Toxoplasma gondii in animal reservoirs and the environment

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Toxoplasma gondii in animal reservoirs and the environment

Toxoplasma gondii in dierreservoirs en het milieu

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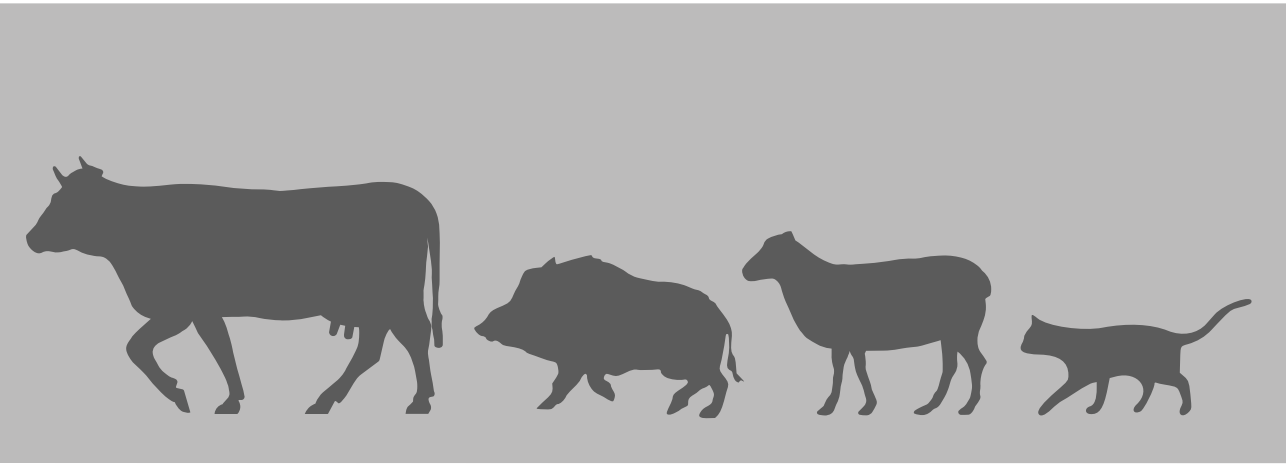
Chapter 1

Introduction



Toxoplasma gondii is one of the most successful parasites worldwide, capable of infecting virtually all warm blooded animals. It is estimated that up to one third of the world's human population is infected (Tenter et al., 2000). Pregnant women and immune-compromised individuals are the main risk groups, although immune-competent individuals may develop ocular disease as a result of infection. Moreover, *T. gondii* infection has been associated with behavioral changes and the development of psychiatric disorders (Flegr, 2007; Yolken et al., 2009). If a woman is infected for the first time during pregnancy the parasite may be transmitted transplacentally to the foetus. This can result in death of the foetus, central nervous system abnormalities, or eye disease, affecting the quality of life of the child throughout its lifetime. Recently *T. gondii* was the subject of disease burden estimations: As a result of the severe clinical symptoms and lifelong implications, the disease burden of *T. gondii* is high (Havelaar et al., 2007; Jones, Holland, 2010; Kortbeek et al., 2009; Mead et al., 1999). In addition, *T. gondii* ranked second on a list of prioritized emerging zoonoses in The Netherlands (Havelaar et al., 2010). All of these facts taken together indicate that toxoplasmosis is a neglected zoonosis in many ways and needs more attention. The parasite is also a major cause of abortion in sheep and goats, and thereby the cause of substantial economic losses (Innes et al., 2009a).

Toxoplasma gondii: the parasite

The obligate intracellular protozoan parasite *Toxoplasma gondii* was discovered more or less simultaneously in 1908 by Nicolle and Manceaux in Tunis and Splendore in Brazil (for translated manuscripts view (Nicolle, Manceaux, 2009) and (Splendor, 2009)). It was named *Toxoplasma gondii* by Nicolle and Manceaux after its bowed shape (toxon = bow, plasma = life) and the original host (the gundi). Carnivorism was suggested as a possible mode of transmission as early as 1937 (Sabin, Olitsky, 1937) and confirmed in 1965 by a high incidence of *T. gondii* infection in a hospital where a raw meat diet was given therapeutically (Desmonts et al., 1965). The infectivity of cat faeces was demonstrated in 1965 (Hutchison, 1965), but the complete coccidian life cycle (Fig. 1.1) is only understood since the identification of sexual development of *T. gondii* in cat intestines in the late sixties, early seventies (Dubey et al., 1970a; Dubey et al., 1970b; Frenkel et al., 1970; Hutchison et al., 1969; Hutchison et al., 1970; Hutchison et al., 1971; Overdulve, 1970; Sheffield, Melton, 1970; Weiland, Kuhn, 1970; Witte, Piekarski, 1970). *T. gondii* is the only known member of the genus *Toxoplasma*, but as a protozoan parasite it belongs to the phylum of Apicomplexa together with other coccidian species, piroplasms and plasmodia. *T. gondii* is present in all geographical regions of the world and infects virtually all warm-blooded species.

Life cycle

The life cycle of *T. gondii* includes both sexual and asexual multiplication (Fig. 1.1). Sexual multiplication of *T. gondii* takes place in the gut of felines, making them the definitive hosts. Many feline species have been shown capable definitive hosts (Dubey, 2009a). If a cat ingests a *T. gondii* infected prey animal or meat, bradyzoites are released from the tissue cysts contained in their meal. In the previously uninfected cat, these bradyzoites invade epithelial cells of the cat's small intestine, where they start multiplying asexually. After five asexual stages of multiplication gametogony begins. Female macrogamonts and male microgamonts are formed, and upon fertilization of the macrogamete by a microgamete, a zygote and an oocyst wall are formed. The nucleus divides twice and two sporoblasts (each with two nuclei) are formed. As the epithelial cells rupture, millions of oocysts containing sporoblasts are discharged into the intestinal lumen of the cat and eventually shed into the environment or cat litter box. Depending on temperature and humidity these sporoblasts sporulate within 1 to 5 days to become infectious sporozoites with a haploid DNA content (4 sporozoites per sporoblast). Sporulated oocysts are infectious to cats (leading to another round of sexual multiplication) (Dubey, 1996a), but even more so to an unequalled range of intermediate hosts: Probably all warm-blooded animals can be infected.

If an intermediate host ingests oocysts sporozoites will be released into the gut lumen and pass through the gut epithelium to enter cells in the lamina propria. In case an intermediate host ingests tissue cysts the released bradyzoites behave similarly to these sporozoites: Both sporozoites and bradyzoites transform into tachyzoites that enter a host cell where they divide rapidly until the cell bursts. Next, they continue to infect neighbouring cells. Tachyzoites disseminate through the body by the circulation mostly intracellularly in leucocytes (Unno et al., 2008), and finally enter various nucleated cells, but especially those in nervous and muscle tissue, where they transform into slowly dividing bradyzoites surrounded by a cyst wall. The fate of these tissue cysts is not entirely clear. Tissue cysts seem to remain present

lifelong in most hosts, although individual cysts are thought to rupture occasionally. This occasional cyst rupture is considered responsible for the persistence of antibodies in the host, because the released bradyzoites could stimulate the immune response in the immune-competent host. Released bradyzoites transforming back into rapidly-dividing tachyzoites could explain the reactivation resulting in clinical symptoms or even fatal toxoplasmosis in immune-compromised individuals.

Although intermediate hosts do not shed *T. gondii* they are infectious via carnivorism. Both felines and intermediate hosts are susceptible to infection via tissue cysts, which means that intermediate hosts are also infectious to each other. This ability to complete a cycle without the necessity to pass through the definitive host is quite unique in the world of parasites. Another interesting characteristic of *T. gondii* is the ability to change the behavior of rodents, causing them to specifically loose their aversion for cats (Vyas et al., 2007). This trait provides an evolutionary advantage as it promotes *T. gondii* transmission.

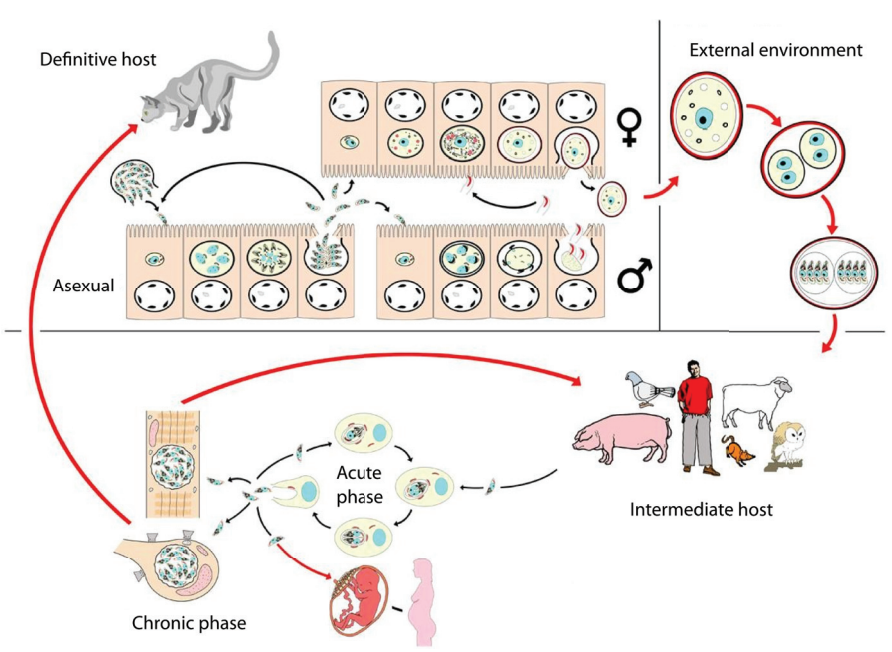


Figure 1.1 The life cycle of *Toxoplasma gondii*.
Reprinted from Trends in Parasitology, Vol. 18 / No. 8, Ferguson, D.F.P., *Toxoplasma gondii* and sex: essential or optional extra?, pp. 355-359, Copyright (2002), with permission from Elsevier.

Besides faecal-oral and meatborne transmission there is even a third option for transmission to the next host: If a host is primary infected during pregnancy tachyzoites may be transmitted transplacentally to the foetus. It has been shown that congenital transmission in mice (Beverley, 1959; de Roever-Bonnet, 1969; Eichenwald, 1948; Owen, Trees, 1998; Rejmanek et al., 2010; Remington et al., 1961) and possibly sheep (Duncanson et al., 2001; Hide et al., 2009; Morley et al., 2007; Williams et al., 2005) is not limited to primary infection, but may happen from chronic infection. Therefore, a population of mice can remain *T. gondii* positive for years without an external source of contamination. These congenitally infected mice may not develop antibodies to *T. gondii* due to immune tolerance (Jacobs, 1964; Owen, Trees,

1998; Rejmanek et al., 2010), and the possible presence of *T. gondii* infected mice that are seronegative (as has been shown in the field (Dubey et al., 1995)) is an important consideration in epidemiological studies. Moreover, congenital transmission is of major clinical importance.

Molecular typing and genome

It has long been known that strains of *T. gondii* vary in virulence for mice, and after the development of molecular typing techniques, the genetic variation in *T. gondii* strains present worldwide has been linked to these phenotypic differences. The first typing experiments showed that mouse virulent strains all belonged to a single clonal genotype, whereas the less virulent ones showed more variability (Sibley, Boothroyd, 1992). Typing more strains showed that the population of *T. gondii* is highly structured and that nearly all strains could be grouped into one of three clonal lineages (type I, II and III) (Darde et al., 1992; Howe, Sibley, 1995; Sibley, Boothroyd, 1992). These three clonal lineages were derived by only a few genetic crosses of ancestral strains (Boyle et al., 2006; Grigg et al., 2001) and probably originated within the last 10,000 years (Su et al., 2003). Direct oral infectivity to other intermediate hosts via tissue cysts may explain their successful expansion (Khan et al., 2006a; Su et al., 2003).

However, typing results were and still are overrepresented by strains isolated from humans and domestic animals in North-America and Europe. Nowadays, several laboratories are focusing on typing strains from more remote areas and wildlife hosts. Chickens are often used as sentinel animals because they easily pick up *T. gondii* from the environment due to their roaming behavior (Lehmann et al., 2006). *T. gondii* from especially South America (Ferreira et al., 2007; Khan et al., 2006b; Pena et al., 2008), but to a moderate extent also Africa (Bontell et al., 2009; Boughattas et al., 2010; Mercier et al., 2010) and the Middle East (Dubey et al., 2010) has been shown genetically more divergent, and a new type X (later subdivided in type A and X (Sundar et al., 2008)) identified from sea otters in California (Conrad et al., 2005; Miller et al., 2004) was afterwards also shown present in terrestrial carnivores (Miller et al., 2008). Most worryingly, severe and even fatal cases of toxoplasmosis in immune-competent humans on the Suriname-French Guiana border were linked to one atypical strain (Carme et al., 2002; Demar et al., 2007). Nowadays high clonality and low diversity is considered associated with a high degree of anthropization and a dominance of domesticated cats and intermediate hosts (Ajzenberg et al., 2004; Mercier et al., 2010). The role of type I as a major lineage is questioned as it is rarely detected in human cases of toxoplasmosis (Ajzenberg, 2010). Instead, newly identified Africa 1 and Africa 3 may represent major lineages (Mercier et al., 2010).

While strain collection and typing was ongoing, whole genome sequencing projects have provided better insight in the genetic make-up of *T. gondii.* The full genome sequence with annotation for the ME49 strain and the genomic sequences for the GT1, VEG and RH strains are now available from toxoDB.org (Gajria et al., 2008). The genome of *T. gondii* is about 65Mbp long, haploid, and spread over 14 chromosomes (Khan et al., 2005b). For most loci only two alleles exist, one of which is shared by two clonal types. Sexual recombination in *T. gondii* leads to mixed genotypes, and therefore the genotype for one locus not necessarily represents genotype of the whole genome. Because of shared alleles and recombinant genotypes, multilocus typing is essential to differentiate the original 3 clonal types from each other and from recombinants or atypical strains. Typing techniques vary widely, but

are usually based on restriction fragment length polymorphism (RFLP) analysis (Khan et al., 2005a; Su et al., 2006) (markers are available at: www.toxomap.wustl.edu/Toxo_Genetic_Map_Table), length polymorphisms for microsatellites (Ajzenberg et al., 2005), or single nucleotide polymorphisms as detected by sequencing (Bontell et al., 2009; Frazao-Teixeira et al., 2011). Sequencing gives the highest resolution to detect polymorphisms, and multilocus sequencing has, for example, shown that many South-American strains previously identified as type I or recombinant by RFLP analysis actually represent atypical strains (Frazao-Teixeira et al., 2011).

Toxoplasmosis in humans

It took several decades until the newly discovered *T. gondii* was recognized as a human pathogen. Probably the first report comes from Janků (Janků, 1923), but it took until 1939 for *T. gondii* to be conclusively identified as a cause of human disease (Wolf et al., 1939). Nowadays, *T. gondii* is well known and even feared for the problems that may arise in the foetus when *T. gondii* is transmitted transplacentally. The classical triad of signs is characterized by chorioretinitis, intracranial calcifications and hydrocephalus. If a woman is infected for the first time during pregnancy, *T. gondii* is transmitted to the foetus in about one third of the cases (Dunn et al., 1999). The probability of transmission increases with gestational age, while the severity of the effects, especially the odds of developing intracranial lesions but not that of eye lesions, decreases (Dunn et al., 1999; Thiebaut et al., 2007). According to the latest review of cohort studies based on universal screening for congenital toxoplasmosis in Europe, transmission occurs in about 15% of women infected at 13 weeks of pregnancy, 44% at 26 weeks, and 71% at 36 weeks (Thiebaut et al., 2007). In this same study, the risk of foetal death (either by induced termination after foetal diagnosis or spontaneous stillbirth) was estimated at 2%, the crude risk of ocular lesions diagnosed in the first year of life was 14%, and that of intracranial lesions at 9% (Thiebaut et al., 2007). About 75% of congenitally infected children are born without clinical symptoms (Thiebaut et al., 2007), but in a different study (Havelaar et al., 2007) the hazard rate of developing chorioretinitis later in life was estimated to be constant at 2% per year based on studies by Gras and Binquet (Binquet et al., 2003; Gras et al., 2001). Due to the severity and duration of congenital toxoplasmosis the disease burden is high (Havelaar et al., 2007; Kortbeek et al., 2009; Mead et al., 1999; Vaillant et al., 2005).

In immune-competent individuals the acute phase of the infection usually passes asymptomatically or signs are limited to a transient lymphadenopathy and mild fever-like symptoms. Consequences such as encephalitis, pneumonitis, myocarditis or disseminated infections are highly unlikely. These consequences are, however, more common and may lead to fatal toxoplasmosis in immune-compromised individuals, such as those receiving corticosteroids or cytotoxic drugs, patients with hematological malignancies, transplants or AIDS. Especially in AIDS patients *T. gondii* was an important cause of death, usually by encephalitis. However, since the introduction of highly active antiretroviral therapy (HAART) this is under control in the developed world. In immune-compromised individuals including haematopoietic stem cell transplant patients, toxoplasmosis is not necessarily caused by primary infection. Recrudescence of a latent infection is a more common cause (Martino et al., 2000). However for patients receiving a solid organ, and especially a heart (muscle tissue is a predilection site for *T. gondii*), the risk of toxoplasmosis is highest in case the donor is positive and the recipient is negative (Derouin, Pelloux, 2008). Toxoplasmosis in transplant

patients can be prevented by serological screening of donor and recipient and, if necessary, prophylactic treatment with co-trimoxazole (often already administered as prophylaxis for pneumocystosis) or pyrimethamine-sulphadioxide (Derouin, Pelloux, 2008).

In addition, *T. gondii* infection is an important cause of chorioretinitis. Previously all cases of ocular toxoplasmosis were considered (late) sequelae of congenital transmission, but because ocular toxoplasmosis was found in siblings (Silveira et al., 1988), after longitudinal follow-up (Silveira et al., 2001), and in relation to outbreaks (Burnett et al., 1998) it was recognized that ocular toxoplasmosis can be a consequence of postnatally acquired infection (Gilbert, Stanford, 2000; Holland, 1999). Although acquired infection cannot be differentiated serologically with absolute certainty, detailed serological work-up seems to confirm the relative importance of acquired infections to the incidence of ocular toxoplasmosis (Montoya, Remington, 1996; Ongkosuwito et al., 1999), and it has been suggested that at least two thirds of ocular toxoplasmosis cases were acquired postnatally (Gilbert, Stanford, 2000). The incidence of eye-disease in immune-competent people acquiring *T. gondii* is uncertain: Estimates vary widely and may depend on *T. gondii* genotype (Khan et al., 2006b).

Because *T. gondii* has a predilection for nervous and brain tissue besides muscle tissue, mental effects have been studied extensively. In mice *T. gondii* infection appears to specifically reduce their fear of cats (Lamberton et al., 2008; Vyas et al., 2007; Webster, 2001). In humans *T. gondii* infection has been associated with schizophrenia (Torrey et al., 2007; Yolken et al., 2009), traffic accidents (Flegr et al., 2002), Alzheimer disease (Kusbeci et al., 2011), and behavioral changes (Flegr, 2007), although the cause-effect chain has not yet been conclusively shown.

As described in the previous section a series of fatal cases in immune-competent people was reported from French Guiana (Carme et al., 2002; Demar et al., 2007). This increased virulence is likely due to a different genetic make-up of the parasite, and shows that newly emerging strains are a potential threat to human health.

Toxoplasmosis in animals

Similarly to *T. gondii* infection in humans, the infection usually remains asymptomatic in most other species. Congenital transmission resulting in abortion or offspring born with abnormalities is the most commonly observed problem. Especially sheep and goats are susceptible to congenital toxoplasmosis and in these animals *T. gondii* is an important cause of abortion (Buxton et al., 2007; Dubey, 2009c). To prevent these abortions, an attenuated live-vaccine, based on a strain (S48) that has lost its ability to develop tissue cysts by continuous passage in mice (Buxton, 1993; Wastling et al., 1993), has been developed and is commercially available (Toxovax®).

There are, however, some species in which *T. gondii* infection can have serious consequences (Innes, 1997). For Australian marsupials (Canfield et al., 1990) and New World monkeys (Epiphanio et al., 2003) primary infection with *T. gondii* is often fatal. Female Pallas cats can transmit *T. gondii* to their offspring when chronically infected, which often leads to fatal toxoplasmosis in kittens, and is a common cause for failure of captive breeding programs (Kenny et al., 2002). Some pigeon breeds or species are highly susceptible to clinical toxoplasmosis, and canaries show an unusually severe eye infection with symptoms varying from blindness to complete ocular atrophy (Dubey, 2002). The high susceptibility for

marsupials, New World monkeys and Pallas cats is considered a result from their evolutionary development separated from cats and *T. gondii*: felines were first introduced in Australia by settlers in the late 18th century, New World monkeys live high up in trees, and the exposure to *T. gondii* in wild Pallas cats and other hosts in Mongolia is very low (Brown et al., 2005). The susceptibility of pigeons and canaries has yet to be explained.

Encephalitis by *T. gondii* is an important cause of mortality in Californian sea otters (Kreuder et al., 2003), and is especially prevalent in areas with heavy freshwater runoff such as Morro bay (Miller et al., 2002). An unusual genotype (type X) was identified with a spatial clustering in the southern half of California and especially around Morro bay (Conrad et al., 2005; Miller et al., 2004). This specific strain may partly explain this apparently high susceptibility of sea otters to *T. gondii*, although no statistically significant association between genotype and pathogenicity was observed (Miller et al., 2004). A more recent study that included sequencing of the GRA6 gene, demonstrated that type X can be subdivided in two types, one of which has been named type A. Type X and A were also identified in sea otters from Washington state although at a lower prevalence (Sundar et al., 2008). Sundar and colleagues could not confirm the importance of *T. gondii* as a cause of mortality. *T. gondii* is also highly prevalent in other marine mammals (Dubey et al., 2003).

Cattle appear to be at the other end: In cattle, natural *T. gondii* infection does not appear to cause clinical disease or abortion (Dubey, 1986). It has also been suggested that cattle are not very susceptible to infection (Munday, Corbould, 1979), and that, if infected, tissue cysts are unlikely to persist. Tissue cysts are only infrequently recovered from experimentally inoculated cattle (Dubey, 1986; Dubey, Thulliez, 1993; Esteban-Redondo et al., 1999), and there are few successful recoveries from naturally infected cattle (Dubey, 1986) or beef samples (Aspinall et al., 2002; Dubey et al., 2005).

Possible sources of acquired infection in humans

Humans, just as many other warm-blooded species, are intermediate hosts of *T. gondii*. Since the elucidation of the life cycle of *T. gondii*, the possible sources of infection for humans are clear: Like all intermediate hosts, humans can be infected by congenital transmission of tachyzoites and ingestion of tissue cysts or oocysts (Fig. 1.2). As congenital transmission occurs when the mother ingests tissue cysts or oocysts, this section will focus on acquired (or non-congenital) infection.

Tissue cysts

Tissue cysts are responsible for meatborne infection. They are most prevalent in muscle and nervous tissue, and therefore organs such as liver and kidney are less likely sources of infection although they cannot be ruled out. Viable tissue cysts have been detected in especially sheep, pigs, and various wild animals, and are considered very rare in cattle (Tenter et al., 2000). If infected meat is consumed without prior freezing (less than -12°C for at least 2 days) or proper heating (core temperature over 67°C) *T. gondii* can be transmitted (Dubey, 1996b; Kijlstra, Jongert, 2008a). In addition to freezing and heating, salting can inactivate *T. gondii* depending on the salt concentration and duration (Kijlstra, Jongert, 2008a). Other treatments such as fermenting, drying and smoking reduce tissue cyst viability but the exact conditions needed seem less well established (Kijlstra, Jongert, 2008a; van Sprang, 1984).

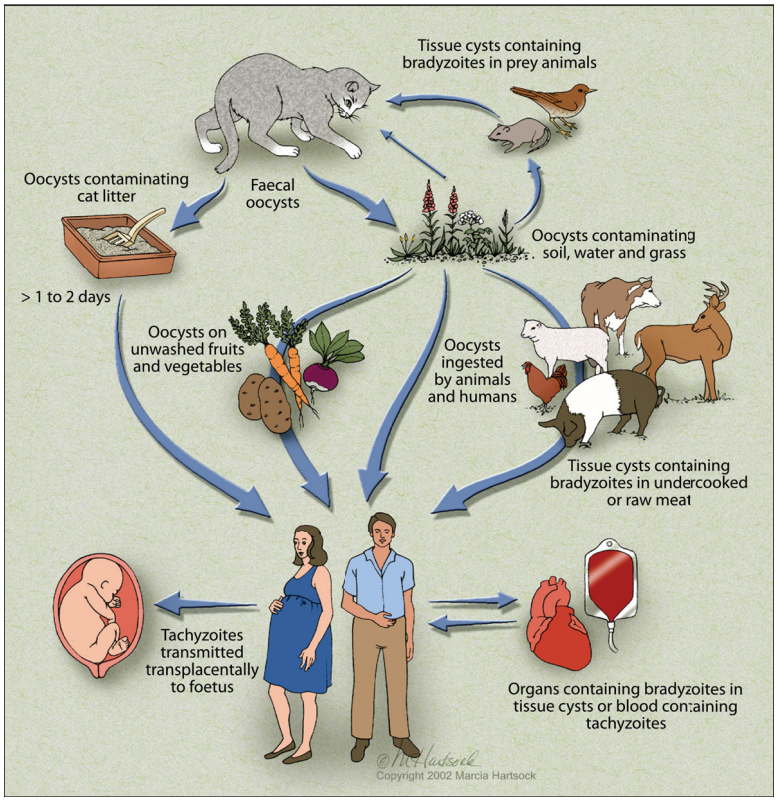


Figure 1.2 Transmission routes leading to human infection with *Toxoplasma gondii*
(© Marcia Hartsock)

Oocysts

Upon primary infection cats shed millions of oocysts into their litter box or the environment. These oocysts sporulate and become infectious within 1 to 5 days after excretion. Thereafter, oocysts remain infectious for up to 18 months in soil depending on humidity, temperature and exposure to direct sunlight (Frenkel et al., 1975; Yilmaz, Hopkins, 1972), and for 6 up to 54 months in water (Dubey, 1998b) and seawater (Lindsay, Dubey, 2009) at temperatures between 4 and 25°C. Oocysts are also resistant to freezing: Experimentally sporulated oocysts survived up to 28 days of freezing at -21°C (Frenkel, Dubey, 1973). From the place of deposition by a cat oocysts can be distributed mechanically, by vectors (Ruiz, Frenkel, 1980), wind or rain, and transported to water by runoff. Based on the amount of excreted oocysts upon infection, the yearly incidence of oocyst excretion by cats, the cat population size, their faecal production, and the percentage of faeces deposited outside, the annual quantity of oocysts deposited on USA soil was estimated at 779-1328 oocyst/m^2 (Dabritz, Conrad, 2010). In combination with their resistance to environmental conditions oocyst contamination of the environment is expected to be large.

The most likely opportunities for humans to ingest sporulated oocysts arise while cleaning the cat litter box more than one day after use (Kapperud et al., 1996), from consuming unwashed fruits and vegetables (Kapperud et al., 1996; Kniel et al., 2002), from contact with soil (Cook et al., 2000) either during or after gardening or playing in the sandbox, and from drinking untreated water (Bowie et al., 1997; Sroka et al., 2006). Additionally, it is conceivable that accidental water intake while swimming in open surface waters can lead to infection. This has, however, never been demonstrated. Possibly because the gravity of oocysts (1.104-1.140) (Dubey et al., 1970b) is such that they are likely to sink to the bottom. Dogs may also play a role in transmission of cat-shed oocysts to humans, as their fur is considered more likely to harbour oocysts than cat fur (Frenkel et al., 1995; Frenkel et al., 2003; Lindsay et al., 1997). Owning a dog has in fact been associated to *T. gondii* infection (Etheredge et al., 2004; Frenkel et al., 1995; Sroka et al., 2010). Shellfish and other filter feeders that are consumed raw are also a potential source of infection: for mussels and oysters the capability to filtrate and retain oocysts from water has been shown experimentally (Arkush et al., 2003; Lindsay et al., 2004; Lindsay et al., 2001); *T. gondii* has been detected by PCR in a wild mussel (Miller et al., 2008), retail oysters (Esmerini et al., 2010) and in farmed oyster and clam species (Putignani et al., 2011); an association between consumption of raw oysters, clams or mussels and *T. gondii* infection within the last 6 months was shown (Jones et al., 2009); and infected shellfish are considered an important source of infection for marine mammals (Conrad et al., 2005; Dubey et al., 2003). Filter feeding fish species have also been implicated in the transmission of *T. gondii* to marine mammals, and presence and persistence of infectious oocysts in the alimentary canals of anchovies and sardines has been demonstrated experimentally (Massie et al., 2010). However, these fishes are, thanks to processing, unlikely to be an important source of infection for humans. Lastly, aerosolized transmission of *T. gondii* oocysts has been suggested in an outbreak in people in a horse-stable in Atlanta (Teutsch et al., 1979).

Milk

A more debated source of infection is the consumption of raw milk. It is known that raw milk from acutely infected animals can contain *T. gondii* parasites. However, it is generally thought that milk will contain mostly tachyzoites, which are less resistant to pepsin digestion (Dubey, 1998a) and therefore considered less infective. Nonetheless, outbreaks or cases in humans due to consumption of raw milk, specifically goat's milk, have been reported (Chiari Cde, Neves, 1984; Riemann et al., 1975; Sacks et al., 1982; Skinner et al., 1990), and consumption of raw goat milk was associated with *T. gondii* seropositivity (Jones et al., 2009). Feeding goat whey was also identified as an important risk of infection for pigs (Meerburg et al., 2006). If camel milk can contain *T. gondii* it could be an important source of infection for nomads that consume their camels' milk raw (Elamin et al., 1992).

Other sources

In addition to these natural routes, there are some purely man-made modes of transmission such as organ transplantation or blood transfusion. Although transmission via transfusion of blood or, more likely, leucocytes is theoretically possible because there is a parasitemic phase in infection, case-reports are scarce (Siegel et al., 1971). Transmission by organ transplantation is most likely with heart transplants, as has been described in the section on toxoplasmosis in humans.

