate buffer

Sodium carbonate	1.59g
Sodium bicarbonate	2.93g

Made up to 1000mls with the addition of dH_2O.

8.1.4 IMDM 5% FCS

Iscove's Modified Dublecco's Media (IMDM) containing 5% Foetal Calf Serum (FCS) was prepared as follows:

IMDM	500mls
FCS	25mls
Penicillin	100 units/ml
Streptomycin	100µg/ml
L-glutamine	292µg/ml

8.1.5 IMDM 2% FCS

IMDM containing 2% Foetal Calf Serum (FCS) was prepared as follows:

IMDM	500mls
FCS	10mls
Penicillin	100 units/ml
Streptomycin	100µg/ml
L-glutamine	292µg/ml

8.1.6 Phosphate buffered saline (PBS) pH7.4

A 10x solution was prepared in 800mls dH_2O:

Sodium chloride	0g
Potassium chloride	2g
Sodium dihydrogen phosphate heptahydrate	26.8g
Potassium dihydrogen phosphate	2.4g

pH was adjusted to 7.4 with HCl; dH_2O added to a final volume of 1000mls then autoclaved. The 10x solution diluted 1:10 with dH_2O (1x PBS).

8.1.7 PBS Tween (PBST)

PBS containing 0.05% Tween 20 was prepared as follows:

PBS (1x)	1000mls
Tween 20	500µl

The final solution was autoclaved.

8.1.8 Saturated salt solution

For preparation of 1000mls saturated salt solution:

Sodium chloride	400g
dH_2O	1000mls

The solution will contain particles of sodium chloride which have not fully dissolved, this is a normal characteristic of a saturated salt solution.

8.1.9 1.2% Sodium bicarbonate

For preparation of 100mls sodium bicarbonate solution

Sodium bicarbonate	1.2g
dH_2O	100mls

This solution is made fresh for use in the mouse bioassay.

8.2 Timescale overview of pig experiment

2012	May	June	July	August	September	October
Monday						1
Tuesday	1					2
Wednesday	2			1		3
Thursday	3			2		4
Friday	4	1		3		5
Saturday	5	2		4	1	6
Sunday	6	3	1	5	2	7
Monday	7	4 -28dpi **Vaccinate**	2 Wk4 PV 0dpi **Challenge**	6 35dpi Wk5 PC	3	8
Tuesday	8	5	3	7	4	9
Wednesday	9	6	4	8	5 Wk3 Bioassay	10
Thursday	10	7	5	9	6	11
Friday	11	8	6	10	7	12
Saturday	12	9	7	11	8	13
Sunday	13	10	8	12	9	14
Monday	14	11 -21dpi Wk1 PV	9 7dpi Wk1 PC	13 42dpi **Cull** Wk6 PC	10	15
Tuesday	15	12	10	14 **Cull**	11	16
Wednesday	16	13	11	15 Begin mouse **Cull** bioassay	12 Wk4 Bioassay	17
Thursday	17	14	12	16 Begin mouse **Cull** bioassay	13	18
Friday	18	15	13	17 Begin mouse **Cull** bioassay	14	19
Saturday	19	16	14	18	15	20
Sunday	20	17	15	19	16	21
Monday	21	18 -14dpi Wk2 PV	16 14dpi Wk2 PC	20	17	22
Tuesday	22	19	17	21	18	23
Wednesday	23	20	18	22 Wk1 Bioassay	19 Wk5 Bioassay	24
Thursday	24	21	19	23	20	25
Friday	25 **Pigs Arrive**	22	20	24	21	26
Saturday	26	23	21	25	22	27
Sunday	27	24	22	26	23	28
Monday	28	25 -7dpi Wk3 PV	23 21dpi Wk3 PC	27	24	29
Tuesday	29	26	24	28	25	30
Wednesday	30 -33dpi	27	25	29 Wk2 Bioassay	26 Wk6 Bioassay **Cull Mice**	31
Thursday	31	28	26	30	27 **Cull Mice**	
Friday		29	27	31	28 **Cull Mice**	
Saturday		30	28		29	
Sunday			29		30	
Monday			30 28dpi Wk4 PC			
Tuesday			31			