Relative importance of the sources of acquired infection in humans

To set-up effective strategies to reduce human risk of infection, not only the possible sources, but also the relative importance of these sources needs to be clarified. There are different approaches to study the relative importance of different sources of infection (as reviewed by (Pires et al., 2009)). In the field of *T. gondii* a classical epidemiological risk-factor analysis is the most common approach. Because infection with *T. gondii* often goes unnoticed, usually chronic rather than acute infections are studied. In that case the effect of a risk-factor can be diluted, as the *T. gondii* infection may have been acquired long before asking about the habits: for example, adults that report no soil contact (gardening) may have been infected when playing in the sandbox as a child, or vegetarians were infected by eating undercooked meat before becoming a vegetarian. Therefore risk factor analyses based on incident cases (usually seroconversions in pregnant women) are more reliable, but they are not often conducted. One European multicentre study that includes centres in Naples, Milan, Copenhagen, Oslo, Brussels, and Lausanne is the key reference (Cook et al., 2000). In this study it was estimated that 30 to 60% of the infections were acquired through the consumption of meat and 6 to 17% was soil-borne. The statistically significant risk factors identified were: consumption of meat other than beef, lamb or pork (OR 4.12); consumption of raw/undercooked lamb (OR 3.13); travel outside Europe, USA or Canada (OR 2.33); contact with soil (OR 1.81); consumption of raw/undercooked beef (1.73); and tasting of meat during cooking (OR 1.52). Consumption of raw/undercooked pork was not significantly associated with *T. gondii* infection (OR 1.4 (95% CI 0.7-2.8)). It also showed that the most important meat source varied by country (which is likely due to differences in consumption habits) and that a large proportion of the infections remained unexplained (14-49%).

Another approach to get an idea of the relative contribution of different sources is by combining information on the prevalence and concentration of infection in the sources with the number of effective contacts with those sources. This quantitative microbial risk assessment (QMRA) approach has been used for example for *Campylobacter* (Evers et al., 2008) and *E. coli* (Kosmider et al., 2010). Regarding meatborne transmission, it is known that *T. gondii* tissue cysts are most frequently present in various tissues of infected pigs, sheep and goats and less frequently in infected poultry, rabbits, cattle, dogs and horses (Tenter et al., 2000). Next to that, it is known that outdoor reared animals are at higher risk of *T. gondii* infection due to exposure to environmental oocysts (Tenter et al., 2000). However, because bioassay (the only method that was available for sensitive detection of *T. gondii* tissue cysts in meat) results are not quantitative, *T. gondii* risk assessments were limited to one qualitative study that has shown the effectiveness of freezing, heating and salting in inactivating *T. gondii* (Mie et al., 2008). It is even more difficult to include oocyst-related infections into a risk assessment because, besides detection using sentinel chickens, detection of *T. gondii* oocysts in soil and water samples is more challenging than detection from meat (Dumetre, Darde, 2003).

A third approach to study transmission is by molecular typing. There may be small differences in the genetic make-up of the pathogen under study, and by comparing the subtypes isolated from different sources with those isolated from humans, transmission patterns can be revealed. This molecular epidemiology approach has been developed and used mostly for bacteria (e.g. for *Salmonella* (Hald et al., 2004) and *Campylobacter* (Sheppard et al., 2010)), for viruses

(examples in virology are given by e.g. Lam et al., 2010; McCormack, Clewley, 2002), and to a lesser extent also for parasites (e.g. *Cryptosporidium* (Wielinga et al., 2008), and apicomplexan parasites (Beck et al., 2009)). As described molecular typing has been very useful in studying the evolution of *T. gondii*, and genetic differences between strains from different continents have been shown. However, because expansion is mostly clonal in humans and domestic animals from Europe and North America, the usefulness of molecular typing techniques in studying transmission on these continents is questionable: For subtyping to work as a method for source attribution there need to be strong associations for the dominant subtypes with specific sources, and each source should have a specific subtype (Pires et al., 2009).

Toxoplasma gondii in The Netherlands

T. gondii infection is common in people all over the world. In 1996 the average prevalence of antibodies against *T. gondii* for the Dutch population was estimated at 40.5%, and for women at reproductive age at 35.2% (Kortbeek et al., 2004). In 2006 the seroprevalence for the overall Dutch population had decreased to 26.0% and for women at reproductive age to 18.5% (Hofhuis et al., 2011). In both studies sera were obtained from a nationwide study to determine the immunization effect of the national vaccination program in the Dutch population (the PIENTER study http://www.rivm.nl/preventie/vaccinatie/PIENTER/). In PIENTER approximately 400 inhabitants between 0 and 80 years from 48 municipalities are randomly selected. Municipalities differ between the 1996 and 2006 studies. Participants donate a blood sample and fill out a questionnaire. Additional questions to address specific risk factors for *T. gondii* infection such as (raw) meat consumption were added to the 2006 questionnaire. The most important risk factors identified for people aged 20 and older in the 1996 survey were: living in the Northwest, having professional contact with animals, living in a moderately urbanized area, being divorced or widowed, being born outside The Netherlands, frequent gardening, and owning a cat (Kortbeek et al., 2004). In the 2006 survey living in the Northwest, living in urban areas, low educational level, consumption of raw pork, keeping a cat, and not having occupational contact with clients or patients were independently associated with *T. gondii* seropositivity (Hofhuis et al., 2011). For younger participants (0-15 years of age), risk factors were: keeping sheep or cattle, consumption of raw unwashed vegetables and putting sand in the mouth (Hofhuis et al., 2011). A decreasing prevalence is observed in many European countries (e.g. UK (Nash et al., 2005), Austria (Edelhofer, Prossinger, 2010), France (Villena et al., 2010)) and is considered an effect the shift towards intensive indoor pig-breeding and the increase in frozen storage of meat products.

For the Dutch situation, the disease burden of congenital toxoplasmosis was calculated and compared to the burdens of other foodborne pathogens based on disability-adjusted life-years (DALYs) (Havelaar et al., 2007). Disability-adjusted life-years are the sum of life-years lost and those lived with disability weighted for the severity of the disability. Using this method, diseases that differ in clinical outcome and incidence (e.g frequent salmonellosis which is usually characterized by self-limiting diarrhea, and congenital toxoplasmosis which is uncommon but may have lifelong consequences) can be compared. To calculate DALYs the incidence of the various clinical outcomes is a key element. First available data were used to estimate the overall incidence of congenital toxoplasmosis. The incidence was estimated at 8.1 per 10,000 live-borns from women seronegative at the start of pregnancy, by repeated serological testing of 15,170 women seronegative at first testing in the Toxoplasma Infection

Prevention (TIP) study (Conyn-van Spaendonck, 1991). The incidence of the various clinical presentations of congenital toxoplasmosis was estimated based on a different set of studies. Based on the combination of these data, the disease burden for congenital toxoplasmosis was estimated at 620 (95% CI 220-1900) DALYs (Havelaar et al., 2007). However, using the increase in seroprevalence observed for women of reproductive age in the 1996 PIENTER study the incidence of congenital toxoplasmosis was estimated at 21 per 10,000 live-borns from women seronegative at the start of pregnancy. Using this incidence estimate the disease burden increased to 1200 DALYs. Therefore a study was set-up to estimate the incidence more precisely using the same study design as was described in Denmark (Schmidt et al., 2006). Over 10,000 Guthrie cards collected in 2006 were tested for the presence of IgG and IgM antibodies against *T. gondii*, and based on the results the incidence of congenital infection was estimated at 2 per 1,000 live-born children. Based on this new incidence estimate the disease burden was estimated at 2300 (95% CI 820-6710) DALYs (Kortbeek et al., 2009). This demonstrates that *T. gondii* is one of the most important foodborne pathogens in The Netherlands. Because ocular toxoplasmosis from acquired infection was not included in these calculations the estimated disease burden still underestimates the total burden from *T. gondii* infections. Based on an estimated 67,000 newly acquired *T. gondii* infections per year in the general population with 14% showing mild symptoms, 0.7% showing severe symptoms and 0.3% developing chorioretinitis, the disease burden for acquired toxoplasmosis was estimated at 1200 DALYs (3-2700) per year and almost entirely attributed to chorioretinitis (Kemmeren et al., 2006). However, once more the uncertainties around the estimated number of infections per year and the incidence of the different health outcomes were large. Therefore new estimations based on more recent data are in preparation.

In The Netherlands no general screening program for congenital toxoplasmosis is in place, neither for pregnant women as in France (Villena et al., 2010) and Austria (Aspock, Pollak, 1992), nor for neonates (as in Poland (Paul et al., 2001) and used to be offered in Denmark (Roser et al., 2010)). The screening program for pregnant women in France consists of a serological test before or early in pregnancy and monthly serological follow-up for those who tested negative (Foulon et al., 2000). Women that seroconvert during pregnancy are offered therapy using spiramycin and tested for transmission to the foetus. If amniocentesis is positive, spiramycin is replaced by pyrimethamine-sulfadiazine treatment until delivery. These foetuses are additionally checked using ultrasound or MRI, and if transmission and severe malformations are shown the pregnancy may be terminated. Because in The Netherlands most women are seronegative at reproductive age (81.5% (Hofhuis et al., 2011)) many would require follow-up during pregnancy and such a program is unlikely to be cost-effective (Conyn-van Spaendonck, 1991), which has more recently been outlined for the situation in the UK (Gilbert, Peckham, 2002). It is, however, not entirely clear how many cases of congenital toxoplasmosis can be prevented by screening and treatment because of the uncertain effect of treatment on transmission and symptoms (Gilbert et al., 2001; Gras et al., 2001; Thiebaut et al., 2007), the time lag between infection and detection, and the inability to detect some of the infections. The psychological burden from false positive test results is another important issue to take into account when considering implementation of a prenatal screening program (Conyn-van Spaendonck, van Knapen, 1992). The prenatal screening program is also a recurring topic for discussion in France (Ancelle et al., 2009; Jeannel et al., 1990). Neonatal screening has the advantage that it requires less testing (there is no follow-

up needed) but the effectiveness of treatment of infected newborns is even more uncertain (Gilbert, Dezateux, 2006). This lack of a demonstrable benefit of treatment was the reason for the recent termination of the program in Denmark (Roser et al., 2010).

Table 1.1 Seroprevalence of *Toxoplasma gondii* per livestock species in The Netherlands

Species	% (*n*)	Year	Assay	Sampling	Reference
Horse	7% (85)	1982	ELISA[a]	At slaughter	(van Knapen et al., 1982)
Horse	0% (15)	1958	SFDT[b] ≥ 1:64 no inactivation	At slaughter, Amsterdam	(de Roever-Bonnet, 1958)
Cattle (dairy, North)	13.1% (6123)	1987-89	ELISA	At slaughter	(van Knapen et al., 1995)
Cattle (dairy, South)	42.6% (618)	1979-80	ELISA	Various farms	(van Knapen et al., 1995)
Cattle (steers, South)	26.4% (235)	1979-80	ELISA	Various farms	(van Knapen et al., 1995)
Cattle (calves, south)	1.2% (156)	1979-80	ELISA	Various farms	(van Knapen et al., 1995)
Cattle	22% (180)	1982	ELISA	At slaughter, various ages and farms	(van Knapen et al., 1982)
Cattle	14% (150)	1969	SFDT ≥1:16	At slaughter, various ages	(Cremers, 1969)
Cattle	6.7% (30)	1958	SFDT ≥1:64 no inactivation	At slaughter, Amsterdam	(de Roever-Bonnet, 1958)
Sheep	75.4% (1032)	1983	ELISA	7 farms	(van Walderveen, de Leeuw, 1983)
Sheep	65% (40)	1991	ELISA	At slaughter, different farms, different ages	(Cremers et al., 1991)
Sheep	30% (115)	1982	ELISA	At slaughter, mainly young, various farms	(van Knapen et al., 1982)
Sheep	92% (100)	1969	SFDT ≥1:16	At slaughter	(Cremers, 1969)
Sheep	89% (175)	1963	SFDT ≥1:16	Ewes at farm	(de Roever-Bonnet, 1963)
Goats (dairy)	47.1% (189)	1996	LA[c], ELISA	10 selected goat farms (3 with abortion problems)	(Antonis et al., 1998)
Swine (fattening, conventional)	0.4% (265)	2004	ELISA	At slaughter, 24 farms	(van der Giessen et al., 2007)
Swine (fattening, free-range)	5.6% (178)	2004	ELISA	At slaughter, 9 farms	(van der Giessen et al., 2007)
Swine (fattening, organic)	2.7% (402)	2004	ELISA	At slaughter, 40 farms	(van der Giessen et al., 2007)
Swine (fattening, conventional)	0% (621)	2001/2002	LA, IFAT[d] and IB[e]	At slaughter, 30 farms	(Kijlstra et al., 2004)

Swine (fattening, free-range)	4.7% (635)	2001/2002	LA, IFAT and IB	At slaughter, 17 farms	(Kijlstra et al., 2004)
Swine (fattening, organic)	1.2% (660)	2001/2002	LA, IFAT and IB	At slaughter, 16 farms	(Kijlstra et al., 2004)
Swine (fattening)	1.8% (994)	1995	ELISA	At slaughter, various slaughterhouses, few farms per slaughterhouse	(van Knapen et al., 1995)
Swine (fattening)	2.1% (23,348)	1989	ELISA	At slaughter, 120 farms in Integrated Quality Control project	(Berends et al., 1991)
Swine (fattening)	0% (196)	1982	ELISA	At slaughter, various farms	(van Knapen et al., 1982)
Swine (fattening)	54% (50)	1969	SFDT ≥1:16	At slaughter	(Cremers, 1969)
Swine (sows)	30.9% (1009)	1995	ELISA	At slaughter	(van Knapen et al., 1995)
Swine (sows)	11% (36)	1982	ELISA	At slaughter, various farms	(van Knapen et al., 1982)
Swine (sows)	86% (50)	1969	SFDT ≥1:16	At slaughter	(Cremers, 1969)
Swine (unspecified)	62% (128)	1963	SFDT ≥1:4	At slaughter, various farms, approx. 8 months old	(Folkers, Perie, 1963)
Swine (unspecified)	16% (25)	1958	SFDT ≥1:64 no inactivation	At slaughter, Amsterdam	(de Roever-Bonnet, 1958)
Poultry (free range hens)	30% (136)	1982	ELISA	At slaughter, >1 year old, various farms	(van Knapen et al., 1982)
Poultry (broilers)	0% (82)	1982	ELISA	At slaughter, 8 weeks old, various farms	(van Knapen et al., 1982)
Poultry (battery hens)	0% (183)	1982	ELISA	At slaughter, approx. 1 year old, various farms	(van Knapen et al., 1982)

[a]ELISA: Enzyme-linked immunosorbent assay; [b]SFDT: Sabin-Feldman dye test; [c]LA: Latex-agglutination; [d]IFAT: Immuno-fluorescense antibody test; [e]IB: Immunoblot

Until effective therapy or vaccination can be offered, prevention of *T. gondii* infection is considered the most effective way to decrease the disease burden in The Netherlands. At the moment, the prevention strategy is based on educating pregnant women about the risks for infection. However, inclusion of other intervention measures may be more effective. To set up targeted prevention strategies, transmission needs to be well understood. Although possible sources are clear from the life cycle, their relative contribution remains unclear and depends on infection rates in the sources as well as contacts with those sources.

Concerning the infection rates in the sources only data on livestock are available. Two independent studies recently showed that the prevalence in pigs is highly dependent on housing system: In conventional housing *T. gondii* is close to absent, whereas the prevalence for pigs raised with outdoor access is approximately 3% (Kijlstra et al., 2004; van der Giessen et al., 2007). For other livestock species the most recent prevalence data available date from the 1980s or earlier, or are based on a limited sample size (Table 1.1).

Aims and outline of this thesis

T. gondii has long been known as a cause of abortion and abnormalities at or after birth, but was only recently recognized as one of the most important foodborne pathogens world-wide. Several relatively recent findings have led to this renewed recognition. First of all, calculating and comparing the disease burden of congenital toxoplasmosis to the disease burden of other foodborne pathogens demonstrated its actual importance (Havelaar et al., 2007; Kortbeek et al., 2009; Mead et al., 1999; Vaillant et al., 2005). In addition to that, *T. gondii* was long perceived harmless in the immune-competent, but outbreak investigations as well as detailed diagnostic work-up of chorioretinitis cases have demonstrated that about two thirds of patients with ocular toxoplasmosis may have acquired the infection postnatally (Gilbert, Stanford, 2000). Also, the evidence for a link between *T. gondii* seropositivity and mental changes or, more specifically, schizophrenia is growing (Yolken et al., 2009). At the same time, there is a tendency towards more animal friendly rearing conditions with outdoor access. This has been shown to increase the prevalence in pigs (Kijlstra et al., 2004; van der Giessen et al., 2007), and will, as a result, also increase human exposure to *T. gondii*. Furthermore, the outbreak of fatal toxoplasmosis in immune-competent individuals in French Guiana that seems related to the genotype (Carme et al., 2002; Demar et al., 2007), demonstrates that the emergence of new strains gives cause for concern.

Together these findings suggest that *T. gondii* should be taken more seriously as a public health issue. Because the effectiveness of therapy is debated (Gilbert et al., 2001; Gras et al., 2001; Thiebaut et al., 2007), prevention of infection is probably the most effective intervention measure. To set up targeted prevention strategies, the relative contribution of the different sources needs to be clearly understood. In 2007 the European Food Safety Authority stressed the importance of monitoring *T. gondii* in food animals (EFSA, 2007). The goal of the research presented in this thesis was to answer the question: What are the most important sources of *T. gondii* infection for the Dutch population? Answering this question will guide evidence-based development of prevention strategies. We describe studies related to production animals and meat, and related to cats and the environment. These studies were designed to meet the following objectives:

1. To quantitatively detect and genotype *T. gondii* tissue cysts in tissue samples by PCR and study the correspondence with serology.
2. To study seroprevalence of *T. gondii* in sheep, cattle and wild boar.
3. To compare the relative contribution of different livestock species to meatborne *T. gondii* infections by quantitative microbial risk assessment.
4. To get more insight in oocyst contamination of the environment and possible intervention measures taken at the definitive host by studying the seroprevalence and risk factors for *T. gondii* infection in cats.

Detection of tissue cysts by PCR was considered inferior to bioassays because tissue cysts can easily be missed due to the small sample used for DNA isolation in combination with the low concentration and inhomogeneous distribution of tissue cysts. On the other hand, bioassays are not desirable for screening purposes because they are expensive and rely on use of considerable numbers of experimental animals. Therefore a sensitive and quantitative PCR-based detection method had to be developed first (Chapter 2). This method was extended to allow genotyping of the gene encoding dense granule protein GRA6. Then the seroprevalence of *T. gondii* in various animal sources was studied (Chapter 3, 4 and 5). Because recent serological information was already available for pigs in The Netherlands (Kijlstra et al., 2004; van der Giessen et al., 2007) studies were not repeated, but data are incorporated in the QMRA model. In contrast, the role of cattle was studied extensively, because in our opinion, even if cattle are less susceptible and not often infected, their role can be of importance as beef is often eaten raw. The data on the different sources was used as input for a quantitative microbial risk assessment (QMRA) model in combination with consumption and food preparation data to determine the most important sources of meatborne *T. gondii* infections in The Netherlands (Chapter 6). In The Netherlands probably only domestic and stray cats (*Felis catus*) are important definitive hosts, although the wild cat (*Felis silvestris*) has been reported incidentally (Canters et al., 2005; Mulder, 2007). As cats are the only species shedding *T. gondii* into our environment, prevention of cat infection could be effective at reducing the environmental infection pressure for both consumption animals and humans. Therefore, risk factors for infection with *T. gondii* in cats were studied to get an idea of possible intervention measures (Chapter 7).

Studying the various sources of *T. gondii* infection for humans will give an indication of the most important sources and thereby contribute to the development of targeted and therefore more effective intervention measures.

Chapter 2

Direct detection and genotyping of *Toxoplasma gondii* in meat samples using magnetic capture and PCR

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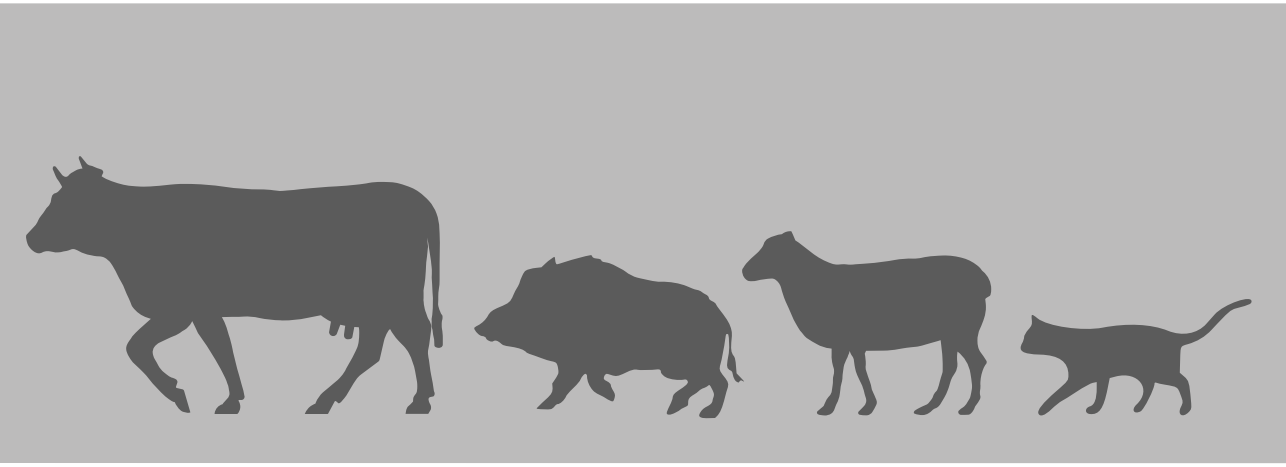

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Different transmission routes, including the ingestion of undercooked meat, can result in *Toxoplasma gondii* infection in humans. The development of effective prevention strategies is hampered by a lack of quantitative information on the contamination level of different types of meat. Therefore, we developed a method for detection and quantification of *T. gondii.* The method involved preparation of crude DNA extract from a hundred gram samples of meat, magnetic capture of *T. gondii* DNA and, quantitative real-time PCR targeting the *T. gondii* 529-bp repeat element. The detection limit of this assay was approximately 230 tachyzoites per 100 g of meat sample. There was a linear relation between the number of parasites added to the samples and Cp-values. Results obtained with the PCR method were comparable to bioassay results for experimentally infected pigs, and to serological findings for sheep. In addition, the *T. gondii* in 50% of the positive sheep samples could be genotyped by sequencing of the GRA6 gene, after isolation of the gene by magnetic capture. Two subtypes of GRA6 type II were identified in the 16 samples from sheep. For seven samples, the identification of *T. gondii* as type II was confirmed by microsatellite typing. The PCR method can be used as an alternative to bioassay for detection and genotyping of *T. gondii,* and to quantify the organism in meat samples of various sources.

Introduction

Infections by the protozoan parasite *Toxoplasma gondii* are widespread in humans and in many warm-blooded animal species. Although most infections in humans are asymptomatic, severe complications may occur in immunocompromised patients and after congenital *T. gondii* infection (Weiss and Dubey, 2009). The incidence of congenital toxoplasmosis in The Netherlands was recently estimated at 2 per 1000 live-born children (Kortbeek et al., 2009). Using these new data the disease burden was calculated at 2300 disability-adjusted life years (Kortbeek et al., 2009), which is almost four times higher than the previous estimate (Havelaar et al., 2007). This high disease burden makes toxoplasmosis one of the most important food-borne infections, and underscores the necessity to intervene. Considering the lack of evidence of the effectiveness of treatment (Thiebaut et al., 2007; Gilbert, 2009), prevention strategies are considered most effective. Humans can contract *T. gondii* via tissue cysts in undercooked meat and via accidental ingestion of oocysts by contact with cat faeces, contaminated soil, water, or vegetables. Even though the sources of *T. gondii* infection for humans are well known, insight in their relative contribution needs to be improved for the development of effective prevention strategies. Risk-factor analysis indicates that 30 to 63% of human infections can be attributed to the consumption of undercooked meat (Cook et al., 2000). However, what kind of meat contributes most to human infections depends on prevalence of *T. gondii* in consumption animals and on eating habits. An indication of the relative contribution of different kinds of meat can be obtained by screening large numbers of meat samples for the presence of *T. gondii.* Genotyping isolated parasites will give further insight into the epidemiology of toxoplasmosis.

The gold standard for detecting *T. gondii* in meat samples is a bioassay using either mice or cats. These bioassays are laborious and time-consuming techniques, which are not desirable for screening large numbers of samples from an animal ethics point of view. Therefore, PCR-based methods to detect *T. gondii* in meat samples have been developed. However, although the PCR itself is usually sensitive in detecting *T. gondii* DNA, when used on meat samples, these methods lack sensitivity in comparison to the bioassay (da Silva and Langoni, 2001; Garcia et al., 2006; Hill et al., 2006). This lack of sensitivity of PCR-based methods is likely due to the inhomogeneous distribution of *T. gondii* tissue cysts, in combination with the small size of the sample: For PCR, DNA is usually isolated from 50 mg of sample at maximum. In the bioassay either up to 500 g of meat is fed to a cat, or fifty to a hundred grams of meat is inoculated into mice after artificial digestion. Clearly, the probability of the presence of a tissue cyst in a 50 mg sample is much lower than in the 50 to 500 g sample used in bioassays. As a consequence, taking fifty milligrams of the homogenate of a large sample, instead of taking a fifty milligram sample randomly, will increase the probability of isolating *T. gondii* DNA. However, it will be present at a low concentration in a high background of host DNA, which might lead to inhibition of the PCR (Bellete et al., 2003). The effects of a low concentration and inhibition can be overcome by sequence-specific magnetic capture, as has been previously described for the detection of mycobacterial DNA in clinical samples (Mangiapan et al., 1996). To detect *T. gondii*, the 529-bp repeat element (Homan et al., 2000; Reischl et al., 2003) is used as target for sequence-specific capture and real-time PCR. Because the *T. gondii* genome is distributed over fourteen chromosomes (Khan et al., 2005b), only part of the genome is isolated using sequence-specific capture. The 529-bp repeat element is highly conserved (Reischl et al., 2003), and therefore not suitable for typing. To enable genotyping,

the GRA6 gene is captured. GRA6 is a dense granule protein of 32 kDa (Lecordier et al., 1995). The single copy GRA6 gene is highly polymorphic (Fazaeli et al., 2000), which makes it a useful marker for typing.

It was our aim to develop a PCR-based method that can be used as an alternative to the bioassay in quantitative screening of large numbers of meat samples. In this paper, a method that combines homogenization of a large sample with sequence-specific magnetic capture to detect and genotype *T. gondii* in meat samples is described. This method simplifies testing large numbers of meat samples, to determine the relative contribution of different kinds of meat in human *T. gondii* infections, while reducing the use of experimental animals.

Materials and methods

Oligonucleotides

All Tox-oligonucleotides are complementary to the 529-bp repeat element (GenBank AF146527) (Homan et al., 2000)), and all GRA6-oligonucleotides are complementary to the GRA6 gene (GenBank L33814) (Lecordier et al., 1995)) (Table 2.1). All oligonucleotides were synthesized by Biolegio (Nijmegen, The Netherlands). Capture-oligonucleotides were designed to capture either both strands of the 529-bp repeat element (Tox-CapF and Tox-CapR), or both strands of the GRA6 gene (GRA6-CapF and GRA6-CapR), from 5' to the primer binding sites. The capture-oligonucleotides were 5' end labelled with a biotin-triethylene-glycol (biotin-TEG) spacer arm to allow capture using M-270 Streptavidin Dynabeads (Invitrogen, Breda, The Netherlands).

Primers Tox-9F, Tox-11R and probe Tox-TP1 were described previously as Tox-9, Tox-11 and Tox-HP-2 (Reischl et al., 2003), but the last nucleotide at the 3' end in the Tox-HP-2 probe has been deleted. Tox-TP1 was 5' end labelled with 6-FAM and 3' end labelled with Black Hole Quencher 1 (BHQ1).

A competitive internal amplification control (CIAC) (Hoorfar et al., 2004) was developed to enable identification of false negative PCR-results. Overhanging primers CIAC-F and CIAC-R were constructed with the binding sites for the Tox-primers added to the 5' end of *Yersinia pestis* Caf1 primer sites (Janse et al., 2010) (Table 2.1). These overhanging primers were used to amplify a *Y. pestis* Caf1 DNA construct. The product was reamplified using the Tox-9F and Tox-11R primer. The second round PCR-product was run on gel (1.5% agarose in TAE) and the 188bp long product was purified (QIAquick Gel Extraction kit, Qiagen, Venlo, The Netherlands). The concentration of the purified product (CIAC) was determined (NanoDrop-1000 spectrophotometer; Thermo Scientific, Waltham, MA). In the PCR reaction, CIAC was amplified using the Tox-primers and detected by the CIAC-probe, which was 5' end labelled with JOE and 3' end labelled with BHQ1. Different concentrations of CIAC were tested in combination with different dilutions of *T. gondii* DNA to assess the optimal amount of CIAC to add to the PCR-reaction mixture. Addition of 0.02 fg of CIAC to 20 µl of PCR mix did not inhibit amplification of *T. gondii* DNA, and gave a consistently positive CIAC-PCR for *T. gondii* negative samples. Although the CIAC-PCR was positive consistently, the Cp-value varied as might be expected for the low concentration added. Therefore, the Cp-value was not informative regarding inhibition, and only a negative CIAC-PCR was regarded indicative for inhibition.

Table 2.1 Oligonucleotide sequences of capture-oligonucleotides, primers and probes used in this study.

Name	Sequence 5'-3'	5' label	3' label	Position[a]
Tox-CapF	cttggagcca cagaagggac agaagtcgaa ggggactaca gacgcgatgc cgctcctcca gccgtcttgg	Biotin TEG	-[b]	173–242
Tox-CapR	aagcctccga ctctgtctcc ctcgccctct tctccactct tcaattctct ccgccatcac cacgaggaaa	Biotin TEG	-	406–475
GRA6-CapF	gatttgtgtt tccgagcagg tgacctgggt cgctttttg aaacagcagg aaaacagctt cgtggtgcca cgtagcgtgc ttgttggcga ctacc	Biotin TEG	-	301–395
GRA6-CapR	gcagttcgta cagattccta cgttcttctc cacgtcggac agtgccgctc tcccggtcgt tgcatggcat cgactacaag acatagagtg cc	Biotin TEG	-	1145–1236
Tox-9F	aggagagata tcaggactgt ag	-	-	243–264
Tox-11R	gcgtcgtctc gtctagatcg	-	-	386–405
Tox-TP1	ccggcttggc tgcttttcct	6-FAM	BHQ1	338–357
GRA6-F1	ttgggagtgt cggcgaaatg gc	-	-	404–425
GRA6-R1	gtggcgcata gacccctgt tttcatc	-	-	1115–1141
CIAC-F	aggagagata tcaggactgt agccagcccg catcact	-	-	NA[c]
CIAC-R	gcgtcgtctc gtctagatcg atctgtaaag ttaacagatg tgctagt	-	-	NA
CIAC-probe	agcgtaccaa caagtaattc tgtatcgatg	JOE	BHQ1	NA

[a] position on GenBank AF146527 for Tox-oligonucleotides, and on GenBank L33814 for GRA6-oligonucleotides; [b] - : no label; [c] NA: not applicable

Magnetic capture and detection PCR (MC-PCR)

Preparation of crude DNA extract

A hundred gram meat sample (free of fat and connective tissue) was cut into pieces of approximately 1 cm^3. Knives and forceps were thoroughly rinsed with hot water and soap, and thereafter cleaned with DNAzap (Ambion, Austin, TX) to prevent cross-contamination. The cut tissue was put into a Stomacher400 bag with filter (Seward, Worthing, UK), and cell lysis buffer containing 100 mM Tris HCl pH 8.0, 5 mM EDTA pH 8.0, 0.2% SDS, 200 mM NaCl, 40 mg/l proteinase K (30 mAnson-U/mg; Merck, Haarlem, The Netherlands) was added at 2.5 ml per gram of sample. The sample was homogenized in the Stomacher400 (Seward, Worthing, United Kingdom) for 2 min at high speed. The bag was sealed, and the sample was incubated overnight at 55°C in a water bath. After incubation, the samples were homogenized for 1 additional minute. Afterwards, 50 ml of homogenate was transferred to a 50-ml tube, and centrifuged for 45 min at 3500 × *g*. Twelve millilitres of supernatant (crude extract) was transferred to a 15-ml polypropylene tube.

Removal of free biotin

Twelve millilitres of crude extract was incubated at 100°C for 10 min to inactivate the proteinase K. Per sample, 50 µl of streptavidin sepharose (binding capacity 300 nmol/ml; GE Healthcare, Amersham, United Kingdom) was washed 3 times in phosphate buffered saline (PBS). After cooling down the crude-extract samples to below 40°C, the washed streptavidin-sepharose was added. The samples were incubated for 45 min at room temperature, while

rotating at 10 rpm, to allow for streptavidin-biotin binding. Afterwards, the tubes were centrifuged for 15 min at 3500 × *g*, and 10 ml of biotin-free supernatant was transferred to a clean 15-ml polypropylene tube.

Sequence-specific magnetic capture

Ten picomoles of Tox-CapF and Tox-CapR (Table 2.1) were added to each supernatant. The supernatants were heated at 95°C for 15 min to denature all DNA. Then, the tubes were transferred to a shaking water bath set at 55°C, and left to allow for hybridization between capture-oligonucleotides and *T. gondii* DNA for 45 min. The tubes were left to cool down to room temperature while rotating at 10 rpm for 15 min. Per sample, 80 µl of M-270 Streptavidin Dynabeads (Invitrogen) was washed in Binding & Washing (B&W) buffer (5 mM Tris HCl pH 7.5, 0.5 mM EDTA pH 8.0, 1 M NaCl) according to the manufacturer's instructions. The washed beads and 2 ml of 5 M NaCl were added to each supernatant sample and the samples were incubated rotating (10 rpm) at room temperature for 60 min. The complex of streptavidin bead and biotin-labelled capture-oligonucleotide with hybridized *T. gondii* DNA was isolated using the Dynal MPC-1 magnet (Invitrogen). The tube was placed in the magnet for 10 min and supernatant was removed with a disposable Pasteur pipette. The beads were washed twice in B&W buffer, and resuspended in 50 µl of distilled water in a 1.5-ml tube. The bead suspension was heated at 100°C for 10 min to release *T. gondii* DNA from the beads. The tube was placed in the Dynal MPC-S magnet (Invitrogen), and the supernatant was immediately transferred to a clean 1.5-ml tube. Beads were discarded. The magnetic-capture procedure is summarized in figure 2.1.

Real-time quantitative PCR on 529-bp repeat element

PCR amplification was performed in 96-wells plates using a LightCycler480 thermal-cycler instrument (Roche, Almere, The Netherlands). The 20 µl reaction mixture consisted of 4 µl 5 × concentrated Taqman master mix (Roche), 0.7 µM of each primer (Tox-9F and Tox-11R), 0.1 µM of Tox-TP1, 0.2 µM of CIAC-probe, 0.02 fg of CIAC, and 10 µl of template DNA. The reaction mixture was initially incubated at 95°C for 10 min to activate FastStart DNA polymerase. Initial incubation was followed by 45 amplification cycles that consisted of a denaturation step at 95°C for 1 s, an annealing step at 58°C for 20 s, and an extension step at 72°C for 20 s. Afterwards, the samples were cooled to 40°C for 5 s. The temperature transition rate was 4.40°C/s for an increase in temperature and 2.20°C/s for a decrease in temperature. Fluorescence at 530 nm (Tox-TP1) and 560 nm (CIAC-probe) was measured at the end of each extension step. A *T. gondii* DNA standard series was included on each run for calculation of the standard curve, and estimation of PCR efficiency (>1.85) and error (<0.05). For each sample, the fluorescence-by-cycle curve was used to calculate the fractional cycle number or crossing point (Cp) at which the second derivative was at its maximum (LightCycler software, Roche). Either Cp-values or scores (positive/negative) were used in the analysis. All samples with a Cp-value that show a smooth exponential-amplification curve were scored positive, all samples without a Cp-value but with a positive CIAC-PCR were scored negative. Samples without a Cp-value and with a negative CIAC-PCR were retested.

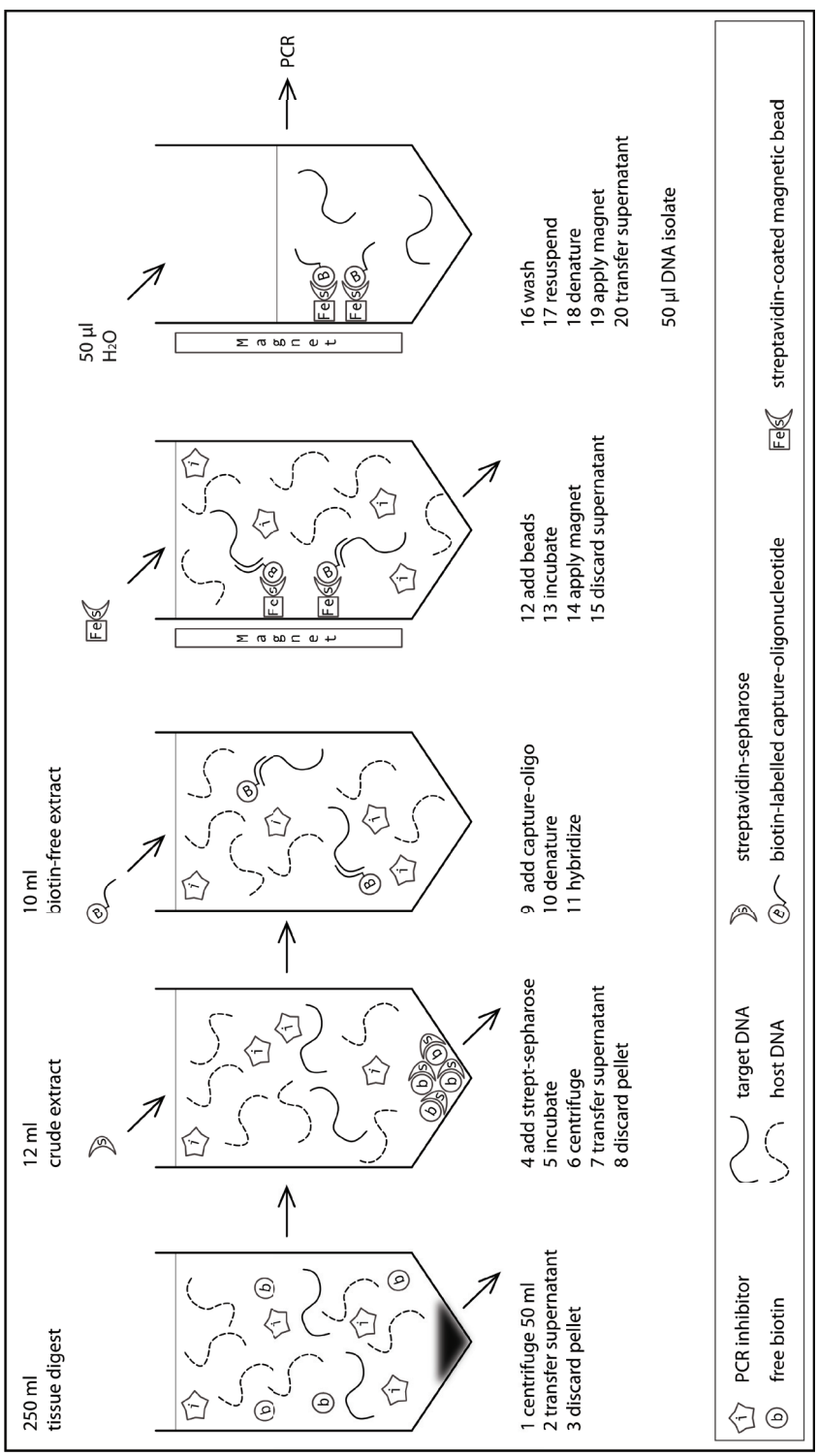

Figure 2.1 Schematic representation of sequence-specific magnetic capture of *T. gondii* DNA from 100 g samples of tissue digested with proteinase K.

Magnetic capture and genotyping

Magnetic capture of GRA6 gene

For field samples positive in MC-PCR, 12 ml of crude extract, prepared as described, was used to capture the GRA6 gene, after removal of free biotin. Capture was performed according to the protocol described with slight modifications: 15 pmol of GRA6-CapF and GRA6-CapR (Table 2.1) were used, and GRA6 DNA was not released from the beads. Instead, 10 µl of resuspended beads was used as template in PCR.

GRA6 PCR

Twenty-five microlitres of PCR reaction mixture contained 12.5 µl of HotStarTaq Master Mix (Qiagen), 10 pmol of each primer (GRA6-F1 and GRA6-R1), 10 µl of template, and RNase-free water. The PCR reaction, performed on a conventional thermal cycler (Hybaid Px2 thermal cycler; Thermo Scientific), was initiated by a heat activation step of 15 min at 94°C, followed by 45 amplification cycles (30 s at 94°C, 30 s at 63°C, and 45 s at 72°C), and a final extension step of 10 min at 72°C. PCR products were visualized by gel electrophoresis, SYBRgold (Invitrogen) staining and UV illumination.

Sequencing of GRA6-PCR products

Positive PCR products were cleaned up using ExoSAP-IT (usb, Staufen, Germany), according to the manufacturer's instructions. The sequencing mixture contained: 7 µl sequence buffer (200 mM Tris HCl, 5 mM $MgCl_2$, pH 9.0), 1 µl BigDye Terminator reagent v3.1 (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands), 4 µl Q-solution (Qiagen), 5 pmol of either primer GRA6-F1 or GRA6-R1, 1 up to 3 µl of cleaned PCR-product (depending on the strength of the band on gel), and milliQ water up to a final volume of 20µl. The program consisted of 25 cycles (30 s at 95°C, 15 s at 50°C, and 4 min at 60°C). Sequencing reactions were purified using BigDye XTerminator Purification kit (Applied Biosystems) and were analysed using the 3730 DNA analyzer (Applied Biosystems). DNA sequences were stored and analysed using BioNumerics 5.1 (Applied Maths, Ghent, Belgium). Representative sequences were submitted to GenBank.

DNA isolation using High Pure PCR template preparation kit

For microsatellite typing, several target sequences should be available in the DNA extract. Therefore, 250 µl of the crude extract (equivalent to 80 to 100 mg of tissue sample and prepared as described) was purified using High Pure PCR template preparation kit (Roche). This kit contains spin columns with two layers of glass fiber fleece for DNA isolation and purification. The manufacturer's protocol for mammalian tissue was used from step 2 (adding binding buffer) onwards (http://www.roche-applied-science.com/pack-insert/1732668a.pdf). Final elution was done in 100 µl. These samples were tested by real-time PCR on the 529-bp repeat element as described. Samples from naturally infected sheep with a Cp-value below 30 were sent to the *Toxoplasma* Biological Resource Center (Limoges, France) for microsatellite typing (Ajzenberg et al., 2009).

Bioassay

To check for the presence of infectious cysts, hundred gram samples of tissue were tested by bioassay in mice. Each sample was mixed with 250 ml pepsin solution (0.8 g/l pepsin, 85 mM NaCl, 0.26% HCl in milli-RO water) and incubated for 2 h at 37°C. The suspension was then centrifuged for 15 min at 600 × *g*. Supernatant was removed and the pellet was resuspended in 8 ml PBS containing 50 µg/ml gentamicin. Five Swiss white mice were each inoculated intraperitoneally with 1 ml of this suspension. Five weeks after inoculation, the mice were euthanized and sera were checked for the presence of anti-*T. gondii* IgG antibodies by indirect immunofluorescence assay. Each serum was diluted 1 in 50 in PBS. Fifty microlitres of serum dilution was applied on slides coated with formalin-treated RH-strain tachyzoites (ToxoSpot IF; BioMerieux, Marcy-l'Etoile, France) and incubated for 30 min at 37°C. The slides were washed with PBS and dried. Thirty microliters of a 1 in 500-dilution of anti-mouse IgG conjugate labeled with Alexa dye 488 (Invitrogen, Merelbeke, Belgium) was applied and incubated for 30 min at 37°C. After washing and drying, the slides were examined using a fluorescence microscope (Carl Zeiss, Göttingen, Germany). If a mouse tested positive, its brain was checked by PCR for confirmation (as described previously, (Kijlstra et al., 2008)). The bioassay was carried out at and according to the guidelines of the Institute of Public Health in Brussels.

Samples

T. gondii tachyzoites and DNA standards

T. gondii RH-strain tachyzoites were cultured on rabbit kidney cells (RK13) until complete lysis of the cells. Harvested tachyzoites were washed three times in PBS and counted using a Bürker-Türk counting chamber. A stock of 2.5×10^6 cultured RH-strain tachyzoites per µl PBS was prepared and aliquots were stored at -20°C. For spiked samples, 100 µl of a dilution of the tachyzoite stock was added to a 100 g meat sample before processing. In addition, DNA was extracted (High Pure PCR Template Preparation kit, Roche) from 33×10^6 tachyzoites and eluted in 50 µl of elution buffer. The DNA concentration was 73.7 ng/µl (NanoDrop-1000 spectrophotometer; Thermo Scientific); one parasite thus corresponds to 112 fg of DNA. Ten microlitres of dilutions of the DNA stock were added to the PCR-mix as positive controls.

Experimentally infected pig samples

Four SPF-pigs infected by oral administration of 500,000 oocysts of the *T. gondii* DX-strain (Astrid Tenter, TiHO, Hannover, Germany), and two negative control pigs, were sacrificed at 60 days post infection. All infected pigs showed an antibody response to *T. gondii*. Samples of brain, heart, tenderloin and a combination of the abdominal muscles were divided into two portions: one was sent to the Institute of Public Health in Brussels (Belgium) to be tested by bioassay while fresh. The other portion was sent to the National Institute of Public Health and Environment in Bilthoven (The Netherlands) to be tested by MC-PCR after storage in the freezer at -20°C. Hundred grams of dorsal muscle, scapular muscle, diaphragm and tongue were tested by MC-PCR only, as resources for bioassay experiments were limited. Pig infections were carried out following the guidelines of the Animal Sciences Group of Wageningen University and Research Centre.

In another experiment, 100 g of heart from each of 37 experimentally infected pigs and 6 negative controls (Bokken et al., submitted) was tested by MC-PCR. All pigs were confirmed seronegative for *T. gondii* at the beginning of the experiment. They were housed indoors without bedding. At eight to nine weeks of age these animals were inoculated orally with either 2000 or 2700 *T. gondii* DX-strain tissue cysts originating from brain tissue of female NMRI mice (Astrid Tenter, TiHO, Hannover, Germany) 11 to 16 weeks post infection. These pigs were housed at and according to the guidelines of the Faculty of Veterinary Medicine, Utrecht University, and sacrificed between 28 and 69 days post infection.

Naturally infected sheep samples

Heart and serum samples were collected from all sheep slaughtered at two Dutch slaughterhouses during one day ($n = 183$). All sera were tested by indirect ELISA for antibodies against *T. gondii*. Briefly, plates were successively incubated for 1 h at 37°C with 100 µl 3.4 µg/ml saponin-octylglucoside solubilized *T. gondii*-RH-antigen (Hughes et al., 1982), with 125 µl 1% BSA in PBS, with 100 µl 1:100 diluted sera, and with 100 µl 1:6000 diluted HRP-labelled polyclonal rabbit anti-sheep immunoglobulins (Dako, Heverlee, Belgium). After each of these incubation steps plates were washed four times in PBS with 0.05% Tween 20. Plates were read after 10 min of incubation with TMB peroxidase substrate (KPL, Gaithersburg, MD, USA). The optical density at 450 nm (OD) was corrected for plate-to-plate variation by linear regression, resulting in a corrected OD-value (ODc-value). The $\log_{10}$ of the ODc-values (logODc-value) was used in statistical analysis. Two cut-off values were chosen to categorize the animals into a high (ODc-value > 0.226), intermediate (ODc-value 0.108–0.226), and low (ODc-value < 0.108) antibody class. A selection of hearts from animals in the high (32/61) and low class (23/104), and all hearts from the sheep with intermediate ODc-values ($n = 18$), were tested. Hearts weighted between 90 and 200 g. A 100 g sample or the whole of each heart was tested by MC-PCR. When the PCR was negative, any remaining heart tissue was tested.

Data analysis

Detection limit and comparison of isolation methods

The detection limit of the PCR was determined by probit analysis (SPSS Statistics 17.0, SPSS Statistics, Chicago, IL) on the results of repeatedly testing *T. gondii* DNA amounts ranging from 2 fg to 500 pg. The detection limit of the complete MC-PCR method was determined by repeatedly testing 100 g meat samples spiked with a known number of tachyzoites (10-fold dilutions from 250,000 to 25; and 125, 63 and 31). In addition, the benefit of the sequence-specific magnetic capture over isolation of total DNA from a fraction of crude extract of a 100 g sample was assessed. The samples from naturally infected sheep ($n = 73$) and experimentally infected pigs that were also tested by bioassay ($n = 4$) were used. The isolation methods were compared by number of positives and Cp-values for the positives (paired t-test).

Quantification of parasites

In real-time PCR the Cp-value decreases linearly with increasing log-transformed DNA concentration. The results with spiked samples (either meat spiked with dilutions of tachyzoites, or PCR reactions spiked with dilutions of *T. gondii* DNA) were used to estimate the formula that predicts the $\log_{10}$(concentration) from the Cp-value and the type of spike (DNA or tachyzoites). This was done by fitting a generalized linear model (SPSS Statistics 17.0).

Results in experimentally infected pigs

A cross-tabulation of MC-PCR and bioassay is presented without further analysis. From the results with the additional 37 experimentally infected pigs the sensitivity of the MC-PCR was estimated (Win Episcope 2.0).

Comparison with serology

To estimate the correlation between ELISA-results and the probability of a positive PCR, a logistic regression model (SPSS Statistics 17.0) was fitted. In this model, the logit of the probability of a positive PCR depends on the logODc-value. The fit of the model was evaluated by Hosmer and Lemeshow test. The predictive ability of the model was evaluated by ROC curve analysis. In addition, the correlation between antibody concentration, as indicated by logODc-value, and parasite load in the PCR positives, as indicated by Cp-value, was checked by linear regression analysis (SPSS Statistics 17.0).

Results

Detection limit of the magnetic capture-PCR and comparison of isolation methods

The 95% detection limit of the PCR was estimated at 15.7 fg (95% CI: 10.0–55.9 fg) per PCR reaction. The probit regression model adequately fitted the data (Pearson's χ^2 (df = 13) is 10.752, p = 0.632). Using probit analysis on the results with spiked meat samples, the 95% detection limit of the method including the magnetic capture was estimated at 227 tachyzoites per 100 g sample (95% CI: 107–3094). Model fit was adequate (Pearson's χ^2 (df = 7) is 10.152, p = 0.180).

Seventy-three sheep and 28 pig samples were tested using two DNA isolation methods. Out of these 101 samples, 41 tested positive after magnetic-capture isolation, while only 34 tested positive using the DNA sample isolated using the High Pure kit. None of the samples found negative after magnetic-capture isolation tested positive after High Pure isolation. For the 34 samples that tested positive after both isolation methods, the Cp-values for High Pure isolated samples (mean 31.94) were significantly higher (difference: mean 1.06, SD 1.29, $p < 0.05$) than those for magnetic-capture isolated samples (mean 30.88).

Quantification of parasites

Observed Cp-values for samples with known *T. gondii* DNA or tachyzoite-concentration were used to fit a generalized linear model (Fig. 2.2). The best fitting model was described by $Cp = 43.88 - 13.759\ type - 3.413 \log_{10}(concentration)$, in which $type = 1$ for pg DNA and $type = 0$ for tachyzoites spikes. The intercept with the y axis (43.88, 95% CI: 43.427; 44.332), decreased by 13.759 (95%CI: -14.194;-13.324) if DNA spikes were used. The slope (-3.413, 95% CI: -3.530; -3.295) was equal for tachyzoites and DNA spikes as the interaction term between type and Cp-value was not significant (95% CI:-0.429; 0.131) and did not contribute to the fit of the model.

The rewritten formula ($\log_{10}$(*tachyzoites*) = (43.88 – *Cp*)/3.413) can be used to estimate the number of tachyzoite-equivalents from the Cp-value in field samples. The graph (Fig. 2.2) shows that the variation in Cp-value increased as concentration of the spike decreased. Therefore, the uncertainty in the calculated number of tachyzoites increases at higher Cp-values.

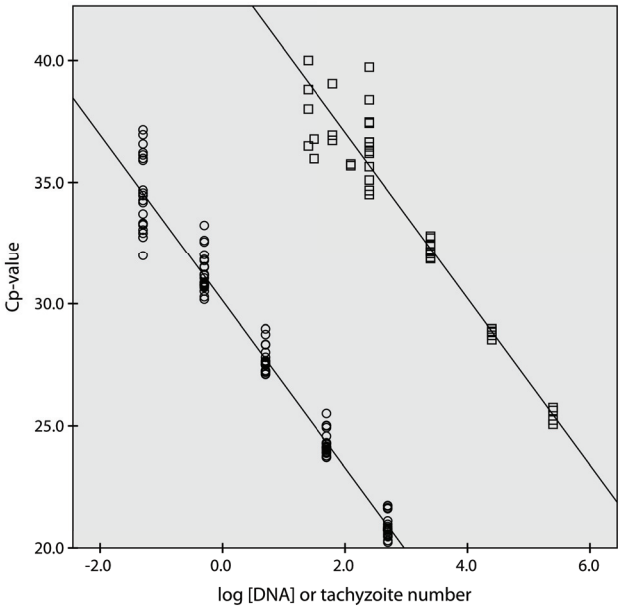

Figure 2.2 Plots of $\log_{10}$ values for the amount of *T. gondii* DNA (pg) added to PCR-mixes (circles) or the number of tachyzoites added to 100 g meat samples (squares) against Cp-values. Circles and squares indicate observed values. The lines show the results predicted using a generalized linear model (Cp = 43.88 – 13.759 *type* – 3.413 $\log_{10}$(*concentration*), in which *type* = 1 for pg DNA and *type* = 0 for tachyzoites spikes).

Magnetic capture PCR on samples from experimentally infected pigs

For six pigs a comparison with bioassay was made. As the tenderloin samples for MC-PCR of animal 1, 2 and 3 were too large (> 140 g) to process as one, they were subdivided into two portions that were tested simultaneously. Results using the MC-PCR were comparable to the bioassay results, with the exception that a different type of tissue tested positive for animal 2 (Table 2.2). All samples from the negative control pigs tested negative in both assays. CIAC was positive for all *T. gondii* PCR-negative samples. For positive samples, the Cp-value was used to calculate the number of parasites. These calculated numbers of parasites were divided by the amount of sample used to calculate the number of parasites per gram (Table 2.2).

In addition, hearts and tenderloins of 37 experimentally infected pigs and 6 negative control pigs were tested by MC-PCR. Out of the 37 infected pigs, 33 tested positive (for 5 pigs heart

only, for 28 pigs heart and tenderloin) (sensitivity 89.2%, 95% CI 79.2–99.2%). All 6 negative control pigs tested negative (specificity 100%).

Table 2.2 Comparison of bioassay (bio) results with MC-PCR (PCR) results and parasite load for experimentally infected pigs.

Pig		Brain	Heart	Tenderloin	Abdominal muscle	Dorsal muscle	Scapular muscle	Diaphragm	Tongue
1	bio	P[c]	P	P	N	np[e]	np	np	np
	PCR	P	P	P/N	N	N	N	P	P
	g[a]	21	130	81/87	104	106	102	102	102
	p/g[b]	1234	52	2/-	-[f]	-	-	1	44
2	bio	N[d]	P	N	N	np	np	np	np
	PCR	N	N	P/N	N	N	N	N	N
	g	30	139	96/101	105	104	102	100	102
	p/g	-	-	1/-	-	-	-	-	-
3	bio	N	P	N	N	np	np	np	np
	PCR	np	P	N/N	N	N	N	N	N
	g		92	98/97	102	104	102	101	101
	p/g		26	-/-	-	-	-	-	-
4	bio	N	N	N	N	np	np	np	np
	PCR	N	N	N	N	N	N	N	N
	g	23	83	132	103	101	102	100	100
	p/g	-	-	-	-	-	-	-	-

[a] g: grams of tissue for MC-PCR; [b] p/g: calculated number of parasites per gram; [c] P: positive; [d] N: negative; [e] np: not performed; [f] none detected

Comparison of magnetic capture PCR and serology in naturally infected sheep

Correlation between logODc-value and qualitative MC-PCR results

To evaluate the ability of MC-PCR to detect *T. gondii* in field samples hearts from slaughtered sheep were tested. Results were compared to ELISA results. Twenty-six sheep with high ODc-values (> 0.226) tested positive in MC-PCR (26/32 = 81.3%), whereas only one sheep with a low ODc-value (< 0.108) tested positive (1/23 = 4.3%). In the intermediate ODc-group, seven sheep tested positive (7/18 = 38.9%). CIAC was positive for all *T. gondii*-PCR negative samples.

To determine whether the probability of a positive PCR (P) can be predicted from logODc-value, a logistic regression model ($\ln(P/(1\text{-}P)) = 3.071 + 5.268\ logODc$) was fitted (Fig. 2.3). To check the fit of the model, six logODc-value categories were constructed. The observed probabilities for these six categories were plotted with the predicted probability per sheep (Fig. 2.3). Observed and predicted probabilities were close, indicating that the model fitted adequately. In addition, the Hosmer and Lemeshow test was not-significant (χ^2 (df = 8) is 11.031, p = 0.20). The PCR outcome (pos/neg) could be predicted accurately from the logODc-value using the model (ROC-curve analysis AUC = 0.897 (95% CI: 0.823–0.971)).

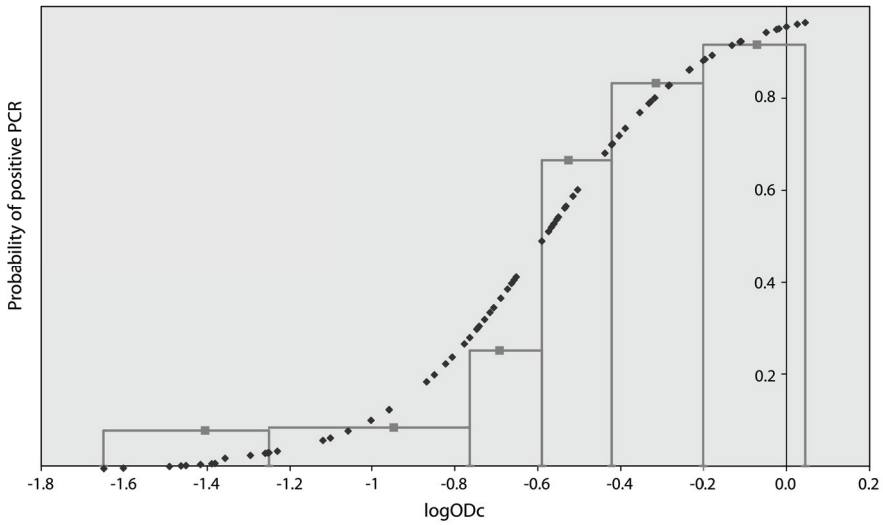

Figure 2.3 The relationship between the probability of positive PCR and the anti-*T. gondii* antibody concentration in sheep; Squares: observed probabilities per logODc-value category, with categories indicated by the widths of bars; Diamonds: probabilities predicted by the logistic regression model ($\ln(P/(1-P)) = 3.071 + 5.268\ logODc$).

Correlation between logODc-value and quantitative MC-PCR results

In order to study the correlation between logODc-value and Cp-value, a linear regression model ($Cp = 29.8 - 4.6\ logODc$) was fitted to the data from the 34 PCR-positive sheep (Fig. 2.4). The antibody concentration, as indicated by logODc-value, explained only a small proportion of the variation in Cp-value, a proxy for the number of parasites present in the heart (adjusted $R^2 = 0.139$). Nevertheless, the Cp-value decreased significantly as logODc-value increased (the 95% confidence interval for the slope ranges from -8.4 to -0.87).

GRA6 genotyping

For all 34 *T. gondii* positive samples from slaughtered sheep the GRA6 capture and PCR was performed. The GRA6 PCR was positive for all thirteen samples that had a Cp-value below 30 in the MC-PCR, and for four out of ten that had a Cp-value between 30 and 33. None of the eleven samples with a Cp-value above 33 were positive in GRA6 PCR. Sequencing was successful for sixteen out of seventeen GRA6-PCR positives. The sequences were aligned with all available GRA6 sequences from GenBank (July 2009). Our sixteen sequences were unambiguously identified as *T. gondii* type II. Fourteen isolates (GenBank GU325790) were identical to the BEVERLEY (GenBank AF239284) and ME49 (GenBank AF239285) strains (Fazaeli et al., 2000). Two showed one G to A replacement (GenBank GU325791). Nine samples that had a Cp-value below 30 after magnetic capture were sent to Limoges, as they also had a Cp-value below 30 for the DNA extract isolated using Roche High Pure PCR template preparation kit. Two samples were not amplified in any of the reactions; the other seven were classified as type II (Table 2.3), which confirmed our typing result.