8.3 Detection of oocysts from cat faeces

Initial detection from ~ 1g faeces

Apollo

Date	Days P.I	Faeces Weight (g)	Oocyst Yield
24-Mar-12	3	55	-
25-Mar-12	4	23	-
26-Mar-12	5	28	95
27-Mar-12	6	57	64 / field
28-Mar-12	7	12	25 / field
29-Mar-12	8	30	68 / field
30-Mar-12	9	22	15 / field
31-Mar-12	10	16	122 / trav
01-Apr-12	11	18	38 / trav
02-Apr-12	12	32	22 / trav
03-Apr-12	13	22	5 / trav
04-Apr-12	14	26	2 / trav
05-Apr-12	15	26	-
06-Apr-12	16	pool* 37	-
07-Apr-12	17	pool* 73	-
08-Apr-12	18	pool* 21	-
09-Apr-12	19	no sample	n/a
10-Apr-12	20	pool* 108	-

Zeus

Date	Days P.I	Faeces Weight (g)	Oocyst Yield
24-Mar-12	3	37	-
25-Mar-12	4	23	-
26-Mar-12	5	25	37
27-Mar-12	6	22	113 / field
28-Mar-12	7	24	56 / field
29-Mar-12	8	23	36 / field
30-Mar-12	9	20	13 / field
31-Mar-12	10	18	99 / trav
01-Apr-12	11	24	23 / trav
02-Apr-12	12	25	0.8 / trav
03-Apr-12	13	19	-
04-Apr-12	14	19	-
05-Apr-12	15	19	-
06-Apr-12	16	pool* 37	-
07-Apr-12	17	pool* 73	-
08-Apr-12	18	pool* 21	-
09-Apr-12	19	no sample	n/a
10-Apr-12	20	pooled* 108	-

* = pooled sample from both animals

8.4 Research ethics committee approval

Lothian NHS Board

South East Scotland Research Ethics Committees
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12 April 2011

Miss Alison C Burrells
PhD Student
Moredun Research Institute
Pentlands Science Park
Bush Loan
Penicuik
EH26 9BH

Dear Miss Burrells,

Study title: **Evaluation of Toxoplasma infections in humans by PCR and Immunohistochemistry**
REC reference: **11/AL/0113**
Protocol number: **N/A**

The Research Ethics Committee reviewed the above application at the meeting held on 06 April 2011. Thank you for attending to discuss the study.

Ethical opinion
Toxoplasma is a small parasite which can infect all warm blooded animals and humans. In this research study the researchers planned to use DNA based technologies to detect the parasite in tissues. From this data they would determine the frequency of people infected and if infection frequency changes with age, gender and cause of death. Different brain sections of infected people will also be tested for the parasite to find out if it has a preference for infecting different parts of the brain. Tissues will be made available from the MRC Sudden Death Brain and Tissue Bank. The researchers confirmed that this tissue bank had local Research Ethics Committee approval and worked within the legal framework of the Human Tissue (Scotland) Act 2006. The researchers provided reassurance over the storage of the study samples and they also confirmed that any excess unchanged tissue would be returned to the Tissue Bank. The Committee considered this to be an interesting and well presented study and had no significant ethical concerns with it. The members of the Committee present gave a favourable ethical opinion of the above research on the basis described in the application form, protocol and supporting documentation, subject to the conditions specified below.

The members of the Committee present gave a favourable ethical opinion of the above research on the basis described in the application form, protocol and supporting documentation, subject to the conditions specified below.

INVESTOR IN PEOPLE

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Chair Dr Charles J Winstanley
Chief Executive Professor James J Barbour O.B.E.
Lothian NHS Board is the common name of Lothian Health Board