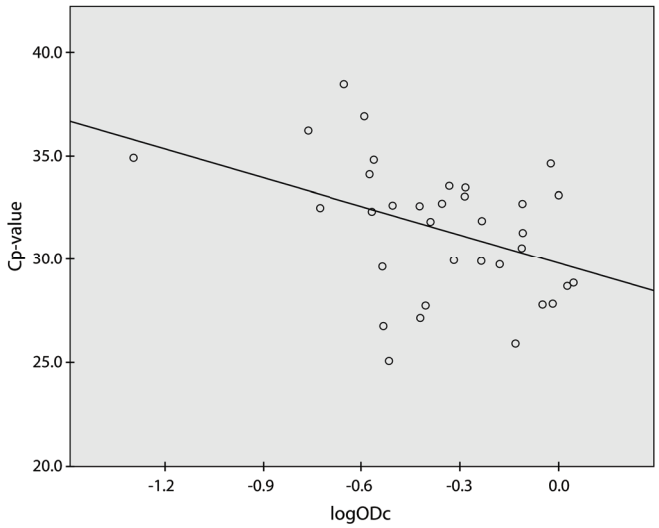

Figure 2.4 Correlation between anti-*T. gondii* antibody concentrations and Cp-values for sheep positive for *T. gondii* by PCR; Circles: observed values; the line was fitted by linear regression (*Cp* = 29.8 - 4.6 *logODc*).

Table 2.3 Cp-values and genotyping results for slaughtered sheep that were positive in MC-PCR with a Cp-value below 30.

Cp-value		GRA6	Microsatellite markers						
MC[a]	HP[b]	Type	Type	TUB2[c]	W35[c]	TgM-A[c]	B18[c]	B17[c]	M33[c]
25.07	26.82	II	II	2.3	2.3	2	2	2.3	1.2
25.91	27.45	II	II	NA[d]	2.3	2	2	2.3	1.2
26.75	28.96	II	II	2.3	2.3	NA	2	2.3	1.2
27.14	27.75	II	II	2.3	2.3	2	2	2.3	1.2
27.74	29.74	II	II	2.3	2.3	NA	2	2.3	1.2
27.79	29.49	II	II	2.3	NA	NA	NA	NA	1.2
27.83	29.93	II	II	2.3	2.3	NA	NA	2.3	1.2
28.69	33.75	II	np[e]	-[f]	-	-	-	-	-
28.85	30.79	II	np	-	-	-	-	-	-
29.63	29.15	II*	NA	NA	NA	NA	NA	NA	NA
29.74	32.23	II	np	-	-	-	-	-	-
29.90	29.73	II*	NA	NA	NA	NA	NA	NA	NA
29.94	30.99	II	np	-	-	-	-	-	-

[a] MC: isolated using magnetic capture; [b] HP: isolated using Roche High Pure PCR template preparation kit; [c] Allelic polymorphisms of markers TUB2, W35, TgM-A, B18, B17 and M33 are expressed in numbers relative to classical typing of *T. gondii* (allele 1, 2, 3 are reserved for clonal lineages I, II and III; allele 1.2 means that types I and II share the allele; allele 2.3 means that types II and III share the allele); [d] NA: not amplified; [e] np: not performed; [f] no data obtained; II*: these two isolates showed a G to A replacement compared to the other GRA6 type II isolates.

Discussion

This paper describes a PCR-based assay for detecting and genotyping *T. gondii* tissue cysts in hundred gram meat samples using sequence-specific magnetic capture of *T. gondii* DNA followed by real-time PCR targeting the 529-bp repeat element.

To develop this method, first a sensitive real-time PCR for the 529-bp repeat element (Reischl et al., 2003) was adapted to the available PCR-system and a competitive internal amplification control was added. The detection limit of 16 fg corresponds to 0.14 tachyzoite-equivalents as the DNA stock contained 112 fg per tachyzoite. This detection limit is comparable to the detection limit of 20 fg or 0.25 parasite-equivalents reported previously (Reischl et al., 2003), and demonstrates that the PCR is sensitive. Although specificity of the PCR primers and probe used has not been confirmed by large scale screening of DNA from other sources, it is expected to be high, especially in combination with capture using 70bp long capture oligonucleotides, as the 529-bp repeat element is not present in DNA from human, mouse, *Echinococcus granulosus*, *Giardia duodenalis*, *Plasmodium falciparum*, *Sarcocystis* spp., *Trichinella spiralis*, *Trichomonas vulgaris* and *Neospora caninum* (Homan et al., 2000).

Next, the protocol for sequence-specific DNA extraction of the 529-bp repeat element using magnetic capture was developed, and the complete MC-PCR procedure was optimized and evaluated using spiked samples. The sensitivity was substantially increased by removal of free biotin and the use of long capture-oligonucleotides (70 instead of 20 bp long). The detection limit of the optimized MC-PCR protocol is approximately 230 parasites per 100 g of meat sample. Because older tissue cysts may contain hundreds of bradyzoites (Dubey et al., 1998), in most cases one cyst per 100 g should be detectable. In addition, spiking of different numbers of tachyzoites showed that the method can be used quantitatively. That is, the number of tachyzoite-equivalents present can be estimated, although the number of tissue cysts they derive from remains unknown.

The detection limit was determined using free tachyzoites. However, since infected animals carry tissue cysts containing bradyzoites, this might have an effect on efficiency of the method, and therefore the MC-PCR was additionally evaluated using samples from infected animals. First, the MC-PCR was compared to bioassay in mice by testing samples from four experimentally infected pigs. Because only a limited number of samples were available, no statistical analysis was performed. However, results suggest that the sensitivity of the MC-PCR is comparable to that of bioassay in mice. For one pig (number 2) the heart was positive in bioassay, whereas the tenderloin was positive in the MC-PCR. As different portions were tested this was not unexpected; particularly as only one tissue was positive and the calculated number of parasites per gram was low, which means that this animal probably had a low level of infection. Parasites could not be detected by bioassay or MC-PCR in any of the samples from animal 4, even though the animal did show a serological response after experimental infection. This indicates that the sensitivity of both direct assays is below 100%. As only four pigs were tested, it is difficult to draw conclusions about the distribution of parasites but the heart appears to be a predilection site as reported previously (Dubey, 1988). The results show that the MC-PCR is a valuable tool in determining the tissue distribution of parasites. Testing an additional 37 pigs that were experimentally infected confirmed that the sensitivity is high (89.2%).

For experimentally infected animals, there is less variation in infectious dose, time after infection, and host and parasite genetics than with naturally infected animals. Therefore, sheep hearts collected from Dutch slaughterhouses were additionally used to evaluate the new method. The MC-PCR results were compared to ELISA results, because the presence of tissue cysts and serological results should correlate, as both *T. gondii* tissue cysts and antibodies against *T. gondii* persist in sheep (Dubey, 2009c). This correlation has been shown for bioassay and MAT-titre (Dubey et al., 2008). A correlation of 100% is not expected, mainly because cysts can still be missed. To increase the probability of detecting *T. gondii* tissue cysts by MC-PCR, the heart was tested, because this is a predilection site of tissue cyst formation in sheep (Esteban-Redondo and Innes, 1998; Esteban-Redondo et al., 1999). Overall, there was a strong correlation between serology and qualitative MC-PCR results. One animal had a low antibody concentration and still tested positive in PCR. Possibly, this animal had a recent infection, in which there were parasites present but the production of antibodies was not yet activated. The six animals that have a high antibody concentration, but tested negative in PCR, may have harboured cysts in other tissues, or at a level below the detection limit of the MC-PCR. The probability of a positive PCR can be accurately predicted from the logODc-value, which means that, for sheep, serological screening can be used to estimate the number of cyst-harbouring animals. The MC-PCR can be used to study this relation for other species. In sheep, the antibody concentration (logODc-value) was related not only to the probability of a positive PCR, but also to the Cp-value or parasite load. The relationship between logODc-value and Cp-value is, however, not strong enough to predict parasite load from the antibody titre, which means that, although serological screening can be used to estimate the number of infected animals, the MC-PCR is needed to determine the parasite load.

The benefit of magnetic capture over isolation of total DNA from a homogenized large sample was assessed by testing two types of DNA isolates in the real-time PCR. Seven out of 41 samples positive after magnetic-capture isolation (17%), tested negative after High Pure isolation from the crude extract. For samples positive after both isolation methods Cp-values were significantly lower for magnetic-capture isolated samples. This confirms that, in addition to homogenizing a large sample to increase the chance of having a tissue cyst present, sequence-specific magnetic capture does further increase sensitivity, probably by concentrating the *T. gondii* DNA and removal of PCR inhibitors.

Since the options for genotyping are limited after sequence-specific capture on the 529-bp repeat element, a capture protocol for the GRA6 gene was developed additionally. Only seventeen out of 34 samples positive in MC-PCR tested positive for GRA6. This demonstrates that the GRA6 capture and PCR is less sensitive than the MC-PCR. A lower sensitivity for GRA6 was expected as the GRA6 gene is single copy (Lecordier et al., 1995), whereas the 529-bp repeat element is repeated more than 300-fold (Reischl et al., 2003). Nevertheless, being able to type approximately 50% of the positive samples is sufficient to get an idea of the genetic diversity of *T. gondii* within a population. Depending on the aim of typing, the capture and sequencing protocol could be extended with more genes to further differentiate isolates. Again, the magnetic capture procedure increased sensitivity compared to isolation of total DNA from a fraction of the crude extract: More samples could be typed on GRA6 after magnetic capture than with microsatellite typing of the High Pure purified extracts. The finding that all *T. gondii* isolates from sheep were type II is in agreement with previous findings for *T. gondii* isolates from French sheep (Dumetre et al., 2006; Halos et al., 2010) and wild boar (Richomme et al., 2009).

The MC-PCR was developed as a method that can be used to compare the public health risks from different meat sources by comparing the parasite load in meat samples. As the method is quantitative, other applications are possible. To give examples, it could be used to determine the predilection site for *T. gondii* tissue cyst formation in different animals, or to study the effects of infectious dose or administration route on parasite load in experimental infections. However, as hundred gram samples are used, the applicability of the method to human diagnostics is limited. Nevertheless, using MC-PCR can be considered when placenta or post-mortem samples are tested.

Although the costs for the materials used for magnetic capture are high compared to conventional DNA isolation methods, they are less than the costs for bioassay. In addition, MC-PCR samples can be stored frozen until processing, which allows more efficient planning of testing, and thereby reduces labour costs. However, if necessary, samples can be tested without storage and results can be available within two days, whereas the time required for bioassay depends on the time for clinical or antibody responses to appear in mice or oocyst shedding to occur in cats.

In summary, a method that can be used to detect and genotype *T. gondii* in meat or tissue samples was developed. As the MC-PCR is quantitative it would be preferable to bioassay when quantification of the parasite burden is essential. The technique is suitable for the first step in an exposure assessment. That is, for screening and comparing *T. gondii* levels in different types of meat. Use of the technique for exposure assessment will contribute to the development of effective strategies to prevent human infections and improve public health.

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Chapter 3

Evaluation of ELISA test characteristics and estimation of *Toxoplasma gondii* seroprevalence in Dutch sheep using mixture models

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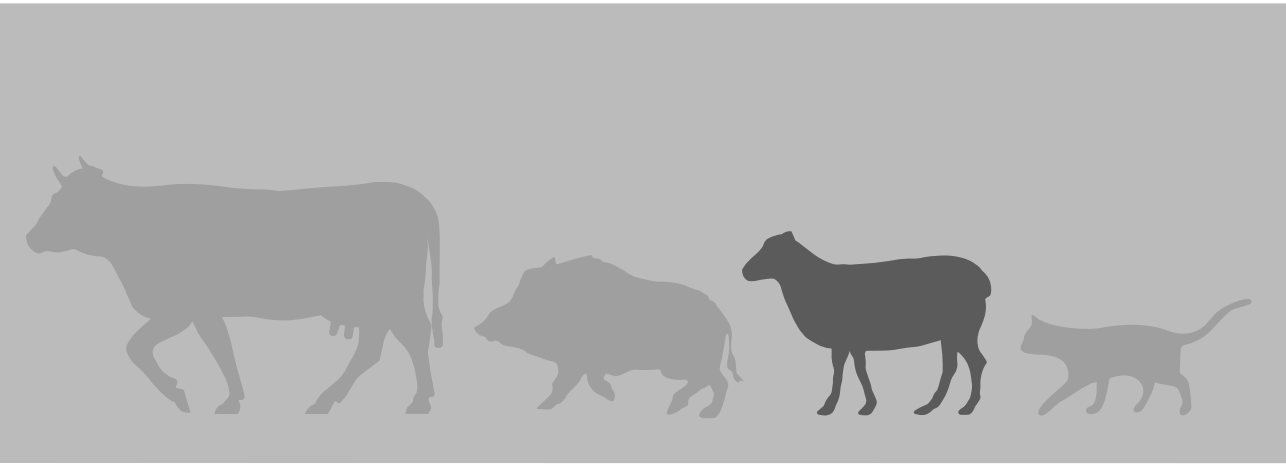

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Lamb and mutton are considered important sources of human *Toxoplasma gondii* infections, but actual data on the prevalence of *T. gondii* in sheep in The Netherlands is lacking. The aim of this study was to investigate the prevalence of *T. gondii* in slaughtered sheep to get more insight in the importance of sheep as a source of human infection. In addition, regional variation in prevalence was studied, as this may indicate differences in environmental contamination. An in-house ELISA that detects antibodies against *T. gondii* was developed and used to test 1179 sera collected from sheep presented at 11 Dutch slaughterhouses between October and December 2007. Since validation of the serological assay was hampered by a lack of appropriate reference sera, the diagnostic performance and seroprevalence were estimated by fitting a binormal mixture model. ROC-curve analysis on the fitted distributions showed high discriminatory power (AUC = 0.995), and high sensitivity and specificity of the ELISA. The overall prevalence was estimated at 27.8% (25.6-29.9%), but was significantly higher in sheep over 1 year old, and in sheep from the central provinces. The high sensitivity and specificity of the in-house ELISA were confirmed by Bayesian analysis together with three commercially available assays: Toxo-Screen DA (bioMérieux), Chekit Toxotest Antibody ELISA (IDEXX), and Toxoplasmosis serum screening ELISA (Institut Pourquier). In conclusion, the binormal mixture model proved a useful method to obtain estimates of diagnostic performance and seroprevalence without use of reference sera. The seroprevalence in sheep was high, and as sheep with antibodies usually carry tissue cysts, this indicates that undercooked lamb and mutton may indeed be important sources of human toxoplasmosis in The Netherlands.

Introduction

Toxoplasma gondii is a protozoon that belongs to the Phylum of Apicomplexa and has a worldwide distribution. Its sexual reproduction takes place in the intestine of the definitive host: members of the family of Felidae, for example domestic cats. Asexual multiplication can take place in a wide-range of intermediate hosts: virtually all warm-blooded animals including humans can be infected. People can get infected with *T. gondii* by ingesting raw or undercooked meat, for example lamb and mutton, containing tissue cysts; by ingesting cat-shed oocysts via contaminated soil, food or water; or congenitally by transplacental transmission of tachyzoites. It has been shown that the overall seroprevalence of *T. gondii* for the Dutch human population was 40.5% but significantly higher in the Northwest in 1995/1996 (Kortbeek et al., 2004). Recently, a similar regional pattern but an overall decreased prevalence of 26.0% was shown for 2006/2007 (Hofhuis et al., 2011). Nevertheless, the incidence of congenital toxoplasmosis in newborns is high: 2 per 1,000 live births (Kortbeek et al., 2009). Based on this incidence estimate, the disease burden of congenital toxoplasmosis is similar to that of the major foodborne pathogen *Campylobacter* (Kortbeek et al., 2009). This high disease burden urges the need for intervention measures and, as the effectiveness of treatment is under discussion (Gilbert, 2009; Thiebaut et al., 2007), the focus is on prevention. To set up specific prevention strategies the relative contribution of the different transmission routes needs to be clarified. Concerning foodborne transmission, the prevalence of *T. gondii* in food animals indicates whether the species can be an important source of infection, although this will still depend on the amount of meat eaten unfrozen and undercooked. This study focuses on the role of sheep.

Sheep are readily infected (Munday, Corbould, 1979) and *T. gondii* is an important cause of abortion (Buxton et al., 2007; Dubey, 2009c). Although it has been suggested that subsequent vertical transmission might be important in sheep (Duncanson et al., 2001; Hide et al., 2009; Morley et al., 2007; Williams et al., 2005) most infections are acquired postnatally through exposure to oocysts in the environment (Innes et al., 2009a). Therefore, differences in *T. gondii* prevalence in sheep may indicate differences in environmental contamination with oocysts. Because both antibodies to *T. gondii* and tissue cysts persist in infected sheep (Dubey, 2009c), sheep carrying parasites usually have detectable antibody levels (Dubey et al., 2008). This implies that serological tests, which are less time consuming and expensive than bioassay or PCR, can be used to estimate the number of animals carrying *T. gondii* tissue cysts in the meat and thereby indicate the risk for public health. This implication holds true only if the serological results are reliable. However, proper evaluation of diagnostic performance of serological tests for *T. gondii* in sheep is thus far limited to one study (Mainar-Jaime, Barberan, 2007).

Limited data show that in The Netherlands in 1982 30% of 115 slaughterhouse sheep were seropositive (van Knapen et al., 1982). However, more recent data on the seroprevalence of *T. gondii* in Dutch sheep are lacking. The objective of this study was to reliably determine the seroprevalence of *T. gondii* in Dutch sheep to get an indication of the potential role of lamb and mutton as sources of human infection in The Netherlands. In addition, regional variation in seroprevalence was studied as it may indicate variation in environmental contamination.

Materials and Methods

Study population

In 2007 the Dutch sheep population consisted of 645,000 ewes, 34,000 rams and 691,000 lambs (CBS, 2007a). The majority of these sheep is kept for meat production (Anon., 2002). They are grazed almost year round and only kept indoors during the lambing season (February and March) (Anon., 2002). In Dutch sheep *T. gondii* is an important cause of abortion: approximately 30% of the aborted foetuses submitted to the Animal Health Service in 2004 and 2005 were *T. gondii* positive (Piet Vellema, Animal Health Service Deventer, personal communication). Vaccination is, however, very uncommon in The Netherlands (Theo Schetters, Intervet, personal communication). In The Netherlands mostly lambs (< 1 year old) are slaughtered: in 2007 82.5% (620,700) of all slaughtered sheep (752,300) were lambs (CBS, 2007b). Data on sheep slaughtered per slaughterhouse are available at the Food and Consumer Product Safety Authority only from 2008 onwards. In 2008 487,000 sheep were slaughtered at 18 slaughterhouses that slaughter over 10,000 sheep per year, 109,000 sheep at 24 slaughterhouses that slaughter between 1,500 and 10,000 sheep per year, and 26,000 sheep at 129 butchers that slaughter less than 1,500 sheep per year.

The study population consists of a sample of the sheep presented at 11 sheep slaughterhouses that were willing to cooperate between October and December 2007. With a population size of 752,300, an expected seroprevalence of 30%, an accepted error of 3%, and a confidence level of 95% the necessary sample size was calculated at 897 using WinEpiscope 2.0 (Thrusfield et al., 2001). A total of 1179 sheep serum samples was collected. Per slaughterhouse sampling was performed on between 1 and 8 days. On those days all sheep presented consecutively were sampled although the number of blood samples per farm was limited to five. Age (under or over 1 year old) was reported by the slaughterhouse and information on originating postal code area was collected from the identification and registration database using the ear tag numbers. According to the seroprevalence studies for the Dutch population (Kortbeek et al., 2004; Hofhuis et al., 2011) postal codes were classified into five regions based on the Dutch provinces (Fig. 3.1): 'Central' (Utrecht and Gelderland), 'Southeast' (Noord-Brabant and Limburg), 'Northwest' (Noord-Holland and Flevoland), 'Southwest' (Zeeland and Zuid-Holland) and 'Northeast' (Groningen, Drenthe, Overijssel and Friesland).

Reference sera

Sera from sheep before, weeks after and months after vaccination with Toxovax® were kindly provided by Intervet (Intervet/Schering-Plough Animal Health, Boxmeer, The Netherlands). These sera were used to optimize the RIVM in-house ELISA protocol by checkerboard titrations. For external validation of the in-house ELISA 97 serum samples from Romanian sheep that had been tested using three commercial tests were tested. These sera were collected August 2007 at one farm in Satu Mare County (Romania). Out of 250 adult sheep and almost 100 lambs present, 49 adults and 48 lambs born that year were selected randomly. These sheep were of local Turcana breed.

Figure 3.1 Map of The Netherlands divided into twelve provinces.

Serological assays

Presence of antibodies against *T. gondii* was determined using the in-house developed indirect ELISA (RIVM ELISA). Briefly, 96-well microtitre plates were coated with 100µl per well of 3.4 µg/ml saponin-octylglucoside solubilized *T. gondii*-RH-antigen (Hughes et al., 1982) in 0.1 M Na_2CO_3 solution (pH 9.6) by incubation for 1h at 37°C, washed four times with washing buffer (PBS-T: 0.07 M phosphate buffered saline (PBS) with 0.05% Tween 20), and tapped dry. Plates were then blocked by incubation for 1h at 37°C with 125 µl 1% bovine serum albumin (BSA) in PBS per well, and washed. Sera were 1:100 diluted in 1% BSA in PBS-T, and per sample 100 µl of serum dilution was added to two wells. Plates were incubated for 1h at 37°C, washed, and incubated with 100 µl 1:6000 diluted conjugate (Horseradish-peroxidase labeled polyclonal Rabbit Anti-Sheep Immunoglobulins, Dako, Heverlee, Belgium) per well. Washing was repeated, a 100 µl of substrate (Sure Blue™ TMB Microwell Peroxidase Substrate, KPL, Gaithersburg, MD, USA) was added to each well, and the plates were incubated at room temperature. The reaction was stopped after 10 min by adding 100 µl of 2 M H_2SO_4. The optical density (OD) at 450nm was read using a microplate reader (EL808, Ultra Microplate Reader, Bio-Tek Instruments, Bad Friedrichshall, Germany).

Six pools of 5 sera with comparable OD-values were made. These serum pools differ in OD-value (from 0.03 to 1.25) and were tested, next to two serum blank samples, in duplicate as controls on each plate. OD-values were corrected for blank measurement and plate-to-plate variation. First, the average OD-value for the blank controls on a plate was subtracted from the OD-values of the sera on that plate. Next, the measured blank-corrected OD-values for the six serum controls on plate x were plotted against their average blank-corrected OD-value over all plates used (Fig. 3.2). Then, the regression line was used to calculate the standardized blank corrected OD values for the test sera on that plate: their ODc-value. Plates were retested if R^2 was less than 0.95 or if duplicates for more than one control had a coefficient of variation (CV = standard deviation of replicates/mean of replicates) above 20%. Individual sera were retested if CV was above 20%, although for sera with OD-values below 0.1 any CV was accepted as long as both OD-values are below 0.1. As some of the ODc-values were negative a standard blank value of 0.05 was added for all sera, and all values were subsequently $\log_{10}$-transformed.

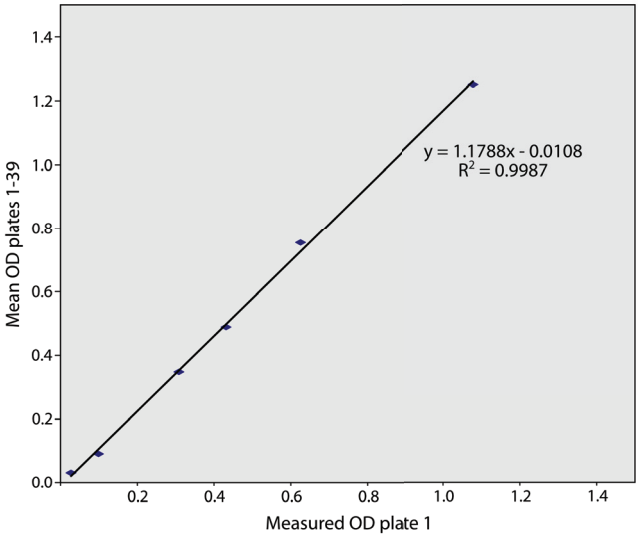

Figure 3.2 Example of correction for plate-to-plate variation in RIVM ELISA for antibodies against *T. gondii* in sheep by linear regression. For each out of 6 controls the mean of the two measured OD-values on the plate is plotted on the x-axis, and the mean OD-value over 39 plates is plotted on the y-axis. The formula for the regression line is used to calculate a corrected OD-value for the test sera on the plate.

The RIVM ELISA was externally validated by comparing the RIVM ELISA results for the Romanian sheep with those obtained using Toxo-Screen DA (Cat. No. 75481, bioMérieux, Marcy l'Etoile, France), Chekit Toxotest Antibody ELISA (Cat. No. TXT1135T, IDEXX Laboratories, Hoofddorp, The Netherlands), and Toxoplasmosis serum screening ELISA (Cat. No. P00710, Institut Pourquier, Montpellier, France) by latent-class analysis. These assays were performed according to the manufacturer's instructions. In IDEXX ELISA both weak positives (>30% reactivity) and positives (>100% reactivity) were considered positive.

In Pourquier ELISA sera with more than 50% reactivity were considered positive. In Toxo-Screen DA sera were tested at 1:40 and 1:4000, sera positive at either dilution were considered positive.

Data analyses

Estimation of seroprevalence in Dutch sheep

Since antibody titres are usually lognormally distributed (Jacobson, 1998; Thrusfield, 2005), ODc-values were log-transformed to allow analysis assuming normal distributions. Under the assumption that the sampled Dutch population consists of a mixture of seropositive and seronegative sheep, the frequency distribution of log-transformed ODc-values should show two components. These components are both assumed to be normally distributed but with different mean and standard deviation: a higher mean is assumed for the positive population. Under these assumptions, a binormal mixture model is fitted to the observed frequency distribution based on maximum likelihood in Mathematica (v7.0, Wolfram Research, Champaign, IL, USA). The model estimates two means, two standard deviations and the mixing parameter. The mixing parameter gives the estimated prevalence, and is equivalent to the area under the curve of the positive distribution divided by the total area under both curves. A 95% confidence interval for the prevalence was constructed using Markov Chain Monte Carlo (MCMC) simulation.

Determination of cut-off value

To study regional differences in seroprevalence and to compare RIVM ELISA results to the results with other serological assays, individual animals needed to be scored positive or negative based on their ODc-value. To obtain a cut-off value a receiver-operating characteristic (ROC) curve was constructed based on the distributions from the binormal mixture model. The ODc-value at which the number of animals scored correctly is maximized, was used as cut-off value. This optimum cut-off was determined by maximizing the sum of sensitivity and specificity using the two components of the binary distribution mixture. In addition, the area under the curve (AUC) for the ROC-curve was calculated as a measure for the discriminatory power of the RIVM ELISA (Greiner et al., 2000).

Comparison of seroprevalence by region

Results with RIVM ELISA were classified as positive and negative using the selected cut-off value. As seroprevalence increases with time spent on pasture, sampling date could be a confounder, and it was tested whether it should be included in the model. As ages may not be equally distributed over the sampling dates, sampling date was checked for multicollinearity with age before entering in the model. Sampling date was recoded into three 1 month intervals. After univariable analysis with serological status dependent on region, multivariable logistic regression analysis with serological status of the animal as dependent variable and region as a categorical explanatory variable will, adjusted for age and, if necessary, sampling date, show whether significant regional differences exist. If crude odds-ratios from univariable analysis change by more than 20% in multivariable analysis, the added variable was considered a confounder and left in the final model. Odds-ratios and their 95% confidence interval based on likelihood ratio statistics are reported. All analyses were performed in SPSS 17.0 (SPSS Statistics, Chicago, IL, USA).

Test evaluation

Since test characteristics from the ROC-curve analysis are based only on the results with the RIVM ELISA itself, a low analytic sensitivity or cross-reactivity might not be identified. For that reason the test was additionally validated externally. As no gold standard test or sufficiently large number of appropriate reference sera were available, the sensitivity and specificity of the RIVM ELISA were estimated using a latent-class model with two subpopulations and 4 tests implemented with Bayesian methods in WinBUGS (Lunn et al., 2000). Since all four tests are detecting the antibody response to a *T. gondii* infection they probably are conditionally dependent. That is, the probability of a positive test result for test 2 is not equal over the 2 possible outcomes of test 1. Ninety-seven serum samples from Romanian sheep were tested: 49 came from adult sheep and 48 from lambs. Because the *T. gondii* prevalence in sheep increases with age, adult sheep and lambs are considered different populations. Therefore a conditional dependence model for four tests in two animal populations was used. Both the logistic regression parameterization and the multinomial parameterization of a previously published model (Engel et al., 2006) were modified to consider four tests and two populations. In both models sensitivity and specificity per test are assumed equal for the two populations. By default, wide normal priors (N(0.0,0.5)) were used for logit-transformed conditional sensitivities and specificities of the tests, and positive valued wide gamma priors (Gamma(0.48,1.0)) were used for the log odds-ratios that model for conditional dependence between tests. Priors on logit-transformed conditional sensitivity and specificity of the RIVM ELISA were further restricted to values above 0, as the Bayesian model initially gave the mirror image of the expected results (prevalence as 1-prevalence, sensitivity as 1-specificity, and specificity as 1-sensitivity) (Engel et al., 2006). For the prevalences uniform priors that exclude only extreme values (U(0.01,0.99)) were used. By default, tests were entered in the following order: RIVM, IDEXX, Toxo-Screen, and Pourquier. To incorporate prior knowledge into the models previous estimates of sensitivity and specificity for Toxo-Screen (Se 92.6% (85.2-96.9), Sp 95.5% (89.9-98.7)) and IDEXX ELISA (Se 90.5% (83.4-95.6), Sp 97.8% (94.2-99.5)) (Mainar-Jaime, Barberan, 2007) were used to set informative prior distributions. As test characteristics are modelled on logit scale, reported estimates for lower and upper limit were first logit-transformed. The mean for these two logit-transformed limits was used as mean for a normal prior distribution. To obtain the precision ($1/sd^2$) for the normal prior distribution, the difference between these two logit-transformed limits was divided by 2×1.96 to obtain the standard deviation (sd). As the logistic regression model assumes a certain order of tests, the influence of test order was examined by varying entry of the three commercial tests: RIVM ELISA was always entered first as this was our main interest. For the multinomial model, which does not allow missing values, the data for one adult sheep had to be omitted. A burn-in of 4000 iterations was discarded, and estimates were based on the next 50,000 iterations. Chain stability was assessed by visual checking of the history plot after 1,000,000 iterations, and comparison of several chains of 50,000 iterations. Model convergence was assessed by visual checking of the kernel density and history plots.

Table 3.1 Location, size, number of sheep and postal codes sampled per region, and number of sheep sampled per month for 11 Dutch slaughterhouses contributing 1179 sheep serum samples for *T. gondii* seroprevalence.

Slaughterhouse			Region							Sampling month		
	Location	Size[b]	Total	NE	C	NW	SW	SE	NA[a]	Oct	Nov	Dec
			samples (postal codes)						samples	samples		
1	SE	medium	157 (59)	21 (16)	17 (9)	0 (0)	9 (5)	81 (29)	29	144	13	0
2	SE	medium	156 (50)	99 (35)	15 (9)	2 (2)	1 (1)	5 (3)	34	156	0	0
3	NW	medium	150 (56)	21 (7)	12 (3)	40 (14)	55 (28)	6 (4)	16	0	150	0
4	C	large	150 (105)	97 (79)	16 (13)	16 (8)	5 (4)	2 (1)	14	0	25	125
5	SW	large	149 (43)	22 (7)	43 (14)	30 (10)	34 (10)	10 (2)	10	0	0	149
6	C	large	149 (44)	105 (37)	11 (5)	3 (2)	0 (0)	0 (0)	30	0	149	0
7	C	medium	124 (43)	24 (15)	67 (19)	9 (5)	3 (3)	5 (1)	16	0	54	70
8	C	small	31 (13)	15 (8)	8 (4)	2 (1)	0 (0)	0 (0)	6	31	0	0
9	C	small	25 (14)	6 (4)	15 (8)	1 (1)	2 (1)	0 (0)	1	25	0	0
10	C	medium	10 (2)	0 (0)	5 (1)	0 (0)	0 (0)	5 (1)	0	10	0	0
11	C	small	5 (1)	0 (0)	5 (1)	0 (0)	0 (0)	0 (0)	0	5	0	0
NA[a]	NA[a]	NA[a]	73 (13)	8 (5)	10 (6)	0 (0)	0 (0)	2 (2)	53	5	23	45
Total			1179 (443)	418 (213)	224 (92)	103 (43)	109 (52)	116 (43)	209	376	414	389

[a] NA: not available; [b] small: 1,500-10,000 sheep/year, medium: 10,000-25,000 sheep/year, large: > 25,000 sheep/year

Results

Descriptive statistics

A total of 1179 serum samples was collected from 11 Dutch slaughterhouses. Seven slaughterhouses were located in the central part of The Netherlands (Table 3.1), which is also the region where almost 50% of all Dutch sheep slaughterhouses is located. Nevertheless, most of the sheep originated from the Northeast (Table 3.1), which is known to be the region with the highest number of sheep (CBS, 2007a). Age was reported for 568 out 1179 sheep. Of these 568, 380 were less than 1 year of age and 188 were above 1 year of age. Postal codes could be obtained for 970 (82.2%) sheep. There were 443 different postal codes (Table 3.1), and although the number of sheep from one postal code varied between 1 and 14 (mean 2.19, median 1.00) 257 postal codes were reported only once, and only 19 postal codes were reported more than five times.

Seroprevalence, cut-off and test characteristics by binormal mixture model

The frequency distribution of the 1179 measured logODc-values clearly showed two components (Fig. 3.3). The binormal mixture that best described the two observed populations was obtained and the fitted distributions were drawn (Fig. 3.3). From the fitted distributions the seroprevalence was estimated at 27.8% (95% confidence interval from MCMC simulation 25.6-29.9%). ROC-curve analysis showed that the test has good discriminatory power (AUC= 0.995) (Fig. 3.4). The optimum cut-off was estimated at a logODc-value -0.569. At this point sensitivity was estimated at 97.8% and specificity at 96.4%.

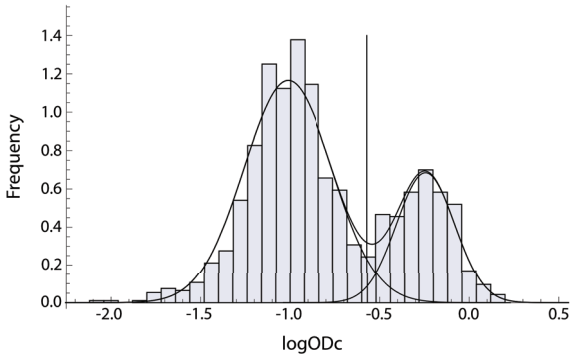

Figure 3.3 Frequency distribution of observed logODc-values in *T. gondii* RIVM ELISA for Dutch sheep (n=1179) (bars) and fitted normal distributions (lines). The vertical line indicates the cut-off value (-0.569) used to classify Dutch sheep as positive or negative for antibodies against *T. gondii*.

Regional variation in seroprevalence

Serological results were dichotomized using the above-mentioned cut-off: 354 out of 1179 sheep (30.0%) scored positive. After correction of this apparent prevalence (*AP*) according to the Rogan-Gladen estimator (*TP = (AP+Sp-1)/(Se+Sp-1)*; Rogan, Gladen, 1978), the true

prevalence (*TP*) was estimated at 28.1%. For 468 sheep both age and postal code was available, hence analysis of regional variation was limited to these sheep. Sampling date and age were highly correlated ($\chi^2 = 64.642$, df = 2, $p < 0.000$): no samples from sheep over 1 year old were collected in December. For that reason, only age was included in the logistic regression model. It was shown that age is indeed an important risk factor, and since it was not homogeneously spread over the regions, also an important confounder. The age-adjusted model shows that seroprevalence is significantly higher in the central provinces (Table 3.2). Dividing The Netherlands into three regions only (North, Central and South) to increase power, showed that not only the higher prevalence in the central region (OR 1.88 (1.11-3.16)) but also the lower seroprevalence in the South (OR 0.54 (0.30-0.96)) was significantly different from the prevalence in the North.

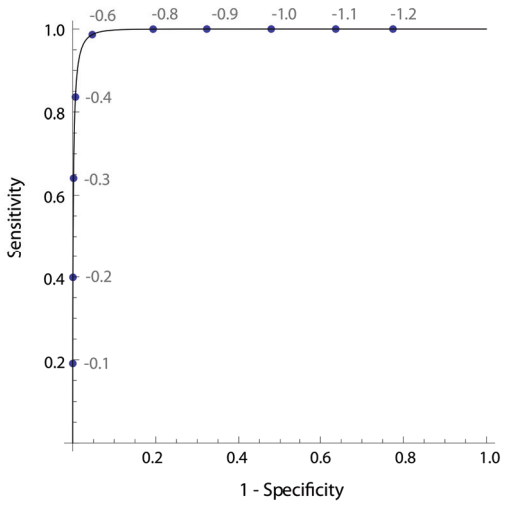

Figure 3.4 ROC-curve for *T. gondii* RIVM ELISA based on the distributions fitted to logODc-values for 1179 Dutch sheep using binormal mixture model. The probability of a false positive (or 1-specificity) on x-axis is plotted against probability of a true positive (or sensitivity) on the y-axis. Accompanying cut-off values (logODc) are indicated along the line.

Table 3.2 Age- and region-specific RIVM ELISA seroprevalence of *T. gondii* in 468 Dutch sheep sampled at 11 slaughterhouses, and odds-ratios for region as a risk factor for RIVM ELISA *T. gondii* seroprevalence.

Region	Age < 1 year	Age ≥ 1 year	Total	Crude OR	Age-adj. OR[a]
	pos/*n* (%)	pos/*n* (%)	pos/*n* (%)		
NE	20/130 (15.4)	24/39 (61.5)	44/169 (26.0)	Reference	Reference
C	32/112 (28.6)	10/15 (66.7)	42/127 (33.1)	1.40 (0.85-2.33)	**1.87 (1.08-3.22)**
NW	6/34 (17.6)	1/2 (50.0)	7/36 (19.4)	0.69 (0.28-1.68)	1.00 (0.39-2.53)
SW	2/27 (7.4)	3/8 (37.5)	5/35 (14.3)	0.47 (0.17-1.30)	0.43 (0.15-1.25)
SE	6/34 (17.6)	25/67 (37.3)	31/101 (30.7)	1.26 (0.73-2.17)	0.57 (0.30-1.07)
Total	66/337(19.6)	63/131 (48.1)	129/468 (27.6)		

[a] age OR 5.76 (3.37-9.87); Statistically significant ORs are printed bold.

Bayesian analysis of test characteristics

A large proportion of Romanian sera (83.5%) tested positive or negative in all four tests. Nevertheless, fewer sera scored positive using Toxo-Screen DA while more sera scored positive using the Pourquier ELISA (Table 3.3). All history plots showed chain stability. The results from the Bayesian analysis using the logistic regression parameterization without prior information indicated that sensitivity (96.4%) and specificity (96.0%) of the RIVM ELISA were high. IDEXX ELISA also had both sensitivity (91.6%) and specificity (97.0%) above 90%. For the agglutination test (Toxo-Screen) a lower sensitivity (75.1%), and for Pourquier ELISA a lower specificity (86.7%) was found (Table 3.4). Adding prior information on sensitivity and specificity of Toxo-Screen and IDEXX ELISA to the model increased the sensitivity of Toxo-Screen (84.7%) and specificity of IDEXX ELISA (98.7%). Estimates for RIVM and Pourquier ELISA were not affected. The model with prior information on test characteristics for Toxo-Screen and IDEXX ran correctly without the constraint on the RIVM sensitivity and specificity prior; this did not affect the estimates, meaning that including this constraint did probably not influence the results in the non-informative model either. Changing the test order had only minor effects, with the largest effect on Toxo-Screen sensitivity. Changing the order to RIVM, Toxo-Screen, Pourquier, IDEXX had the largest effect and estimates are included in Table 3.4. That influence of test order is limited is also indicated by the minor changes in estimates when the multinomial parameterization, in which test order is irrelevant, is used. The multinomial model does indicate a slightly lower prevalence in the adult population and a slightly higher sensitivity of Toxo-Screen, but this is probably caused by omitting the one adult sheep with a missing IDEXX result, as this animal was positive by both RIVM and Pourquier ELISA, but negative by Toxo-Screen (Table 3.3).

Table 3.3 Number of 49 adult and 48 juvenile Romanian sheep with specified combinations of test results on four assays for *T. gondii* antibodies.

RIVM[a]	IDX[b]	TS[c]	PQ[d]	Adult	Young
Pos	Pos	Pos	Pos	29	3
Pos	Pos	Neg	Pos	5	0
Pos	Pos	Neg	Neg	1	0
Pos	Neg	Neg	Pos	2	0
Pos	Neg	Neg	Neg	1	0
Neg	Neg	Neg	Pos	0	6
Neg	Neg	Neg	Neg	10	39
Pos	NA[e]	Neg	Pos	1	0

[a] RIVM: in-house RIVM ELISA; [b] DX: Chekit Toxotest Antibody ELISA (IDEXX); [c] TS: Toxo-Screen DA (bioMérieux); [d] PQ: Toxoplasmosis serum screening ELISA (Institut Pourquier); [e] NA: not available

Discussion

The main objective of this study was to estimate the seroprevalence of *T. gondii* in Dutch sheep to get an indication of their possible role in human *T. gondii* infections. Therefore, an indirect ELISA for the detection of antibodies against *T. gondii* in sheep was developed and validated. The ELISA was subsequently used to test a sample of the Dutch sheep population.

Table 3.4 Median (95% CI) estimates of age-specific *T. gondii* prevalence, and of sensitivity and specificity of four assays for *T. gondii* antibodies in 97 Romanian sheep, estimated using both latent-class logistic and multinomial models, each with two sets of priors.

Parameter	Logistic			Multinomial[a]	
	Default	IDX/TS priors[b]	Test order[c]	Default	IDX/TS priors[b]
Prevalence					
Adult	77.2 (64.0-87.7)	77.1 (63.8-87.6)	77.0 (63.5-87.3)	76.2 (62.7-87.0)	76.1 (62.5-86.9)
Young	7.5 (2.3-17.1)	7.5 (2.3-17.0)	7.4 (2.3-16.9)	7.5 (2.3-16.9)	7.5 (2.3-16.9)
Sensitivity					
RIVM	96.4 (88.6-99.3)	96.4 (88.7-99.3)	96.4 (88.7-99.3)	96.2 (88.3-99.2)	96.2 (88.4-99.2)
IDX	91.6 (80.1-97.7)	92.0 (85.5-95.7)	91.3 (79.2-97.8)	92.2 (81.2-98.0)	91.3 (83.0-95.5)
TS	75.1 (60.3-86.9)	84.7 (74.9-92.0)	76.5 (62.0-87.7)	78.8 (64.8-89.5)	87.0 (77.2-93.2)
PQ	93.2 (82.5-98.3)	93.6 (83.9-98.4)	93.6 (83.6-98.4)	93.8 (84.1-98.4)	93.8 (84.2-98.5)
Specificity					
RIVM	96.1 (88.8-99.2)	95.9 (88.6-99.1)	95.9 (88.5-99.1)	95.7 (88.2-99.1)	95.6 (87.9-99.0)
IDX	97.0 (91.0-99.4)	98.7 (96.0-99.6)	96.9 (90.7-99.3)	97.1 (90.9-99.4)	98.6 (95.9-99.6)
TS	97.0 (91.2-99.4)	97.4 (94.2-99.0)	97.2 (91.3-99.4)	97.2 (91.5-99.4)	97.4 (93.9-99.0)
PQ	86.7 (76.4-94.0)	86.9 (76.4-94.0)	87.0 (76.4-94.0)	87.4 (77.6-94.3)	87.4 (77.2-94.1)

[a] 1 adult sheep was omitted because IDX result was missing; [b] IDX: logit Se N(2.346,7.166), logit Sp N(3.258,3.344), TS: logit Se N(2.596,5.368), logit Sp N(3.258,3.344); [c] order: RIVM, TS, PQ, IDX

But, as no gold standard test or sufficient number of reference sera were available, traditional methods to obtain a cut-off value with estimates of the accompanying test characteristics were inadequate (Jacobson, 1998). Instead, the prevalence was estimated from the frequency distribution of observed logODc-values for the Dutch study population itself, using a binormal mixture model. The model clearly fitted the data, and a precise estimate of seroprevalence was obtained. ROC-curve analysis showed good discriminatory power of the ELISA, and prevalence estimates using the binormal mixture model itself or the cut-off derived from it did not differ significantly. Estimation of test characteristics from the binormal mixture model and the comparison to other tests showed that the RIVM ELISA performed well; indicating that the estimated overall seroprevalence of 27.8% is a reliable estimate. We can, however, not be certain whether we included sheep from all regions proportionally as slaughtering data by sheep origin are not available. But since the slaughterhouses showed wide catchment areas and overall sheep predominantly originated from the sheep-dense Northeast region the selection bias appears limited. The prevalence is in agreement with the apparent seroprevalence of 30% in The Netherlands in 1982 (van Knapen et al., 1982) and it illustrates that mutton and lamb may be important sources of human infection, especially as it has been shown that sheep positive using RIVM ELISA carry tissue cysts (Opsteegh et al., 2010c). The actual risk of a certain animal species as a source of human *T. gondii* infections depends not only on the prevalence of *T. gondii* in the species, but also on the consumed amount of unfrozen and undercooked meat. Although mutton and lamb are not often consumed in The Netherlands (in 2006 1.4 kg/head/year vs. 41.4 kg/head/year for pork; PVE, 2007) it may still be an important source of infection as the seroprevalence in sheep is much higher than the seroprevalence of 0% (van der Giessen et al., 2007) for conventionally raised Dutch pigs.

In The Netherlands, the consumption of mutton and lamb is concentrated in certain ethnic groups (PVE, 2007). The yearly consumption in those groups is much higher than the average 1.4 kg, as may be their risk of infection through sheep meat.

In addition to an overall estimate of seroprevalence, regional variation in seroprevalence in sheep was analysed. Regional variation in seroprevalence was shown for the Dutch population: the highest seroprevalence was observed in the Northwest for people aged 20 years and older (Hofhuis et al., 2011; Kortbeek et al., 2004). Since ethnicity was not an independent predictor of *T. gondii* seropositivity, and urbanization and consumption of raw meat were controlled for in the analysis (Hofhuis et al., 2011), these are less likely explanations for the regional difference. This difference may, however, be related to environmental contamination with oocysts. If regional variation in the level of soil contamination is present it may lead to variation in seroprevalence in sheep as sheep get *T. gondii* infections mainly from ingesting oocysts from the environment (Innes et al., 2009a). Variation in seroprevalence by farm location was shown previously for sheep in Norway, Mexico and France (Caballero-Ortega et al., 2008; Skjerve et al., 1998; Halos et al., 2010), but regional variation for Dutch sheep was not studied previously. Our main goal was to estimate the seroprevalence of *T. gondii* in sheep as a risk for human infection, thus we sampled slaughtered sheep. This limited the number of sheep with information on geographic origin and age, and thereby led to a considerable loss of power to study regional variation. Nevertheless, a significantly higher seroprevalence was found for the central provinces. In general, regional differences in seroprevalence in sheep can result from differences in environmental contamination, or by factors that influence the level of exposure of the sheep to the environment, such as age and farm management. We controlled for confounding by age, but lacked information on farm management. Since larger farms are mostly located in the Northern regions of The Netherlands (LEI and CBS, 2008), it is likely that other farm characteristics also differ by region. Therefore, the regional differences found may be due to a mixture of environmental contamination and farm management. The regional variation found in sheep does not overlap with regional differences found in the human population. A clear overlap, as found in France (Halos et al., 2010), would have strengthened the hypothesis that it is indeed differences in environmental contamination that causes regional variation in seroprevalence in both sheep and humans. But the variation clearly does not overlap, either because region is not a good indicator for environmental contamination in one or both species, or because environmental contamination is not the most important cause of regional variations in seroprevalence. As mentioned, in sheep the regional differences may have been influenced by farm management. For humans consumption of undercooked meat is probably the most important route of infection (Cook et al., 2000), and regional variations therein may not have been fully controlled for as people may not have realized that certain regional meat products are raw (Hofhuis et al., 2011).

Bayesian analysis of test characteristics showed little variation in the estimates between the different models. The posterior estimate for Toxo-Screen sensitivity was influenced by adding prior information on IDEXX and Toxo-Screen test characteristics, because the difference between previously published sensitivity of Toxo-Screen (Mainar-Jaime and Barberan, 2007) and the sensitivity from the model based on only our data was large. Although we used the same protocol and cut-off value as Mainar-Jaime and Barberan (2007), this discrepancy may still be caused by differences in reading the test results as agglutination is scored by an observer. ELISA is less prone to inter-laboratory variation as reading is automated. In our opinion the logistic regression model including the prior information gives the most appropriate

estimates as it is based on the largest dataset. The confidence intervals for the RIVM ELISA test characteristics comprise the estimated characteristics using the binormal mixture model: both analyses showed a high sensitivity and specificity. High test characteristics were also found for the IDEXX ELISA in the Bayesian analysis, but the agglutination test (Toxo-Screen) and Pourquier ELISA showed lower test characteristics. This means that, especially if these two tests are used, and no correction for sensitivity and specificity is applied, seroprevalence may be under or overestimated.

In summary, the seroprevalence of *T. gondii* infection in sheep in The Netherlands was estimated at 27.8%. Regional analysis showed a significantly higher seroprevalence in the central part of the country, which was different from the findings in the human population. The binormal mixture proved a useful method to determine seroprevalence and test characteristics if no large number of reference sera is available.

Acknowledgements

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Chapter 4

Low predictive value of seroprevalence of *Toxoplasma gondii* in cattle for detection of parasite DNA

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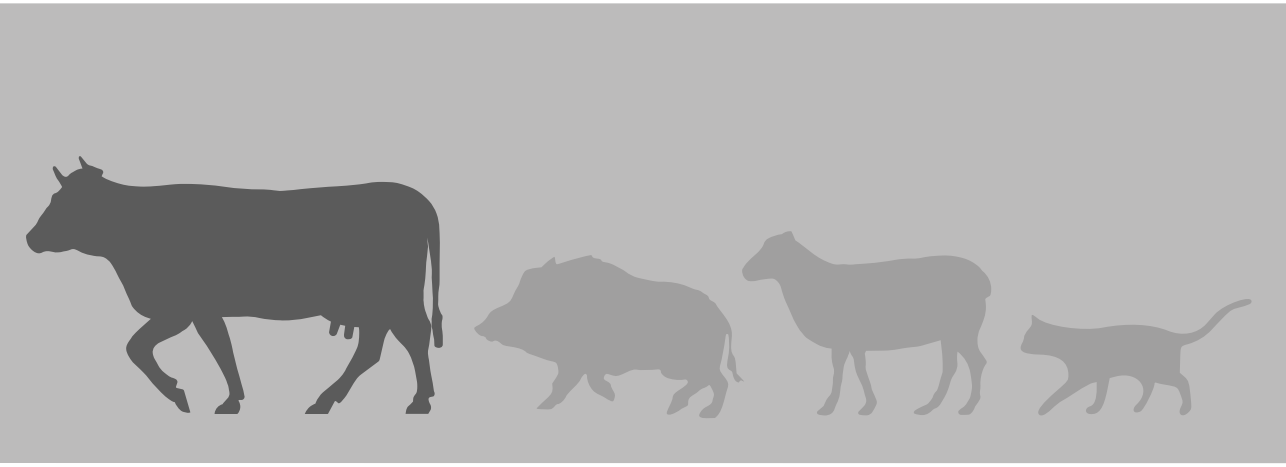

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The role of beef in human infections with *Toxoplasma gondii* is not clear. To get a better understanding of the value of seroprevalence as an indication of the role of beef in human infections with *T. gondii* we studied the seroprevalence of *T. gondii* in Dutch cattle and analysed the correlation between detection of antibodies and parasitic DNA. An indirect ELISA was developed and used to test a sample of the Dutch cattle population. Since validation of the ELISA was hampered by a lack of sufficient bovine reference sera, the results were analysed in two different ways: using a cut-off value that was based on the course of the OD in 27 calves followed from birth until 16 months of age, and by fitting a mixture of two normal distributions (binormal mixture model) to the log-transformed ODs observed for the different groups of cattle in the study population. Using the cut-off value, the seroprevalence was estimated at 0.5% for white veal, 6.4% for rosé veal and 25.0% for cattle. However, using the frequency distributions the prevalences were higher: 1.9% for white veal, 15.6% for rosé veal and 54.5% for cattle. Next, for 100 cattle the results with two different serological assays (ELISA and Toxo-Screen DA) were compared with detection of parasites by our recently developed sensitive magnetic capture PCR. *T. gondii* DNA was detected in only two seronegative cattle. This discordance demonstrates that seroprevalence cannot be used as an indicator of the number of cattle carrying infectious parasites. Demonstrating parasitic DNA in seronegative cattle and not in seropositive cattle suggests that only recent infections are detectable. Whether beef from these PCR-positive cattle is infectious to humans remains to be studied.

Introduction

Toxoplasma gondii is a protozoon that has a world-wide distribution. Infection with *T. gondii* in humans can be severe, especially when contracted transplacentally or in immunocompromised individuals. Also, evidence for deleterious health effects such as chorioretinitis (Burnett et al., 1998; Gilbert and Stanford, 2000) and mental changes (Yolken et al., 2009) in immunocompetent people is accumulating. Cats are the definitive hosts for *T. gondii* and will shed millions of environmentally resistant oocysts after primary infection (Dabritz and Conrad, 2010). Virtually all warm-blooded species, including humans, can act as intermediate hosts. They will develop tissue cysts, especially in muscle and nervous tissue. People can become infected with *T. gondii* by ingesting raw or undercooked meat containing tissue cysts or by ingesting cat-shed oocysts via contaminated soil, food or water. In cattle, natural *T. gondii* infection does not appear to cause clinical disease or abortion (Dubey, 1986). Therefore interest in *T. gondii* in cattle stems mainly from a public health perspective; if cattle carry infectious tissue cysts they may be an important source of human infections since beef is often consumed undercooked.

The role of beef in human *T. gondii* infection is unclear. There is evidence that suggests an important role for beef as a source of human infection. Eating raw beef has been reported as one of the risk factors that predicts acute infection (Baril et al., 1999; Cook et al., 2000; Jones et al., 2009), and in four out of 26 human toxoplasmosis outbreaks between 1965 and 2001 the most probable source was the consumption of raw or undercooked beef (Smith, 1993; AFSSA, 2005). On the other hand, it has been suggested that cattle do not readily acquire infection (Munday and Corbould, 1979), and that tissue cysts are not very persistent in cattle. Tissue cysts are only infrequently recovered from experimentally-inoculated cattle (Dubey, 1986; Dubey and Thulliez, 1993; Esteban-Redondo et al., 1999), and there are few successful recoveries from naturally infected cattle (Dubey, 1986) or beef samples (Aspinall et al., 2002; Dubey et al., 2005). Taking these aspects into consideration, large-scale screening of cattle for the presence of *T. gondii* is necessary to clarify the role of cattle as carriers of *T. gondii* tissue cysts.

Serological assays allow high through-put testing at low costs and are therefore preferred for screening. Although parasites are not detected directly, the seroprevalence can give an indication of the risk of human infection by eating meat from a certain species if the detection of antibodies against *T. gondii* and the presence of tissue cysts have a strong correlation. A strong correlation has been shown in pigs (Gamble et al., 2005) and sheep (Dubey et al., 2008; Opsteegh et al., 2010b), but for cattle the correlation is unclear. It is often not possible to recover tissue cysts from seropositive cattle and sometimes parasitic DNA is detected in serologically negative animals (Gottstein et al., 1998; Wyss, 1999; More et al., 2008; Santos et al., 2010). If the presence of antibodies is not correlated to the detection of tissue cysts because cattle eliminate their tissue cysts, while remaining seropositive, the value of seroprevalence as an indicator of risk for human infection from beef is limited. The infrequent recovery of tissue cysts from seropositive animals can also be the result of a lack of sensitivity of the direct detection method (tissue cysts are present but not detected) or due to a specificity problem with the serological assay used (false positive test results). These issues need to be addressed before any conclusions on the role of beef in human infection can be drawn from the generally high prevalence of antibodies against *T. gondii* in cattle (Dubey, 1986; Tenter et al., 2000).

Although tissue cysts can be detected by bioassay using mice or cats, these assays are not often used for large-scale screening because they are time-consuming and not desirable from an animal ethics point of view. For that reason, PCR-based methods have been developed, but these usually lack sensitivity due to the low concentration and inhomogeneous distribution of tissue cysts in combination with the relatively small sample used. In this study, we use magnetic capture (MC)-PCR, which has an equal sensitivity in comparison with the mouse bioassay (Opsteegh et al., 2010b) for the direct detection of *T. gondii* in bovine samples.

A problem with specificity of the serological assays used is also likely. Several different serological assays with varying cut-off values are currently used to test bovine sera for the presence of antibodies against *T. gondii* (Sabin Feldman dye test (SFDT), modified agglutination test (MAT), latex agglutination test (LAT), IFAT, indirect hemagglutinating antibody test (IHAT), complement fixation test (CFT) and ELISA) and many (SFDT, MAT, LAT, IFAT, IHAT, CFT) have been validated for use with bovine sera by testing sera of experimentally infected animals only (Rommel et al., 1966; Costa et al., 1977; Dubey et al., 1985; Dubey and Thulliez, 1993). This is not sufficient validation for tests applied for screening, because experimental animals are often infected with high doses and sometimes through unnatural routes, which may cause unnaturally high antibody titers. In addition, they may show low background titers with little variation before infection, since they were kept under conditions to keep them free of *T. gondii* and therefore infections with cross-reacting pathogens are also less likely. Using this kind of reference sera, a clear discrimination of ODs for positive and negative animals can be obtained, even though that would not be the case in the target population. Another approach which is common in ELISA-based seroprevalence studies, is to use the mean OD-value for a selection of uninfected animals plus two to four times the SD as a cut-off value. Assuming a normal distribution of OD-values for negative sera, these cut-off values would result in a specificity of 97.7% to almost 100%. However, OD-values for uninfected animals are usually not normally distributed but skewed to the right, making the assumption of 97.7%-100% specificity invalid and, usually, an over-estimation (Jacobson, 1998). In addition, the number of negative sera used is often limited or not representative of the sampling population, making the cut-off value dependent on the sera selected. Further, since only negative sera are used to select a cut-off value, the sensitivity of the assay remains unknown. This approach was used for ELISA-based *T. gondii* seroprevalence studies in cattle (van Knapen et al., 1995; Gottstein et al., 1998; Wyss, 1999). To estimate the sensitivity and specificity of a serological assay, it is recommended to test at least 300 samples from animals known to be infected and 1000 samples from animals known to be uninfected (Crowther et al., 2006), which represent the target population (Jacobson, 1998). A cut-off value can then be selected based on accompanying test characteristics by receiver-operating characteristic (ROC) analysis (Greiner et al., 1995), but obtaining these numbers of reference sera is seldom feasible. Therefore, methods to estimate the seroprevalence without a cut-off value based on the use of reference sera have been developed. If the disease under study is endemic in the target population and the serological test is sufficiently discriminatory, the frequency distribution of antibody titers should show two distributions: one for uninfected and one for infected animals. In that case, the seroprevalence can be estimated directly by distribution analysis (Greiner et al., 1994; Teunis et al., 2009; Opsteegh et al., 2010c) and correction for sensitivity and specificity is unnecessary. These methods have not yet been used to estimate the seroprevalence of *T. gondii* in cattle. In this study, we use a binormal mixture model (a

mixture of two normal distributions; Opsteegh et al., 2010c), in addition to a cut-off that is based on reference sera obtained from calves followed longitudinally, to estimate the seroprevalence of *T. gondii*.

It was our aim to develop a serological test that accurately detects antibodies against *T. gondii* in cattle and subsequently estimate the seroprevalence of *T. gondii* in Dutch cattle and investigate the correlation between seropositivity and the detection of *T. gondii* by the newly developed, sensitive MC-PCR. Because binormal mixture analysis is a useful method to estimate test characteristics when reference sera are lacking, and since ELISA results are well suited for binormal mixture analysis, we chose to modify and validate our in-house ELISA which works well for pigs (van der Giessen et al., 2007) and sheep (Opsteegh et al., 2010c) for use with bovine sera. As the MAT appears best accepted for use with bovine sera, we additionally analysed the correlation between MAT and MC-PCR, and compared ELISA and MAT results for a selection of sera.

Materials and methods

Samples

Control serum samples

Reactivity of the ELISA was confirmed using three *T. gondii*-positive and three negative control sera (Uggla et al., 1987; Dubey and Thulliez, 1993). Cross-reactivity with related parasites, such as *Neospora caninum* (Harkins et al., 1998) and *Sarcocystis* spp. (in cattle *Sarcocystis cruzi, Sarcocystis hirsuta* or *Sarcocystis hominis*) (Uggla et al., 1987; Savini et al., 1994, 1997) has been reported using different *T. gondii* antigen preparations and was therefore evaluated. Cross-reactivity with *N. caninum* was evaluated using serum series from three experimentally *N. caninum*-infected cattle (Schares et al., 1999). To evaluate cross-reactivity with *Sarcocystis* spp., the sera of two calves at 88 days p.i. with *S. cruzi* (Uggla et al., 1987) were tested.

Serum samples - longitudinal study

To study the natural course of the antibody response to *T. gondii* infections in cattle and obtain sera that could be used as reference sera for ROC curve analyses, a group of 27 calves that were born between April and July, 2004 and reared conventionally were followed for 16 months from birth onwards. The first serum sample was collected before colostrum intake and all calves except two steers (numbers 459 and 1375) were moved to pasture from May until October 2005. All samples were stored at -20°C until required for testing.

Serum samples - cross-sectional study

In 2006, 13,000 heifers, 0.5 million dairy cows, 67,000 beef cattle and 1.3 million calves were slaughtered in The Netherlands (CBS, 2006). Based on this population size, the required sample size for calves with an expected prevalence of 1% (based on historical data (van Knapen et al., 1995)) was calculated at 380 (1% accepted error, 95% confidence interval (CI)) (WinEpiscope 2.0 (Thrusfield et al., 2001)). For cattle over 12 months of age, historical data for dairy cattle from the north and south of the country (van Knapen et al., 1995) were combined to estimate an expected prevalence of 20%. Using 20% as expected prevalence, a population size of 568,000, a 3% accepted error and a 95% CI, a sample size of 683 was calculated.

The Dutch Food and Consumer Product Safety Authority (VWA) collected 396 serum samples at four calf slaughterhouses and 676 serum samples at seven cattle slaughterhouses between September and December 2006. A maximum of five animals from any one farm was allowed. Serum samples were sent to the Dutch National Institute for Public Health and the Environment (RIVM) and stored at -20°C until required for testing. All samples were coded with an identification (ID) number linked to the ear tag information. Ear tag information was used to retrieve the age and sex of each animal. Animals that could not be traced were excluded, leaving 187 samples of calves less than 8 months of age, 178 samples of calves between 8 and 12 months of age, and 645 samples of cattle over 12 months old that could be used in the analyses.

Samples for correlation between detection of antibodies and detection of parasites

Serum and a 100 g sample from the heart were collected from the first 100 cattle over 12 months old presented at the slaughterhouse in Nijmegen, The Netherlands, on 27 October, 2009. The knife used to collect samples was rinsed in hot water to prevent cross-contamination of samples. Again ear tag information was used to retrieve the age and sex of the cattle.

Serological assays

Antigen preparation for *Toxoplasma gondii* ELISA

T. gondii RH-strain tachyzoites were cultured on a pre-cultured monolayer of rabbit kidney (RK) 13-cells in RPMI 1640 with 3% FCS, 100 U penicillin/ml and 0.1 mg/ml streptomycin. Culture was continued until all RK13 cells were lysed and free tachyzoites were visible in the medium. Medium with tachyzoites was centrifuged for 10 min at 675 *g*. Supernatant was discarded, the pellet was washed twice in 50 ml of PBS and finally resuspended in 25 ml of PBS. Parasite concentration was determined using a Bürker-Türk counting chamber and after centrifugation (10 min at 675 *g*) the pellet was suspended in extraction buffer (50 mM sodium phosphate pH 7.6, 1 mM phenylmethanesulfonylfluoride (PMSF), 2 mM EDTA pH 7.5, 2 µg/ml pepstatin) to a final concentration of 10^9 parasites/ml. Saponin and octylglucoside were added to the suspension at a final concentration of 0.5% of each. The suspension was incubated for 16 h on a blood-tube rotator and then centrifuged for 30 min at 50,000 *g* at 4°C. The protein concentration of the supernatant was determined using a Micro BCA Protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA) and used as antigen for ELISAs.

***Toxoplasma gondii* ELISA - 1:100 serum dilution**

Flat bottomed polystyrene 96-well microtitre plates (microlon, medium binding, Greiner bio-one, Alphen a/d Rijn, The Netherlands) were coated with 100 µl/well of 3.4 µg/ml *T. gondii* RH antigen in 0.1 M Na_2CO_3 solution (pH 9.6) and incubated for 1 h at 37°C. Plates were washed four times with washing buffer (0.07 M PBS/0.05% Tween 20 (PBS-T)) using an automated plate washer (ELx405 Auto Plate Washer, Bio-Tek Instruments, Bad Friedrichshall, Germany) and tapped dry. Wells were blocked by incubation for 1 h at 37°C with 125 µl 1 × blocking reagent (BR) (blocking reagent for ELISA, Roche Diagnostics, Penzberg, Germany) in demineralized water. Afterwards washing, as above, was repeated. Serum was 1:100 diluted in 1 × BR in PBS-T and 100 µl/well were added. Plates were incubated for 1 h at 37°C and then washed. Conjugate (Horseradish peroxidase-labelled polyclonal Rabbit Anti-cow Immunoglobulins, Dako, Heverlee, Belgium) was diluted 1:7000 in 1 × BR in PBS-T and 100 µl of the solution were added to each well. Plates were incubated for 1 h at 37°C and then washed. Substrate

(100 µl; Sure Blue™ TMB Microwell Peroxidase Substrate, KPL, Gaithersburg, MD, USA) was added to each well and incubated for 10 min at room temperature. The reaction was stopped by the addition of 100 µl of 2 M H_2SO_4. The OD at 450 nm was read using a microplate reader (EL808, Ultra Microplate Reader, Bio-Tek Instruments, Bad Friedrichshall, Germany).

Toxoplasma gondii ELISA - 1:2200 serum dilution

To lower the background OD values for negative sera and thereby obtain a better discrimination between negative and positive sera, sera were tested at a higher dilution (1:2200). The concentrations of antigen (6.8 µg/ml), conjugate (1:4000) and blocking buffer (2% cold water fish gelatin (GCF) (Sigma-Aldrich, St. Louis, MO, USA) were optimized to work with this new serum dilution. The dilution buffer was prepared with 2% GCF instead of BR. The same protocol was used to test sera with a corrected OD (ODc)-value above 0.150 for the 1:2200 serum dilution protocol without antigen-coating. In that case one well per serum sample was coated with antigen and one was incubated only with carbonate buffer for 1 h. All sera that showed reactivity above 0.1 for the well without antigen-coating were excluded from further analyses.

Toxoplasma gondii ELISA correction for plate-to-plate variation

In the 1:100 serum dilution protocol a serum sample with a high OD-value was tested in serial dilution from 1:100 to 1:12,800. Six serum samples with low OD-values and test sera were tested in duplicate. In addition six blank controls without serum were included on each plate. In the 1:2200 serum dilution protocol six serum samples with varying OD-values and two blank controls were included in duplicate. In both protocols OD-values were corrected for blank measurement and plate-to-plate variation by linear regression as described elsewhere (Opsteegh et al., 2010c), resulting in their corrected OD (ODc)-value. Repeatability of results was confirmed from the co-efficients of variance of controls included on each plate.

Toxo-Screen DA

Sera from the 100 cattle that also contributed a heart sample and a random selection of sera from the cross-sectional study (n = 170) were additionally tested using Toxo-Screen DA (bioMérieux, Marcy l'Etoile, France). Toxo-Screen DA is a commercially available version of the MAT and it was performed according to the manufacturer's instructions. Sera were tested at 1:40 and 1:4000 dilution and sera which tested positive at either dilution were scored as positive.

IDEXX HerdChek *Neospora* Antibody ELISA test kit

A random selection of 192 sera from the cross-sectional study was sent to the Dutch Animal Health Service to test for *N. caninum* reactivity with an IDEXX HerdChek *Neospora* Antibody ELISA test kit (IDEXX Laboratories, Hoofddorp, The Netherlands).

NS3-blocking ELISA for bovine viral diarrhea virus (BVDV)

As the *T. gondii* tachyzoites for preparation of the ELISA antigen are cultured on RK13 cells in RPMI containing FCS, there is a risk of infection with BVDV (Bolin et al., 1991, 1994). If viral proteins end up in the *T. gondii* antigen preparation, sera containing antibodies against BVDV may react with those. For that reason the *T. gondii* ELISA was also checked for cross-reactivity with antibodies against BVDV. Twenty sera that were positive by ELISA and negative by Toxo-Screen DA, 12 sera negative by both assays and nine sera positive by both

assays were tested. The presence of antibodies against BVDV was determined at the Dutch Animal Health Service by using a NS3-blocking ELISA (CEDI Diagnostics, Lelystad, The Netherlands) (Kramps et al., 1999; Mars and Van Maanen, 2005).

PCR-based detection of *T. gondii*

Detection of *T. gondii* was performed by MC-PCR as described elsewhere (Opsteegh et al., 2010b). Briefly, 100 g of heart were cut and digested. *T. gondii* DNA was isolated from the crude extract using MC and used as template in quantitative PCR (qPCR), targeting the 529 bp repeat element. All samples with a positive internal amplification control but no amplification of *T. gondii* DNA, were scored as negative. All samples with positive *T. gondii* PCR results and an appropriate amplification curve were scored as positive.

Statistical analyses

Correlation between log-transformed OD values in IDEXX HerdChek *Neospora* and *T. gondii* ELISA

To assess cross-reactivity with *N. caninum,* the variation in $\log_{10}$-transformed ODc-values in *T. gondii* ELISAs compared with the $\log_{10}$-transformed OD-values in the IDEXX HerdChek *Neospora* was evaluated by Pearson's correlation co-efficient (SPSS 18.0, PASW Statistics, Chicago, IL, USA).

Correlation between serological status for BVDV and *T. gondii*

Sera which gave discordant *T. gondii* ELISA and Toxo-Screen DA results (n = 20) were possible cross-reactors. Prevalence of antibodies against BVDV in these sera was compared with the seroprevalence in a selection of sera that were either negative (n = 12) or positive in both assays (n = 9) using a χ^2-test (SPSS 18.0, PASW Statistics, Chicago, IL, USA).

Determination of a cut-off value for *T. gondii* ELISA

To allow comparison of seroprevalence between different age groups in the cross-sectional study, a cut-off value was determined by ROC-curve analysis (Greiner et al., 1995) (SPSS 18.0, PASW Statistics, Chicago, IL, USA). Since no large number of reference sera (representative of the target population) was available, sera from animals in the longitudinal study that showed a clear seroconversion or clearly remained negative were used as reference sera. Besides a limitation in age and time after infection, these calves represent the target population. Samples taken prior to seroconversion were used as negative reference sera, and those taken after seroconversion were used as positive reference sera. Single peak values between two negative samplings were regarded as false positives and included in ROC curve analyses as negatives. Samples with a slightly increased ODc-value that were followed by a sample showing a strong increase were not included as reference sera. The sample taken prior to colostrum intake was not included if it showed a high ODc-value which decreased at the next sampling. Since the calves have to be scored as either seropositive or seronegative at a certain time point, samples were always scored based on the pattern in both dilutions.

Seroprevalence estimation using a cut-off value

All serum samples in the cross-sectional study were classified as positive or negative, as defined by the cut-off value. Apparent prevalence (*AP*) (positive samples/sample size) was calculated separately for different age categories. *AP* was corrected for test sensitivity (*Se*) and specificity

(*Sp*) using the Rogan-Gladen-estimator resulting in true prevalence (*TP*) (*TP = (AP+Sp-1)/(Se+Sp-1)*) (Rogan and Gladen, 1978). Estimates for *Se* and *Sp* of the *T. gondii* ELISA at 1:100 and 1:2200 serum dilutions were obtained from ROC curve analyses. The test characteristics for the combination of test results (regarding only those that test positive at both dilutions as positive) were calculated as $Se = Se1 \times Se2$, and $Sp = Sp1+Sp2-Sp1 \times Sp2$ (Dohoo et al., 2003). The CI for the *TP* was estimated using the normal approximation $TP \pm 1.96\sqrt{var(TP)}$, with $var(TP) = AP(1-AP)/nJ^2$ and $J = Se+Sp-1$ (Greiner and Gardner, 2000). Whether differences in *AP* between age categories were present was assessed using a χ^2-test (SPSS 18.0, PASW Statistics, Chicago, IL, USA).

Seroprevalence estimation using a binormal mixture model

Because the number of sera useful for ROC-curve analysis was limited, additional analyses of the frequency distributions of $\log_{10}$-transformed ODc-values observed in the cross-sectional study were performed as described previously (Opsteegh et al., 2010c). The frequency distribution for calves younger than 8 months, calves between 8 and 12 months old, and cattle over 12 months of age were drawn and analysed separately, as these populations are slaughtered for different types of meat: white veal, rosé veal and beef, respectively.

Test agreement between *T. gondii* ELISA and Toxo-Screen DA

For the 268 sera additionally tested by Toxo-Screen DA, the results were compared with those from the *T. gondii* ELISAs by a Pearson's χ^2-test (SPSS 18.0, PASW Statistics, Chicago, IL, USA) and test agreement was evaluated using κ-statistics (WinEpiscope 2.0 (Thrusfield et al., 2001)).

Results

Validation of the serological assay

The *T. gondii*-positive control sera had high ODc-values, whereas the negative control sera had low ODc-values (Fig. 4.1). The ratio between average ODc-value for positive control sera ($n = 3$) and negative control sera including the *S. cruzi*-positive sera ($n = 5$) increased from 1.5 to 4.4 by using the 1:2200 serum dilution protocol. For both protocols the co-efficient of variation (CV) for the raw OD-values of the control sera was less than 20% over all plates used, declining to below 10% after correction for plate-to-plate variation. For each plate the correlation (R^2) between the measured ODs for the controls on the plate and their overall mean ODs was at least 0.95.

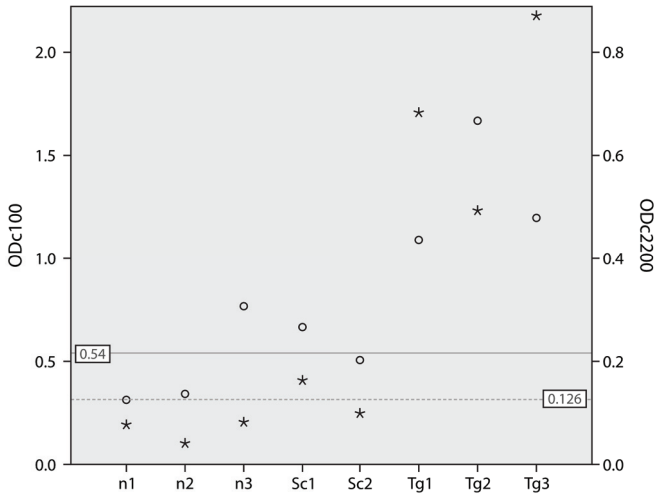

Figure 4.1 *Toxoplasma gondii* ELISA corrected OD-values (1:100 serum dilution, circles; 1:2200 serum dilution, stars) for control sera: n1-n3 are negative controls, Sc1 and Sc2 are calves 88 days post-experimental infection with *Sarcocystis cruzi* (calves C1 and C2, respectively; Uggla et al., 1987), Tg1-Tg3 are experimentally infected with *T. gondii*. Tg1 was collected 228 days p.i. and Tg2 399 days p.i. (steers 1 and 2, respectively; Dubey and Thulliez, 1993). N3 and Tg3 are the same calf at 3 days prior to and 133 days after infection with *T. gondii* (calf C3; Uggla et al., 1987). Cut-off values are indicated by horizontal solid and dashed lines (at 0.54 for 1:100 serum dilution and at 0.126 for 1:2200 serum dilution).

The three cows experimentally infected with *N. caninum* (Schares et al., 1999) showed a rise in *T. gondii* ODc-value simultaneously with the rise in *N. caninum* IFAT-titer (Fig. 4.2 A, B, and C). For one cow the rise was still substantial at a 1:2200 dilution (Fig. 4.2 B).

The sera from the cross-sectional study which were additionally tested for *N. caninum* (n = 192) using IDEXX HerdChek *Neospora*, showed a small but negative correlation (Pearson's correlation coefficient = -0.203, p = 0.05) between *T. gondii* logODc-value at 1:100 serum dilution and *N. caninum* IFAT log-transformed OD-value, and no significant correlation (p = 0.114) was observed between the *T. gondii* logODc-value at 1:2200 serum dilution and *N. caninum* log-transformed OD-value.

The calves experimentally infected with *S. cruzi* showed ODc-values close to the cut-off value (Fig. 4.1).

A selection of sera from the cross-sectional study was tested for BVDV. Of 20 sera which tested positive for *T. gondii* by ELISA but were negative in Toxo-Screen DA, 12 (60%) tested positive for BVDV. For the 21 sera that were either positive or negative by both *T. gondii* ELISA and Toxo-Screen DA 13 (62%) were positive for BVDV. These BVDV seroprevalences do not differ significantly (χ^2 = 0.016, p = 0.901).

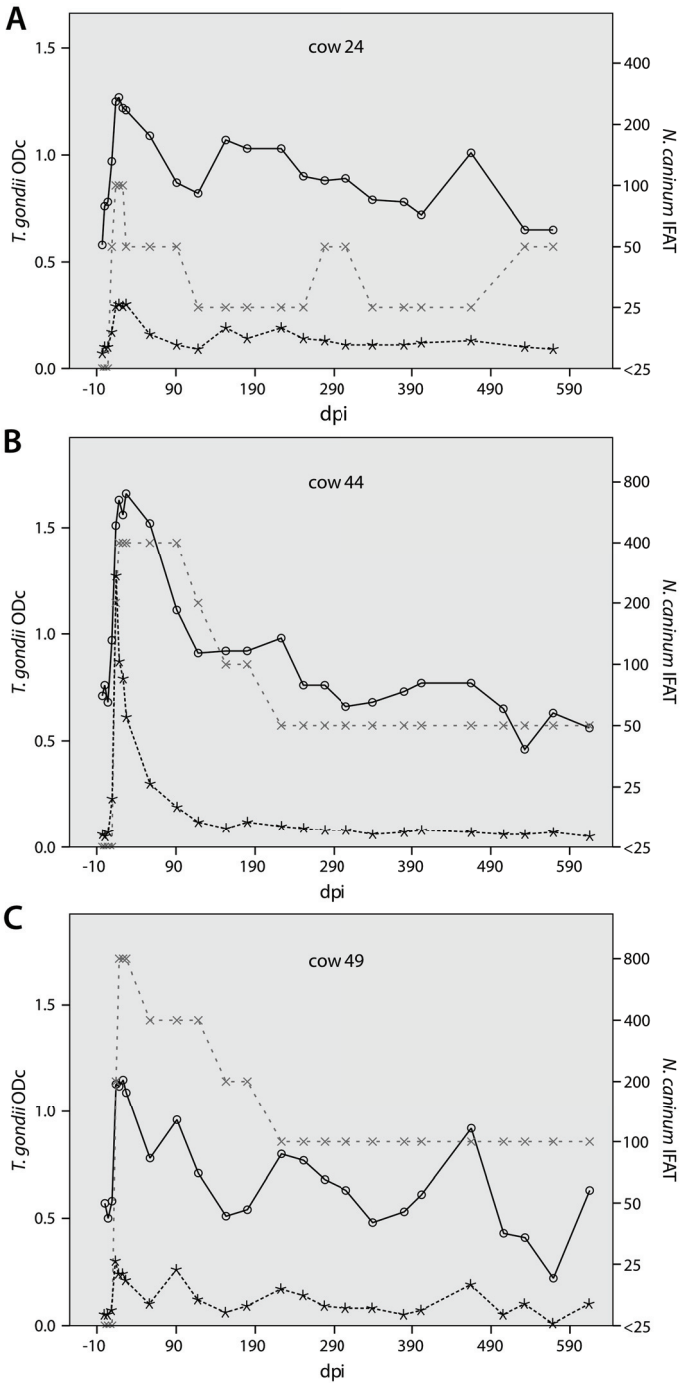

Figure 4.2 Time course of *Toxoplasma gondii* corrected OD-values at 1:100 serum dilution (circles, solid line) and 1:2200 serum dilution (stars, dashed line), and reciprocal *Neospora caninum* IFAT titer (crosses, shaded dashed line) for cows 24 (A), 44 (B) and 49 (C) after experimental infection with *N. caninum* (Schares et al., 1999).

Longitudinal study

For 24 out of 27 calves that were followed longitudinally, samples from at least three time points could be identified as positive or negative based on the course of the ODc-value in both serum dilutions (Fig. 4.3). In total 43 positive and 141 negative reference sera could be identified and 86 samples remained inconclusive. ROC curve analyses with the 184 acclaimed reference sera indicated an ODc-value of 0.540 at serum dilution 1:100 (Se 95.3%, Sp 98.6%), and an ODc-value of 0.126 at dilution 1:2200 (Se 95.3%, Sp 96.5%) as the optimal cut-off values (Fig. 4.4).

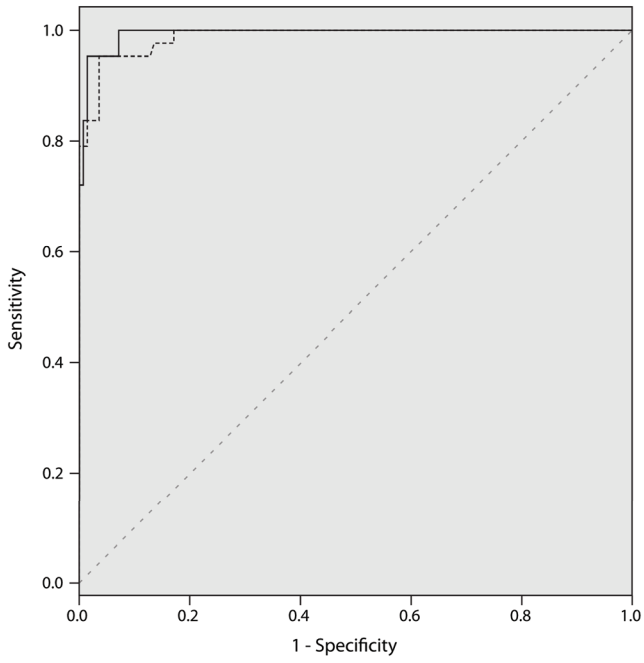

Figure 4.4 Receiver-operating characteristic (ROC) curve analysis for *Toxoplasma gondii* ELISA at 1:100 (solid line) and 1:2200 (dashed line) serum dilution based on the 43 sera that were identified as positive and the 141 sera that were identified as negative out of 270 samples from 27 calves followed over time (Fig. 4.3).

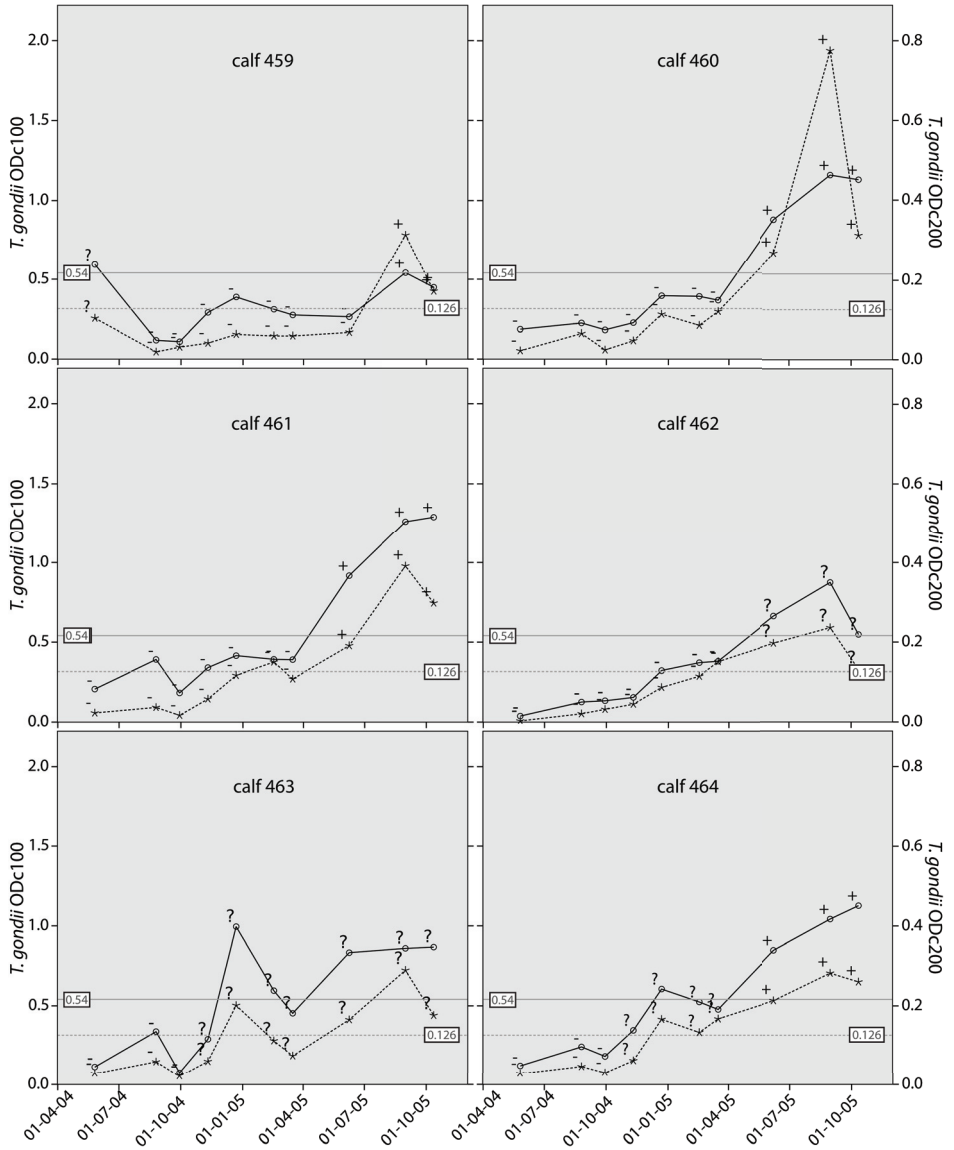

Figure 4.3 Time course of corrected OD-values from before colostrum intake up to 16 months of age in 27 calves by *Toxoplasma gondii* ELISA at 1:100 serum dilution (circles, solid line) and 1:2200 serum dilution (stars, dashed line). Samples used as reference samples in receiver-operating characteristic (ROC) curve analyses are labeled positive (+) or negative (-); samples labeled doubtful (?) were not included in ROC curve analyses. Cut-off values are indicated by shaded horizontal solid and dashed lines (at 0.54 for 1:100 serum dilution and at 0.126 for 1:2200 serum dilution). All calves except numbers 459 and 1375 were turned out to pasture from May until October 2005.

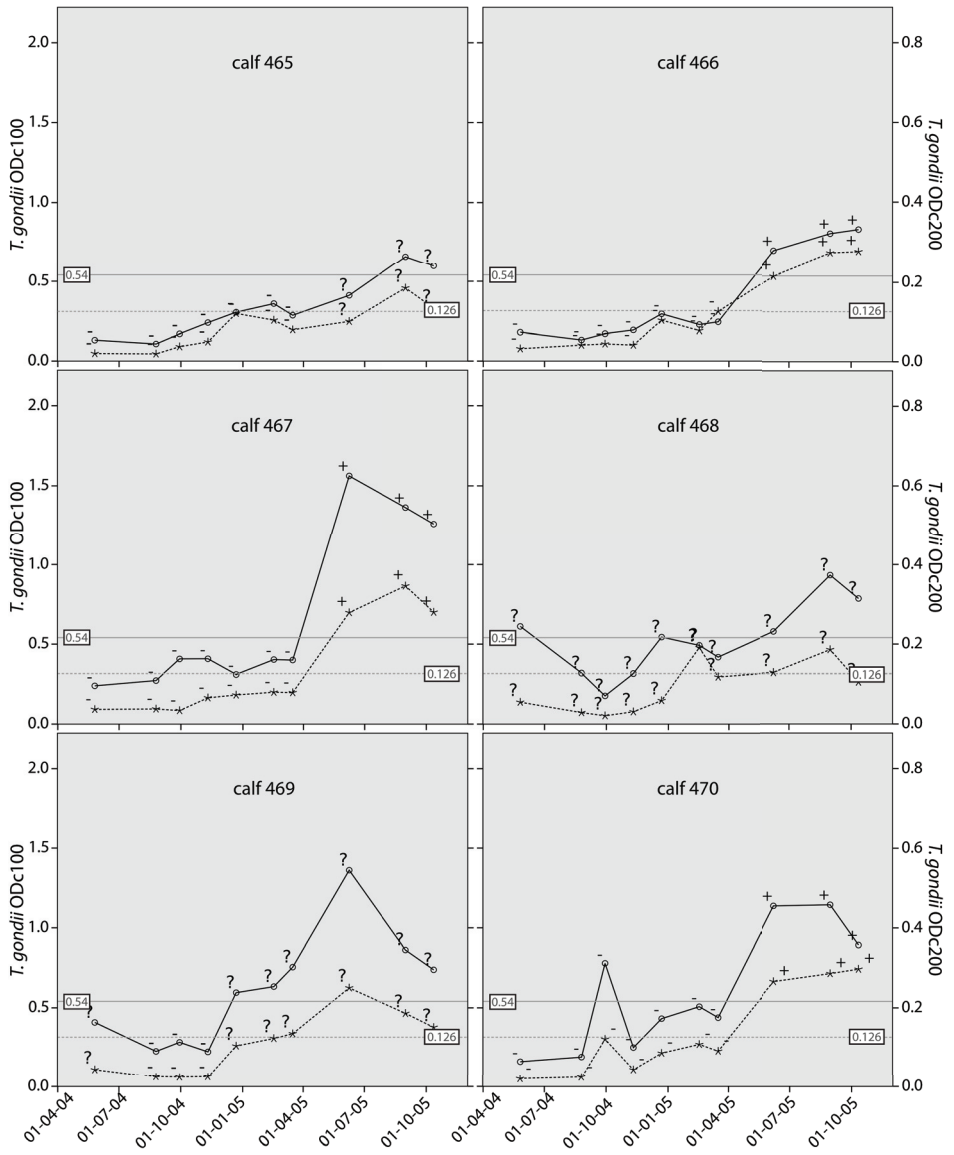

Figure 4.3 continued [Time course of corrected OD-values from before colostrum intake up to 16 months of age in 27 calves by *Toxoplasma gondii* ELISA at 1:100 serum dilution (circles, solid line) and 1:2200 serum dilution (stars, dashed line). Samples used as reference samples in receiver-operating characteristic (ROC) curve analyses are labeled positive (+) or negative (-); samples labeled doubtful (?) were not included in ROC curve analyses. Cut-off values are indicated by shaded horizontal solid and dashed lines (at 0.54 for 1:100 serum dilution and at 0.126 for 1:2200 serum dilution). All calves except numbers 459 and 1375 were turned out to pasture from May until October 2005.]

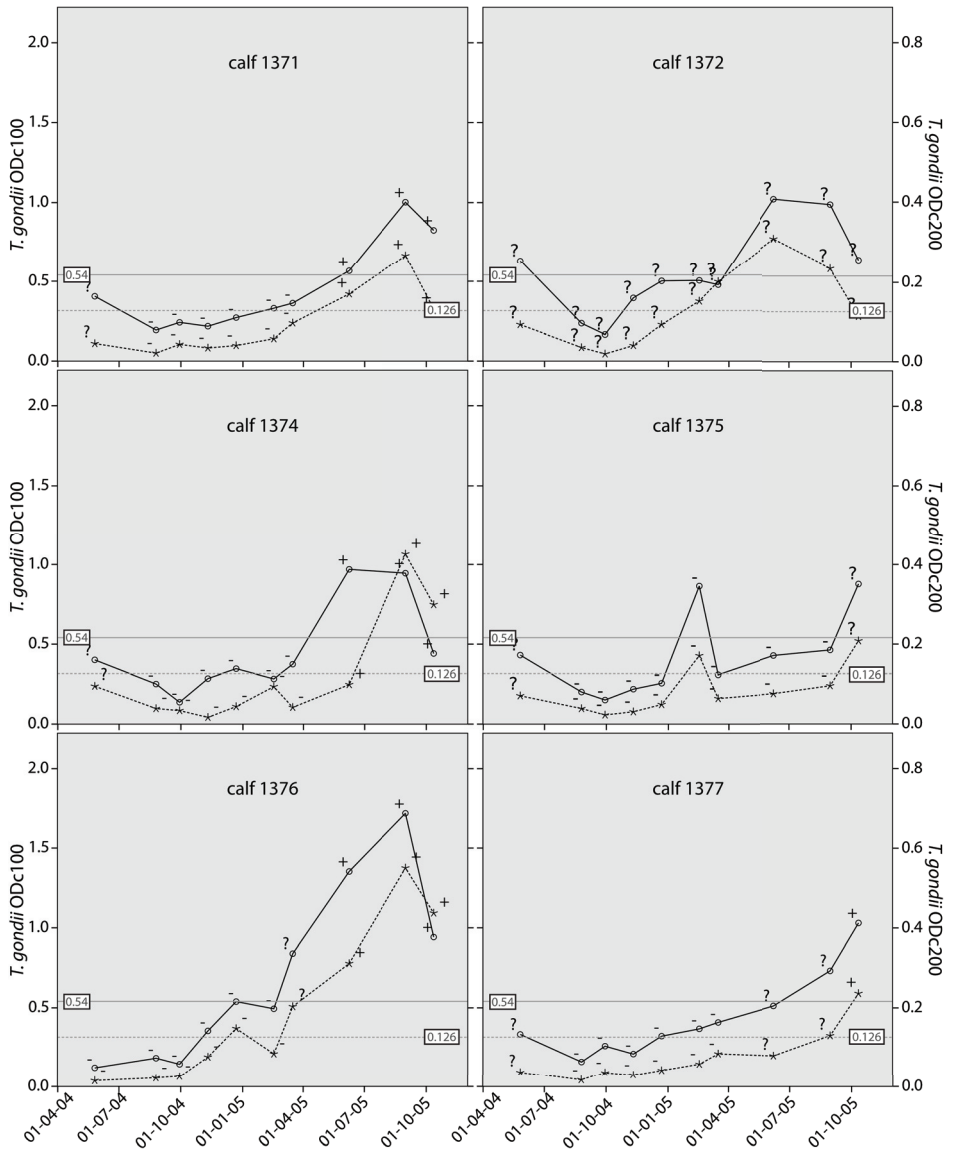

Figure 4.3 continued

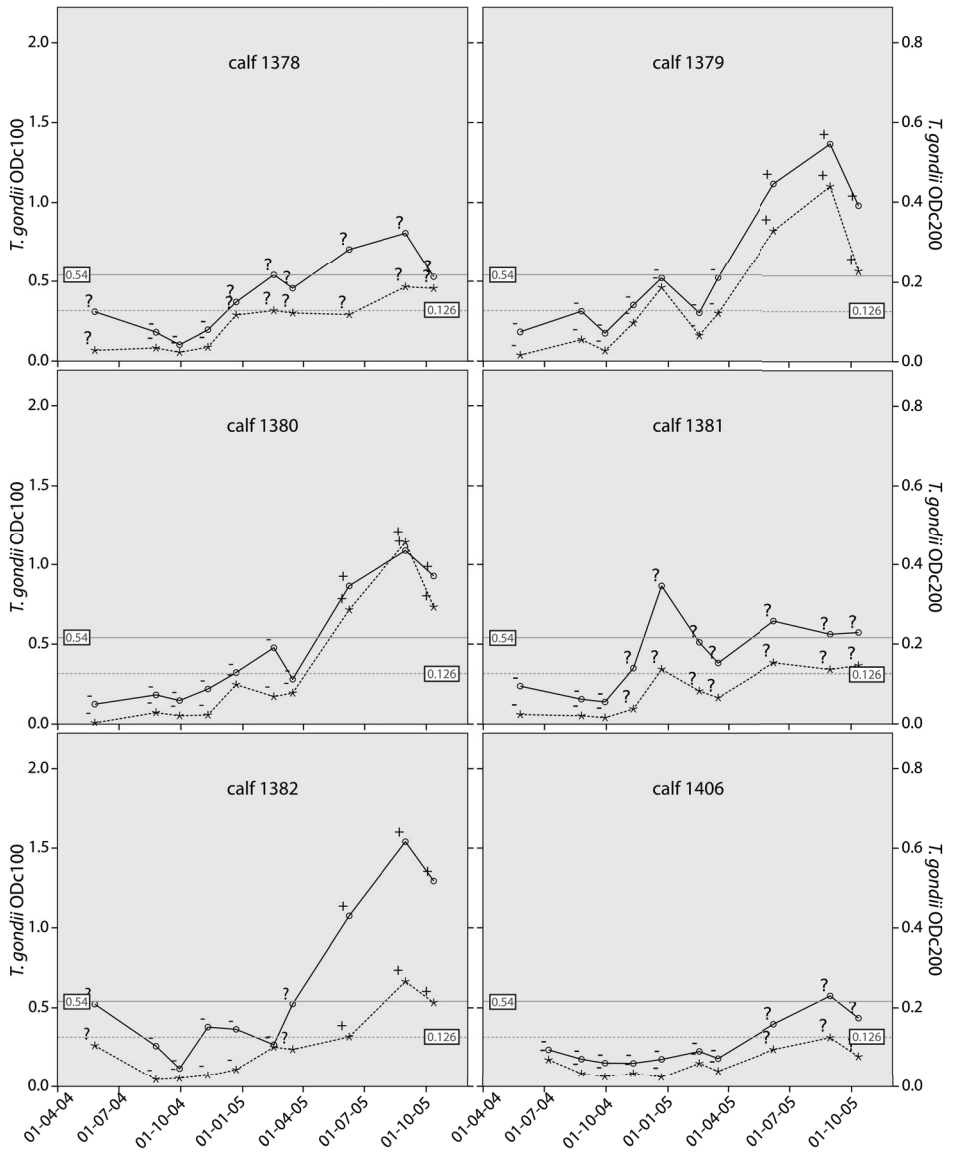

Figure 4.3 continued [Time course of corrected OD-values from before colostrum intake up to 16 months of age in 27 calves by *Toxoplasma gondii* ELISA at 1:100 serum dilution (circles, solid line) and 1:2200 serum dilution (stars, dashed line). Samples used as reference samples in receiver-operating characteristic (ROC) curve analyses are labeled positive (+) or negative (-); samples labeled doubtful (?) were not included in ROC curve analyses. Cut-off values are indicated by shaded horizontal solid and dashed lines (at 0.54 for 1:100 serum dilution and at 0.126 for 1:2200 serum dilution). All calves except numbers 459 and 1375 were turned out to pasture from May until October 2005.]

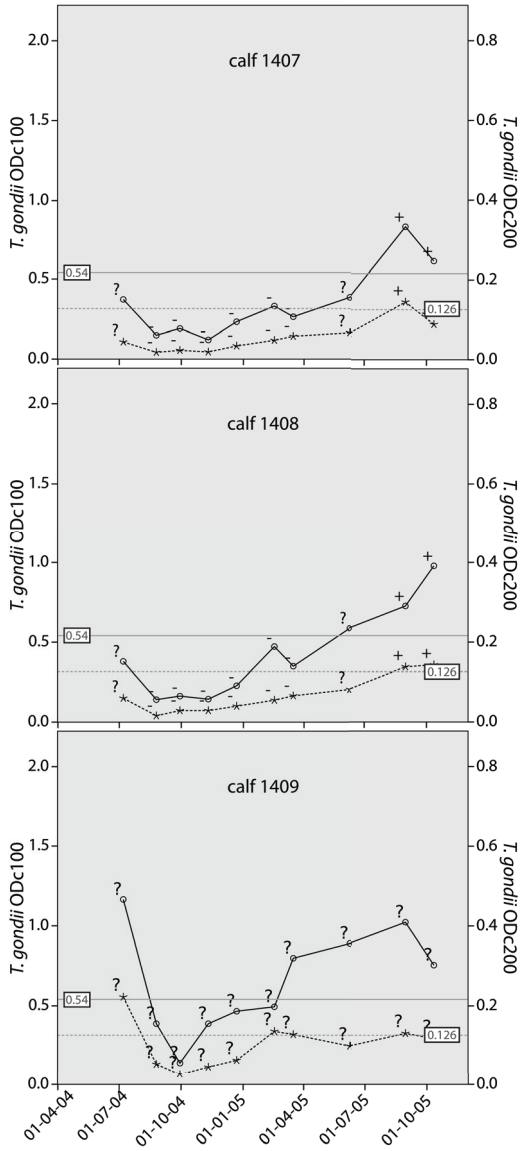

Figure 4.3 continued

Cross-sectional study

Seroprevalence estimation using a cut-off value

A total of 1010 cattle from the cross-sectional study could be traced based on ear tag number and their serum samples were tested by ELISAs. Fifteen sera that were positive at a level above 0.150 at 1:2200 serum dilution showed background ODc-values above 0.100 if tested without antigen coating; seven were above 0.300. Because the *T. gondii* infection status could not be determined for these 15 sera, they were excluded from further analyses.

After classifying the remaining 995 sera as positive or negative as determined by the cut-off value (0.540 at 1:100 and 0.126 at 1:2200) or the combination of test results, the seroprevalence was calculated for different age categories (Table 4.1). Additionally, cattle over 12 months old were sub-grouped according to age and sex (Table 4.2). The apparent prevalence was higher in cattle over 12 months old (22.7%), than in fattening calves less than 8 months of age (0.5%) and calves between 8 and 12 months of age (5.9%) ($\chi^2 = 68.8$, $p < 0.000$). For cattle over 12 months old, apparent prevalences did not differ per age category ($\chi^2 = 9.070$, $p = 0.170$).

Table 4.1 Number of bovine serum samples which tested positive for *Toxoplasma gondii* by ELISA (at 1:100 and 1:2200 serum dilution and combination of test results), apparent prevalence (AP), and true prevalence (TP) with 95% confidence interval (CI) by age group.

Age	*n*	Number of positive results (AP)			TP (95% CI)		
(months)		1:100[a]	1:2200[b]	both[c]	1:100[a]	1:2200[b]	both[c]
<8	187	1 (0.5)	1 (0.5)	1 (0.5)	0.0 (0.0-0.2)	0.0 (0.0-0.0)	0.5 (0.0-1.7)
8-12	170	16 (9.4)	16 (9.4)	10 (5.9)	8.5 (3.9-13.2)	6.4 (1.7-11.2)	6.4 (2.5-10.3)
>12	638	209 (32.8)	183 (28.7)	145 (22.7)	33.4 (29.5-37.3)	31.9 (27.9-35.8)	25.0 (21.4-28.6)
all	995	226 (22.7)	200 (20.1)	156 (15.7)	22.7 (19.9-25.5)	18.1 (15.4-20.8)	17.2 (14.7-19.7)

[a] Positive if corrected OD-value > 0.54; [b] Positive if corrected OD-value > 0.126; [c] Positive if corrected OD-value 1:100 >0.54 and corrected OD-value 1:2200 > 0.126

Table 4.2 Number and percentage of serum samples from cattle over 12 months old, which tested positive for *Toxoplasma gondii* by ELISA at both serum dilutions, by age group and sex.

Age	Female			Male			Total		
(years)	*n*	pos[a]	%	*n*	pos[a]	%	*n*	pos[a]	%
1-2	5	1	20.0	40	5	12.5	45	6	13.3
2-3	37	10	27.0	23	2	8.7	60	12	20.0
3-4	95	30	31.6	3	0	0.0	98	30	30.6
4-5	107	29	27.1	1	1	100.0	108	30	27.8
5-6	118	27	22.9	2	0	0.0	120	27	22.5
6-7	75	15	20.0	2	1	50.0	77	16	20.8
>7	129	24	18.6	1	0	0.0	130	24	18.5
all	566	136	24.0	72	9	12.5	638	145	22.7

[a] Positive (pos) if corrected OD-value > 0.540 at 1:100 serum dilution and corrected OD-value > 0.126 at 1:2200 serum dilution

Seroprevalence estimation using binormal mixture model

For the 995 sera included in the analyses the frequency distributions of the log-transformed ODc-values at 1:100 and 1:2200 serum dilution were analysed per age category. At 1:100 serum dilution the frequency distributions of log-transformed ODc-values were best described by assuming a mixture of two populations (Fig. 4.5). The seroprevalence was estimated at 1.9% (95% CI: 0.7-3.5%) for the calves less than 8 months old, at 15.6% (95% CI: 10.3%-21.0%) for calves between 8 and 12 months and at 54.5% (95% CI: 46.6-65.1%) for cattle over 12 months old. Testing at 1:2200 decreased the observed log-transformed ODc-values and a mixture of two distinctive distributions could not be identified (Fig. 4.6).

Correlation antibodies and detection of parasites

Of the 100 cattle tested for *T. gondii* DNA, two tested positive by MC-PCR, both with a high Cp value (40.00 and 37.29), indicating a low concentration of parasitic DNA. Both were negative by ELISA and Toxo-Screen DA (Table 4.3). One was an 8-year old dairy cow; the other was a 3.5-year old bull. All 15 ELISA-positive and three Toxo-Screen DA-positive cattle were negative by MC-PCR.

Table 4.3 Number of Toxo-Screen DA and magnetic capture (MC)-PCR positive cattle for cattle (n = 100) grouped by combination of *Toxoplasma gondii* ELISA results at different dilutions.

ELISA results		No. of cattle	Toxo-Screen DA	MC-PCR
1:100[a]	1:2200[b]		pos[c]	pos[d]
neg	neg	66	0	2
neg	pos	3	0	0
pos	neg	14	1	0
pos	pos	15	2	0
aspecific binding		2	0	0

[a] Positive (pos) if corrected OD-value > 0.540, negative (neg) if not; [b] Positive (pos) if corrected OD-value > 0.126, negative (neg) if not; [c] Positive (pos) if agglutination is observed at 1:40 and/or 1:4000 serum dilution; [d] Positive (pos) if amplification was detected

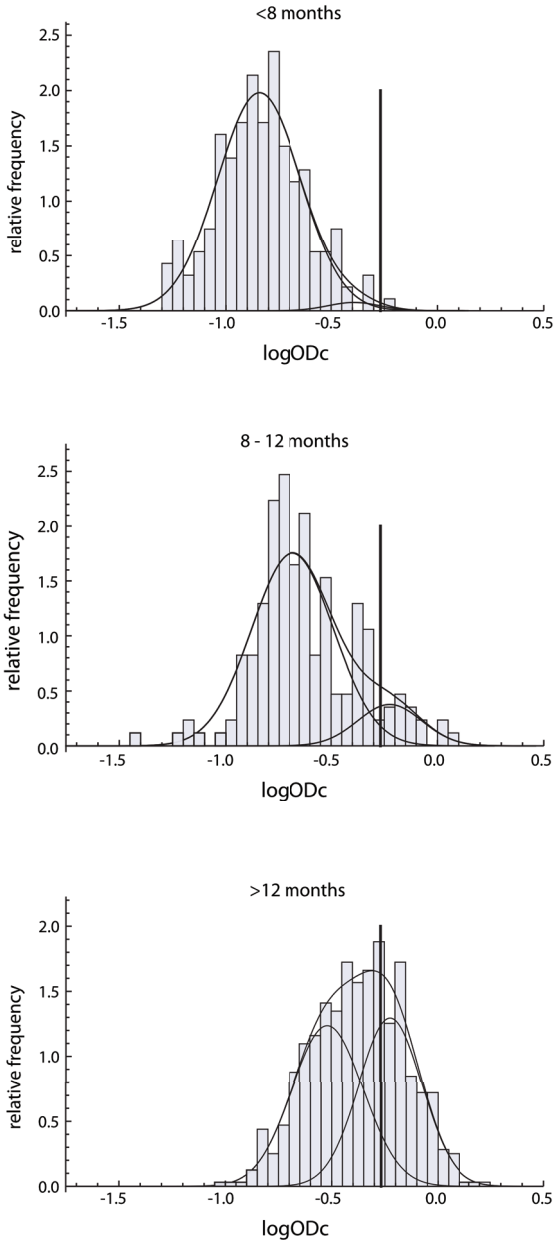

Figure 4.5 Relative frequency distribution of log-transformed corrected OD-values from *Toxoplasma gondii* ELISAs at 1:100 serum dilution (bars), fitted normal distributions (curves), and cut-off value (vertical line) for calves less than 8 months old (n = 187), calves between 8 and 12 months old (n = 170), and cattle over 12 months old (n = 638).

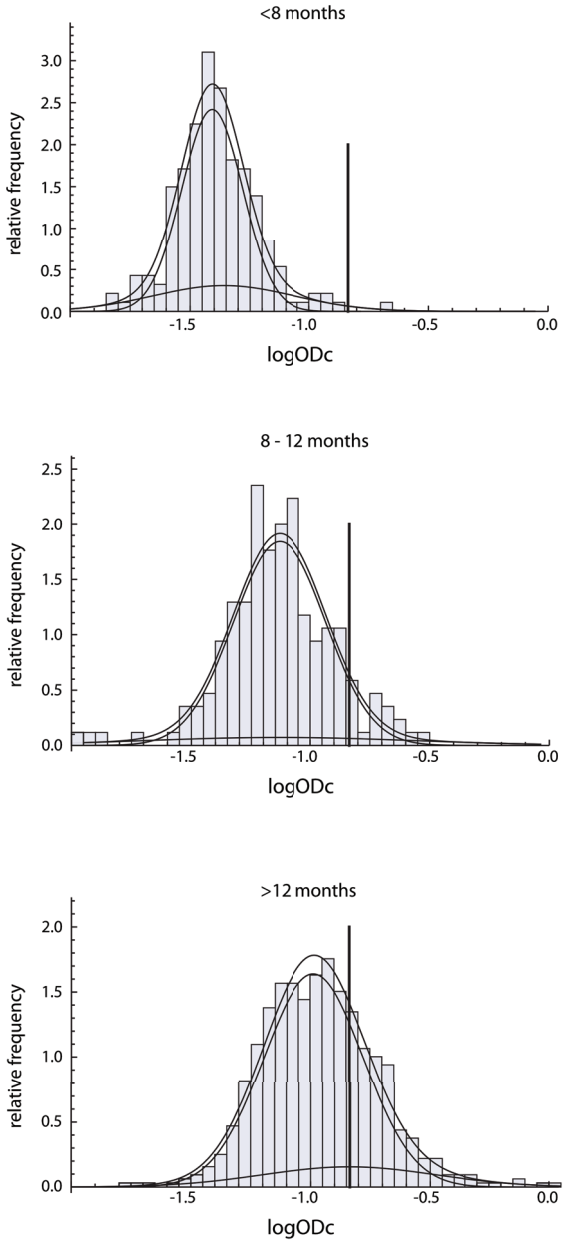

Figure 4.6 Relative frequency distribution of log-transformed corrected OD-values from *Toxoplasma gondii* ELISAs at 1:2200 serum dilution (bars), fitted normal distribution (curves), and cut-off values (vertical lines) for calves less than 8 months old (n = 187), calves between 8 and 12 months old (n = 170), and cattle over 12 months old (n = 638).

Test agreement between *T. gondii* ELISA and Toxo-Screen DA

All sera from cattle for which heart samples were tested by MC-PCR that did not show aspecific binding (n = 98) and sera from 170 cattle in the cross-sectional study were additionally tested by Toxo-Screen DA. Although samples positive by ELISA at both serum dilutions were more likely to test positive by Toxo-Screen DA than ELISA-negative samples (Table 4.4, χ^2 = 10.0, p = 0.007) the agreement between tests was poor (κ = 0.084).

Table 4.4 Cross-tabulation of *Toxoplasma gondii* ELISA and Toxo-Screen DA results for 268 bovine serum samples.

Toxo-Screen DA	ELISA[a]		
	Neg	Pos	Total
Neg	153	68	221
Doubtful	19	4	23
Pos	10	14	24
Total	182	86	268

[a] Positive (Pos) if corrected OD-value > 0.540 at 1:100 serum dilution and corrected OD-value > 0.126 at 1:2200 serum dilution, negative (Neg) if not

Discussion

In this study the seroprevalence of *T. gondii* in Dutch cattle was studied using an ELISA, and the correlation of the ELISA with the presence of parasites was subsequently studied using a sensitive PCR method. The ELISA was validated using several different control sera. The repeatability of the ELISA was good. Cross-reactivity with *N. caninum*-positive sera was investigated in two ways. For cattle experimentally infected with *N. caninum,* a rise in ODc-value in the *T. gondii* ELISA was observed simultaneously with the rise in IFAT-titer for *N. caninum,* suggesting cross-reactivity. On the other hand, for 192 sera from the cross-sectional study, the *T. gondii* ELISA did not show an increase in ODc-value with increasing OD-value in the *Neospora* ELISA. Possibly the cattle used for the *N. caninum* infections were not *T. gondii*-free and a rise in their *T. gondii* titer is measured after *N. caninum* infection. A boost of *T. gondii* antibodies by a *N. caninum* infection has been described previously for sheep vaccinated against *T. gondii* (Innes et al., 2001). It could also be that cross-reactivity with *N. caninum* occurs, especially in experimental infection. The cattle were infected by i.v. inoculation with 1×10^6 *N. caninum* tachyzoites (Schares et al., 1999), possibly leading to very high antibody levels and antibodies directed to epitopes that are not exposed during natural infection. In conclusion, results with the experimentally infected cows and the field sera indicate that although there may be some cross-reactivity with *N. caninum,* it did not significantly influence the results of *T. gondii* ELISAs for field sera.

Cross-reactivity with *Sarcocystis* spp. could be a more important issue as the prevalence in cattle in The Netherlands is assumed to be high. A small study (n = 91) in the 1980s demonstrated 100% positive results using artificial digestion (van Knapen et al., 1987), and, more recently, 94% of 67 Belgian minced beef samples tested positive for *Sarcocystis* spp. (Vangeel et al., 2007), whereas *N. caninum* has a seroprevalence of 9.9% in Dutch dairy cows and 13.0% in Dutch beef cattle (Bartels et al., 2006). Since an assay for antibodies against *Sarcocystis* spp. in cattle is not available we were limited to testing the sera of two calves experimentally infected

with *S. cruzi* in our *T. gondii* ELISA. For these two samples ODc-values close to the cut-off value were shown at the 1:100 and 1:2200 serum dilution. Consequently cross-reactivity with *Sarcocystis* spp., may have influenced our results although it is likely that, as with *N. caninum*, these experimentally infected calves show more cross-reactivity than naturally infected animals would.

Cross-reactivity with antibodies against BVDV was ruled out as the BVDV seroprevalence in possibly cross-reactive cattle (positive by *T. gondii* ELISA but negative by Toxo-Screen DA) (60%) did not differ from the seroprevalence in cattle with corresponding ELISA and Toxo-Screen DA results (62%). The observed seroprevalence of BVDV is similar to the 65% that has been reported previously for Dutch cattle (Kramps et al., 1999).

In addition to some cross-reactivity, aspecific binding to a plate without antigen coating was observed. Problems with unexplained false-positives have been reported previously (Mars and Van Maanen, 2005). As this aspecific binding is not related to the *T. gondii* antigen it is something that should be considered in every ELISA testing bovine sera (it was not observed with sheep or swine sera). In this case, the problem was solved by excluding these samples from the analyses.

The ELISA was used to test sera from a group of calves that were sampled regularly and 183 sera could be used as reference sera to establish a cut-off value by ROC-curve analysis. After using the course of the ODc-value in these calves to establish a cut-off value, the ELISA was used to test a sample of the Dutch cattle population to investigate the seroprevalence of *T. gondii*. The ELISA results were first analysed using the cut-off value from the longitudinal study. Seroprevalences obtained accordingly were comparable to the 1.2% for fattening calves, 26.4% for steers and 12.8% to 42.6% for dairy cattle reported previously (van Knapen et al., 1995). The seroprevalence did not increase with age for cattle over 12 months old. This has been shown previously on farms with cats (Gilot-Fromont et al., 2009) and suggests that antibodies do not persist lifelong. Since the cut-off value was established with a low number of reference sera, we decided to additionally estimate seroprevalence without the use of a cut-off value by binormal mixture analysis. At the 1:100 serum dilution the frequency distributions for all age groups were best described by a mixture of two distributions, and the seroprevalences could be estimated. However, the frequency distributions also indicated a lack of discriminative power of the ELISA. The two distributions that were recognized showed considerable overlap. This lack of discriminative power was unexpected as the ELISA, of which only the blocking protein and the conjugate had been replaced, has given good results for pigs (van der Giessen et al., 2007) and sheep (Opsteegh et al., 2010c). This lack of discriminative power could also explain the difference between seroprevalences estimated using the mixture model and the cut-off value, especially for cattle over 12 months old. As the distributions for the positive and negative populations are not well separated, a small difference between the estimated and true optimal cut-off value will have a large effect on the estimated prevalence. The overlap in the frequency distributions also shows that the Se and Sp were overestimated by ROC-curve analysis using this limited number of reference sera. In our opinion the estimates from the binormal mixture model are more reliable. In the frequency distributions for the 1:2200 serum dilution no distinctive distributions were identified. It seems that, although the 1:2200 serum dilution protocol increased the ratio between ODc-values for positive and negative control sera, it decreased the discriminative power in naturally infected cattle. Positive control sera probably have titers that are still above the minimum concentration that can be detected

after 1:2200 dilution, but positive field sera may have titers that become undetectable at this dilution. This demonstrates the importance of developing and validating serological assays using sera that represent the target population.

Although it has been recommended to graphically present test results (Greiner and Gardner, 2000), other studies that present a frequency distribution with the results for *T. gondii* antibodies in cattle are scarce. In Serbia, cattle (n = 611), sheep (n = 511) and pigs (n = 605) were tested for *T. gondii* antibodies using a MAT (Klun et al., 2006). Animals were classified as seropositive with a MAT titer ≥ 1:25, but in the frequency distributions a split can be observed at 1:200 for sheep and pigs. For cattle, similar to our findings, no split is present and high titers are not detected. In Argentina, cattle (n = 90) were tested for *S. cruzi*, *N. caninum* and *T. gondii* by IFAT (More et al., 2008). For all three assays the cut-off was set at ≥ 1:25. The distributions for *S. cruzi* and *N. caninum* showed a split at 1:50. The distribution for *T. gondii* did not show any split and again, no high titers against *T. gondii* were detected. In addition, even more authors report that the highest titers for cattle are low compared with the highest titer for *T. gondii* in other animal species (Bekele and Kasali, 1989; Matsuo and Husin, 1996; Cabannes et al., 1997; Pita Gondim et al., 1999). The findings in these papers demonstrate that the problem with the lack of discriminative power is not limited to our ELISA, but happens with different types of serological assays. Maybe cattle become infected but are capable of clearing the infection, after which their antibody titer declines. In that case you would still expect to find some recently infected animals at their peak antibody concentration, but it has been described that cattle infected later in life only develop low titers (Dubey et al., 1985). Possibly, the cell-mediated IFN-γ response, which is known to be important in immunity against *T. gondii* in mice (Suzuki et al., 1988; Suzuki and Remington, 1988, 1990; Parker et al., 1991) and sheep (Innes et al., 1995), predominates in older cattle. Consequently, the distribution for the positive population will show a relatively low mean logODc-value as it consists of animals that were infected early in life in which the titers have declined, and of animals that got infected later in life in which a high antibody titer does not develop. Discriminatory power may be further reduced by an increased mean logODc-value for the negative population due to a high prevalence of cross-reactive pathogens.

Since our main goal was to gain an indication of the role of beef in human infections with *T. gondii* and as antibodies are not infectious, the correlation between the presence of antibodies and detection of parasites by PCR was studied. Although serological results and detection of parasitic DNA correlates well in sheep (Opsteegh et al., 2010b) they were found completely discordant in cattle for both ELISA and Toxo-Screen DA. PCR detection of *T. gondii* in seronegative cattle has been described previously. Santos et al. (2010) reports two *T. gondii* PCR-positive brains from serologically negative cattle. More et al. (2008) found two heart samples positive by PCR although one was negative in a MAT, Gottstein et al. (1998) found four fetal brains positive by PCR, although only two were serologically positive and Wyss (1999) reports nine PCR-positive cattle out of which only two were also positive in ELISA. Possibly only recently infected animals, which have not yet developed antibodies, have a parasite load high enough to be detectable by direct assays. Seropositive animals may either have cleared their infection, have parasites present in unusual locations such as the intestines (Dubey, 1992), or have parasites present at a concentration below the detection limits of the current assays. This hypothesis of recent infections is supported by the fact that all detection of *T. gondii* in seronegative cattle was by PCR and not by bioassay (Dubey and Streitel, 1976; Dubey et al., 2005). In PCR the initial phase with tachyzoites would be detectable however, because

tachyzoites are less infective for cats (Dubey and Frenkel, 1976) and only occasionally survive the artificial digestion that precedes mouse inoculation (Dubey, 1998a), they are less likely to be detected by bioassay. Another difference between PCR and bioassay detection is the ability to detect non-viable parasites by PCR. In our opinion, the results nevertheless demonstrate that viable parasites can be present in cattle as parasites must have been multiplying before becoming detectable in the heart. It does, however, mean that the prevalence of PCR-positive cattle is likely to over-estimate the prevalence of cattle carrying live parasites. This over-estimation depends on the duration of live versus dead parasites being detectable, which is unknown at this point but could be studied by development of an mRNA detection system to differentiate live from dead parasites. If it is indeed predominantly tachyzoites, and not tissue cysts, that are present viably in infected cattle, it remains to be studied whether beef from these cattle is infectious to humans.

In conclusion, we have shown that it is difficult to develop a serological assay that is sufficiently discriminatory to reliably detect the presence of antibodies against *T. gondii* in cattle. This is probably caused by the fact that cattle, in contrast to other animals, do not show high titers against *T. gondii*. This could be an effect of the alleged capability of cattle to clear the infection. This idea that cattle can clear their infection could also explain the finding that cattle positive for *T. gondii* by direct detection are more likely to be seronegative as clearance is likely to occur in line with seroconversion. Our results suggest that the risk of human infection from seropositive cattle is low and that, if detected parasite DNA represents the presence of infectious *T. gondii*, the risk of infection is higher from seronegative cattle. In cattle the seroprevalence of *T. gondii* cannot be used as an indicator of the number of cattle carrying tissue cysts.

Acknowledgements

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Chapter 5

Age-related *Toxoplasma gondii* seroprevalence in Dutch wild boar inconsistent with lifelong persistence of antibodies

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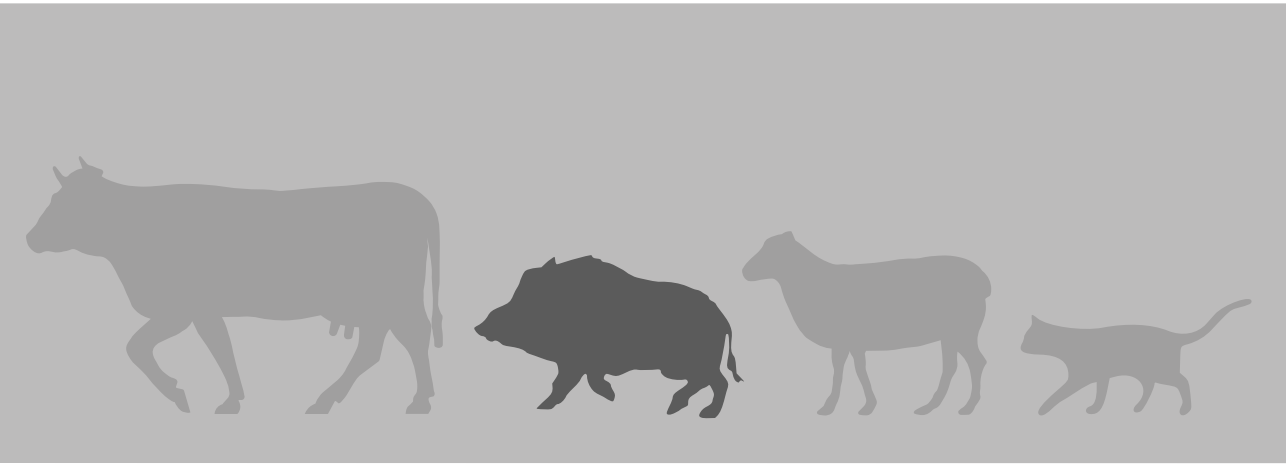

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Toxoplasma gondii is an important zoonotic pathogen that is best known as a cause of abortion or abnormalities in the newborn after primary infection during pregnancy. Our aim was to determine the prevalence of *T. gondii* in wild boar to investigate the possible role of their meat in human infection and to get an indication of the environmental contamination with *T. gondii*. The presence of anti-*T. gondii* antibodies was determined by in-house ELISA in 509 wild boar shot in 2002/2003 and 464 wild boar shot in 2007. Most of the boar originated from the "Roerstreek" (n = 673) or the "Veluwe" (n = 241). A binormal mixture model was fitted to the log-transformed optical density values for wild boar up to 20 months old to estimate the optimal cut-off value (-0.685) and accompanying sensitivity (90.6%) and specificity (93.6%). The overall seroprevalence was estimated at 24.4% (95% CI: 21.1-27.7%). The prevalence did not show variation between sampling years or regions, indicating a stable and homogeneous infection pressure from the environment. The relation between age and seroprevalence was studied in two stages. Firstly, seroprevalence by age group was determined by fitting the binary mixture model to 200 animals per age category. The prevalence showed a steep increase until approximately 10 months of age but stabilized at approximately 35% thereafter. Secondly, we fitted the age-dependent seroprevalence data to several SIR-type models, with seropositives as infected (I) and seronegatives as either susceptible (S) or resistant (R). A model with a recovery rate (SIS) was superior to a model without a recovery rate (SI). This finding is not consistent with the traditional view of lifelong persistence of *T. gondii* infections. The high seroprevalence suggests that eating undercooked wild boar meat may pose a risk of infection with *T. gondii*.

Introduction

Toxoplasma gondii is an important zoonotic protozoan with a worldwide distribution that may cause abortion or abnormalities in the newborn. Cats are the definitive host of *T. gondii* and shed millions of oocysts into the environment after a primary infection. *T. gondii* infection is probably of limited clinical importance in wild boar: Severe clinical toxoplasmosis is considered rare in pigs (Dubey, 2009b), and although decreased reproductive performance was observed in *T. gondii* seronegative—and therefore at risk for primary infection—wild boar (Ruiz-Fons et al., 2006), there are no reports of clinical toxoplasmosis in wild boar. However, infected wild boar are a source of infection for people if their meat is eaten undercooked (Choi et al., 1997). In addition, the prevalence in wild boar gives an indication of the environmental contamination, since they acquire their infection from contact with soil or by ingesting infected rodents or birds.

The *T. gondii* seroprevalence for the Dutch human population has decreased from 40.5% in 1995/1996 to 26.0% in 2006/2007 (Hofhuis et al., 2011). This is thought to be an effect of the decreased prevalence in consumption animals, especially in pigs, due to increased intensive indoor farming. A stable infection pressure from the environment is suggested by the unchanged seroprevalence in sheep when compared to studies in the eighties (Opsteegh et al., 2010c). However, differences may have been missed due to methodological differences between studies, for example the cut-off value used in the serological assay or the number of confounders corrected for in the analysis. Therefore, we chose to compare the seroprevalence of *T. gondii* in wild boar for two years within the same study.

An in-house ELISA was used to test sera from 973 hunted wild boar originating from 2002/2003 and 2007. Because of a lack of appropriate reference sera a cut-off value was selected from a binormal mixture model fitted to the log-transformed optical density-values (Opsteegh et al., 2010c), and used to score wild boar positive or negative. Seroprevalence over sampling years and regions was subsequently compared by logistic regression analysis. The age-dependent seroprevalence was additionally estimated by fitting the mixture model per age category, and interpreted by fitting various compartmental infection models.

Materials and Methods

Study population and samples

Wild boar are omnivorous animals, although their diet consists mostly of vegetable matter (Schley, Roper, 2003). They eat, for example, mast, roots, green plant matter, berries, and agricultural crops, but also fungi, earthworms, insects, eggs, small rodents and birds. Rooting behavior takes up much of their time resulting in intensive soil contact. In The Netherlands the breeding season starts around September, and between 1 and 11 piglets are born approximately 115 days later. In The Netherlands wild boar populations are tolerated in only two areas: 60 wild boar in the "Roerstreek" in the south on the border with Germany, and between 600 and 800 wild boar on the "Veluwe" in the centre of The Netherlands. In both areas the population is controlled by hunting, and in other areas all wild boar are shot. The landscape is similar in both areas, and is characterized by forest, moors and heath, pools and

drift sand. Although *Felis silvestris* has been spotted incidentally in The Netherlands (Canters et al., 2005; Mulder, 2007) we assume that domestic and stray cats are the predominant source of oocysts in both areas.

Since 1994, serum samples of 60-80 animals randomly selected from the thousands of wild boar hunted on the Veluwe, and of all wild boar hunted in the Roerstreek are collected yearly at the Animal Health Service in Deventer. These sera are tested for antibodies against SVD-, PR-, FMD-, and CSF-virus and sent to the RIVM to test for antibodies against *Trichinella spiralis* (Elbers et al., 2000). At the RIVM these samples are stored at -20°C. Location, sex, and age in months as estimated from dental development are recorded. All samples available from the years 2002-2003 (n = 509) and 2007 (n = 464) were included in this study. Most samples originated from the "Roerstreek" (n = 673) and the "Veluwe" (n = 241). A small number of samples (n = 30) came from other areas, mostly from wild boar roaming up North from the Roerstreek, or across the German border into Gelderland and Overijssel.

Serological assay

Sera were tested by in-house indirect ELISA and optical density (OD)-values corrected for plate-to-plate variation as described previously (Opsteegh et al., 2010c), but with the conjugate replaced by 1:12.500 diluted polyclonal rabbit anti-swine HRP-labeled immunoglobulins (Dako, Heverlee, Belgium). The six control sera included on each plate varied in OD-value from 0.10 to 1.25.

Data analyses

Binormal mixture model to estimate cut-off

A binormal mixture model (Opsteegh et al., 2010c) was fitted to the $\log_{10}$-transformed corrected OD-values for wild boar up to 20 months of age (n = 722) to estimate the cut-off value at which the number of correctly scored animals is highest, and accompanying sensitivity and specificity. Analysis was limited to wild boar up to 20 months of age, as initial analysis including all wild boar did not satisfactorily fit the data.

Logistic regression analysis to compare seroprevalence by region and year

The cut-off value obtained from the binormal mixture model was subsequently used to score wild boar as positive or negative. The apparent prevalence ($AP = pos/n$, where 'pos' is a random variable representing the number of positive animals in a sample of size n from the wild boar population) was calculated and corrected for sensitivity (Se) and specificity (Sp) using the Rogan-Gladen estimator, yielding the true prevalence ($TP = (AP + Sp - 1) / J$, with $J = Se + Sp - 1$) (Rogan, Gladen, 1978). Confidence intervals were calculated using the normal approximation $TP \pm 1.96 \sqrt{var(TP)}$, with $var(TP) = AP(1-AP) / nJ^2$ (Greiner, Gardner, 2000).

Seroprevalence was compared over years and regions. The relation between apparent prevalence and sex, age and sampling season was additionally studied as these factors are possible confounders. Animals were classified into 10 equal percentile age categories for univariable analysis, but age was included as a continuous variable in logistic regression analysis. Seroprevalence was checked univariably for differences among groups by Pearson's χ^2-test. Region, year, and all factors significant at the 85% confidence level, were included in logistic regression analysis (SPSS 18.0, SPSS Statistics, Chicago, IL, USA). Odds-ratios with 95% confidence intervals based on likelihood ratio statistics are reported.

Mathematical model for relation between age and seroprevalence

Using the obtained binormal mixture model a direct estimate of the true seroprevalence per age category was obtained, making correction for sensitivity and specificity unnecessary. First the *n* animals were sorted by age. Then age groups of between 100 and 200 animals were composed according to *A(i)={animals* max*(i-100,1) to* min*(i+99,n)}*, where *1< i<n*. For each group *A(i)* we determined the average age *a(i)*. Between every increase of *i*, animals of the same age were shuffled randomly in order to compensate for systematic bias. The seroprevalence *c(i)* for each age group *A(i)* was determined by fitting the binormal mixture model with means and standard deviations as obtained before, but leaving the mixing parameter to be estimated. For each group *A(i)* the estimated mixing parameter *c(i)*, i.e. the seroprevalence, was plotted against their mean age *a(i)*.

Various compartmental infection models (Fig. 5.1: SI, SIS, SI/SR, SIR, SIRS; with S being susceptibles, I being infecteds, i.e. seropositive animals, and R being animals resistant to infection without antibodies) were fitted against the observed age-seroprevalence data *(a(i),c(i))* using a least squares approach.

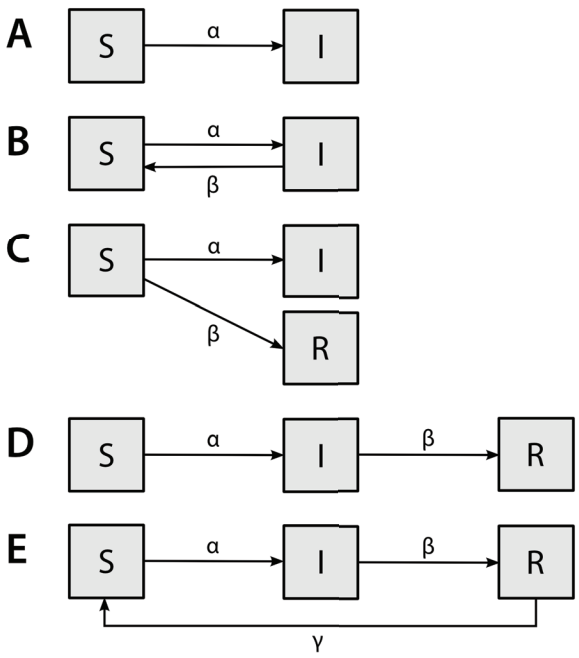

Figure 5.1 The compartmental infection models considered. a) SI model; only infection, b) SIS model; reversion to susceptible possible, c) SI/SR model; either infection or resistance, d) SIR model; some time after infection resistance occurs, e) SIRS model; it is possible to lose resistance and regain susceptibility.

In steady state, the age- and time dependent dynamics and initial conditions for the SIR-model (Diekmann, Heesterbeek, 2000) are described by

$$\left(\frac{\partial}{\partial a}+\frac{\partial}{\partial t}\right)S(t,a)=-(\mu(a)+\alpha)S(t,a), \qquad S(t,0)=B, \quad S(0,a)=S_0(a),$$

$$\left(\frac{\partial}{\partial a}+\frac{\partial}{\partial t}\right)I(t,a)=\alpha S(t,a)-(\mu(a)+\beta)I(t,a), \quad I(t,0)=0, \quad I(0,a)=I_0(a),$$

$$\left(\frac{\partial}{\partial a}+\frac{\partial}{\partial t}\right)R(t,a)=\beta I(t,a)-\mu(a)R(t,a), \qquad R(t,0)=0, \quad R(0,a)=R_0(a),$$

Here, the transition rates α ($S\rightarrow I$) and β ($I\rightarrow R$) are time and age independent, B is the number of births per month, and $\mu(a)$ is an age-dependent death rate. The quantities at time zero are considered known, but not needed later. We assume that the population is in steady state, i.e.

$$\begin{aligned} s'(a)&=-\alpha s(a), & s(A)&=B/N(A)=1,\\ i'(a)&=\alpha s(a)-\beta i(a), & i(A)&=0,\\ r'(a)&=\beta i(a), & r(A)&=0. \end{aligned}$$

every quantity is time-independent. Also, define $N=S+I+R$ and set $s(a)=S(a)/N(a)$, $i(a)=I(a)/N(a)$ and $r(a)=R(a)/N(a)$, then

Note that the birth rates and death rates have conveniently dropped out of the equations. Also note that we have set the fraction of susceptibles equal to one at age A, the age at which newborns are first exposed to the environment (this involves the slight approximation $B=N(A)\approx N(0)$). The solution to this system of equations for the seroprevalence $i(a)$ is found to be

$$i(a)=\frac{\alpha}{\alpha-\beta}\left(e^{(A-a)\beta}-e^{(A-a)\alpha}\right).$$

Transition rates α and β and age A at which seroprevalence was 0% were estimated using a least-squares fit to the seroprevalences grouped by age. Model fit was assessed by calculation of R^2 values. The other compartmental models were obtained by reduction of the described SIR-model (the fit to the SIRS model reduced to a SIS model).

Results

Cut-off and seroprevalence using binormal mixture model

After correction for plate-to-plate variation and log-transformation, the frequency distribution of optical density (OD) values was drawn. The observed distribution of log-transformed OD-values was best described assuming a mixture of two distributions (Fig. 5.2). Based on this mixture, the seroprevalence for the wild boar included (up to 20 months of age) was estimated at 20.5%. The cut-off value at which the number of animals scored correctly is maximal was

estimated at -0.685, with an AUC for the ROC-curve of 0.975, a sensitivity of 90.6% and a specificity of 93.6%.

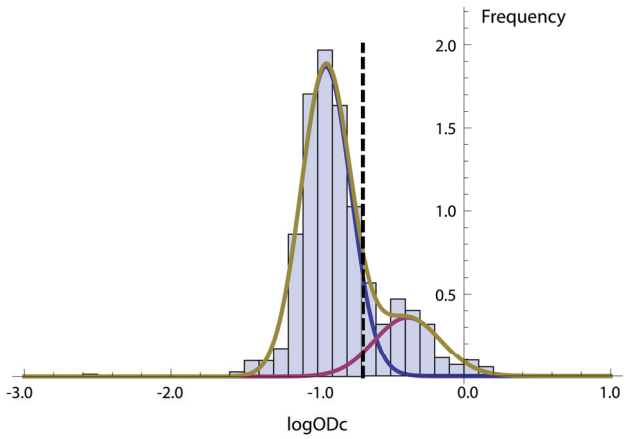

Figure 5.2 Frequency distribution of observed $\log_{10}$-transformed ODc-values in *T. gondii* ELISA for wild boar up to 20 months of age (n = 722) (bars), distributions fitted using the binormal mixture model (lines), and cut-off value (logODc = -0.685) (vertical dashed line).

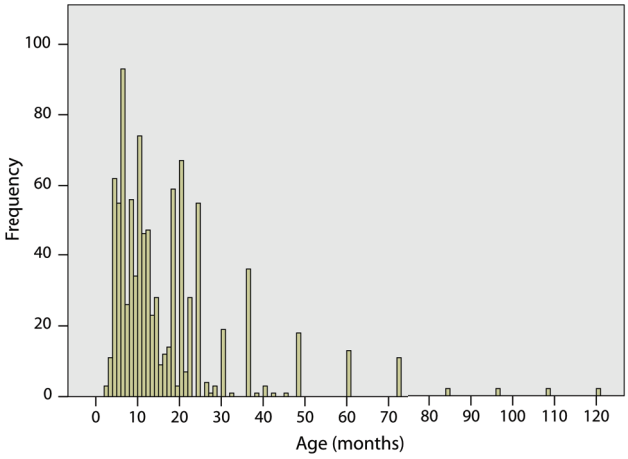

Figure 5.3 Frequency distribution for estimated age in months for 932 wild boar

Seroprevalence and risk factors using logistic regression analysis

Estimated age was available for 932 animals (Fig. 5.3), region of origin and sampling season for 944, and sex for 762 animals. For all wild boar tested (n = 973) the apparent prevalence was 26.9% and true prevalence 24.4% (95% CI: 21.2-27.7%). Univariable analysis showed significant differences in seroprevalence by age category (Table 5.1, $p < 0.0005$), and a significant relation with region and sampling season at the 85% confidence level (Table 5.2).

Sampling season was not significant in the logistic regression analysis ($p = 0.053$) and left out of the final model. The final model with region, year and age including all wild boar showed a significant increase in seroprevalence with age in months (OR 1.02, 95% CI: 1.01-1.03), but no regional or temporal differences (Table 5.3). The model fitted the data (Hosmer and Lemeshow test (Hosmer, Lemesbow, 1980), $p = 0.068$).

Table 5.1 Wild boar serum samples positive for antibodies against *T. gondii* and mean age (months) by age category.

Age category	Mean age	Positive (%)	Total
≤ 5	4.3	13 (9.9)	131
6	6.0	17 (18.3)	93
7-8	7.7	24 (29.3)	82
9-10	9.7	32 (29.6)	108
11-12	11.5	24 (25.8)	93
13-15	13.8	13 (21.7)	60
16-19	17.6	26 (29.5)	88
20-22	20.6	30 (29.4)	102
23-30	25.7	38 (46.3)	82
≥ 31	51.9	30 (32.2)	93
Total	16.4	247 (26.5)	932

Table 5.2 Wild boar serum samples positive for antibodies against *T. gondii* by sampling year, region, sampling season and sex; and *p*-values for Pearson's χ^2-statistic per variable.

Variable	Category	Positive (%)	Total	*p*-value
Sampling year	2002/2003	134 (26.3)	509	
	2007	128 (27.6)	464	0.658
Region	Roerstreek	171 (25.4)	673	
	Veluwe	67 (27.8)	241	
	other	15 (50.0)	30	0.011
Sampling season	winter	54 (24.7)	219	
	spring	17 (22.4)	76	
	summer	75 (24.4)	308	
	autumn	107 (31.4)	341	0.119
Sex	male	113 (28.7)	394	
	female	100 (27.2)	368	0.350

Age-seroprevalence relation

The seroprevalence by mean age per age group showed a steep increase at first, but seemed to stabilize at approximately 35% at 10 months of age (Fig. 5.4). The fitted compartmental models are shown in Figure 5.4 and the estimated transition rates, age with prevalence 0%, and model fit in Table 5.4. The SIRS model is not shown; the fit yielded γ=1, and other parameters the

same as the SIS model. Then, the expression for the prevalence is the same as that for the SIS model. Also, parameter estimates for the SI/SR were such that again we obtained equivalency to the SIS model. The SIS model, in which animals can revert to susceptible state, fitted the data best (R^2-value of 0.88, Table 5.4). Using this model the incidence rate is estimated at 0.050 per month, while each month 0.11 of infected animals become susceptible again. This results in an average time being seropositive of 9 months. Using this model, the seroprevalence is estimated at 0% until 2.5 months of age.

Table 5.3 Odds ratios for sampling year, region and age in months as risk factors for *T. gondii* seroprevalence in wild boar (n = 932).

Variable	Category	OR	95% CI	p-value
Sampling year	2002/2003	reference		
	2007	1.07	0.79-1.45	0.647
Region	Roerstreek	reference		
	Veluwe	1.05	0.75-1.49	0.768
	other	2.33	0.92-5.89	0.074
Age (months)		1.02	1.01-1.03	0.001

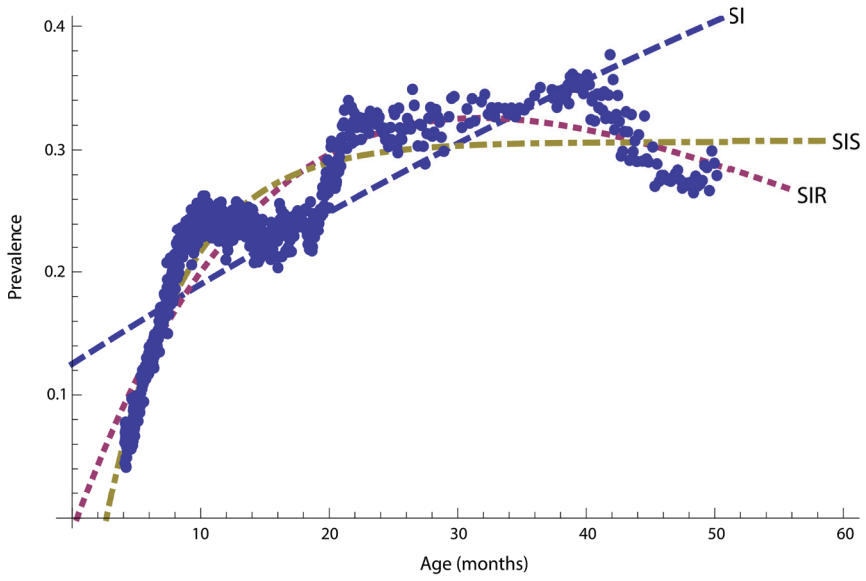

Figure 5.4 *T. gondii* seroprevalence in wild boar by age as estimated by fitting the binormal mixture model to age groups including 100-200 animals plotted at the mean age of the group (dots); and predicted seroprevalence by age using SI, SIR and SIS compartmental infection models.

Table 5.4 Transition rates (α, β), age in months with seroprevalence 0% (A), average time in preceding state in months (T), model fit (R^2), and prevalence equation for SIR, SI, and SIS-model fitted to the age-related *T. gondii* seroprevalence in 932 wild boar.

Model	Parameter	Value	T	R^2	Prevalence
SIR	Rate of infection	α=2.8e-2	36	0.85	$i(a)=\frac{\alpha}{\alpha-\beta}\left(e^{(a-A)\alpha}-e^{(a-A)\beta}\right)$
	Rate of resistance	β=3.5e-2	29		
	Age with prevalence 0%	A=2.3e-1			
SI	Rate of infection	α=7.6e-3	131	0.63	$1-e^{(A-a)\alpha}$
	Age with prevalence 0%	A=-17.6			
SIS	Rate of infection	α=5.0e-2	20	0.88	$\frac{\alpha}{\alpha+\beta}\left(e^{(A-a)(\alpha+\beta)}-1\right)$
	Rate of reversion	β=1.1e-1	9		
	Age with prevalence 0%	A=2.5			

Discussion

It was our aim to study the seroprevalence in wild boar and study temporal and regional variations therein. We used our in-house ELISA to test sera and selected a cut-off value with accompanying test characteristics by binormal mixture analysis. Logistic regression analysis showed that the prevalences found did not differ significantly over the sampling years or between regions. This indicates a stable and homogeneous infection pressure from the environment. The observed seroprevalence in wild boar (24.4%) is much higher than the seroprevalence of approximately 3% in Dutch outdoor-reared pigs (Kijlstra et al., 2004; van der Giessen et al., 2007), but is much lower than the seroprevalence of almost 60% in fattening pigs in The Netherlands in the sixties (van Knapen et al., 1982), and is comparable to the seroprevalence in wild boar from Champagne-Ardenne (Richomme et al., 2009), Czech republic (Bartova et al., 2006), and Austria (Edelhofer et al., 1996).

Using the binormal mixture model, estimates of true prevalence were obtained directly for the different age groups. Plotting these seroprevalences against the mean ages of the groups showed a steep increase in seroprevalence up to 10 months, but a stable situation thereafter. *T. gondii* infection is generally believed to persist lifelong in most hosts (Tenter et al., 2000), and stable OD-values and persistence of tissue cysts has been demonstrated in pigs up to 1 to 2 years post experimental infection (Dubey et al., 1997a). However, the SI-model, that assumes a constant incidence rate among susceptibles and lifelong immunity, did not fit the observed seroprevalence by age curve well. The SIS-model, that includes reversion to susceptible after infection, fitted the data much better. This suggests a loss of antibodies that may have been preceded by a loss of tissue cysts. However, a model with the same fit was also obtained by moving animals from susceptible into either infected or resistant (SI/SR-model), and could probably also arise by incorporating age-dependent parameters, or a combination of these effects. We prefer the SIS model over the SI/SR or SIR model, for the reason that the 'R' compartment is hard to interpret: The animal is supposed to be immune without presence of antibodies. In addition, the rate at which resistant animals become susceptible again is estimated at one in the SIRS-model, which rejects the hypothesis of temporary resistance to infection after antibodies have waned. Including age-dependent parameters in the model (of

unknown age dependency!) adds to the complexity – a simpler model with good explanatory power may be preferred. Temporal variation in infection pressure from the environment can also influence the age-prevalence relation: For example, the prevalence in older people may be higher as they have consumed meat from animals with a high prevalence from before animal husbandry was industrialized. However, as the seroprevalence was shown stable over sampling years that are further apart than the life-expectancy of wild boar, such an effect is unlikely here, and therefore the use of compartmental infection models that inherently assume a constant infection pressure is appropriate. Observations inconsistent with lifelong persistence have been reported previously: Several authors report that no statistically significant effect of age on seroprevalence of *T. gondii* in wild boar was observed (Antolova et al., 2007; Diderrich et al., 1996; Dubey et al., 1997b; Gauss et al., 2005; Richomme et al., 2010), whereas only one study did find a significantly higher prevalence in adult wild boar (Ruiz-Fons et al., 2006). In addition, tissue cysts were detected by mouse bioassay in the heart of only 50% of 20 seropositive (MAT titer ≥ 1:24) wild boar in France (Richomme et al., 2009), and although paired results per wild boar are not presented, the prevalence of *T. gondii* by bioassay (2%) was much lower than by Sabin Feldman Dye test (15%) in a Czech study (Hejlicek et al., 1997).

Seroprevalence could only be calculated for animals of at least 5 months of age as not enough younger boar were sampled. There are two reasons why the predicted age-prevalence relation cannot be extrapolated to younger ages. Firstly, abandoning the nest and weaning occur gradually (Jensen, 1986; Jensen, Recén, 1989). Therefore, exposure to environmental oocysts and, consequently, the infection rate are still increasing at very young age. Secondly, young piglets may be protected against infection by maternal antibodies, leading to a measurable but transient antibody titer that protects them against infection but is not the result of infection. As weaning of the entire litter is complete at on average 17.2 weeks after birth for domestic pigs in a semi-natural environment (Jensen, Recén, 1989) both an estimated seroprevalence of 0% until 2.5 months of age (SIS-model), and a seroprevalence of 13% at age 0 (SI-model) due to maternal antibodies are possible, but, as explained, the actual seroprevalences may differ.

In conclusion, we have shown a high seroprevalence of *T. gondii* in wild boar that was equal over the sampling years and regions. As the seroprevalence is high, consumption of raw or undercooked wild boar meat may pose an important risk of infection. The stable seroprevalence indicates a constant infection pressure from the environment. In addition, we found an age-seroprevalence relation that is inconsistent with a constant infection rate in combination with lifelong immunity. A model including reversion to susceptible state fitted the data nicely. This may mean that a negative serological test does not exclude prior exposure to *T. gondii* and, if the loss of antibodies is preceded by a loss of tissue cysts, that wild boar can clear their infection. But before drawing these conclusions, the actual mechanism behind the stabilization of seroprevalence at around 35% requires further investigation, for example by longitudinal follow-up of infected wild boar regarding presence of antibodies and tissue cysts.

Acknowledgements

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Chapter 6

A quantitative microbial risk assessment for meatborne *Toxoplasma gondii* infection in The Netherlands

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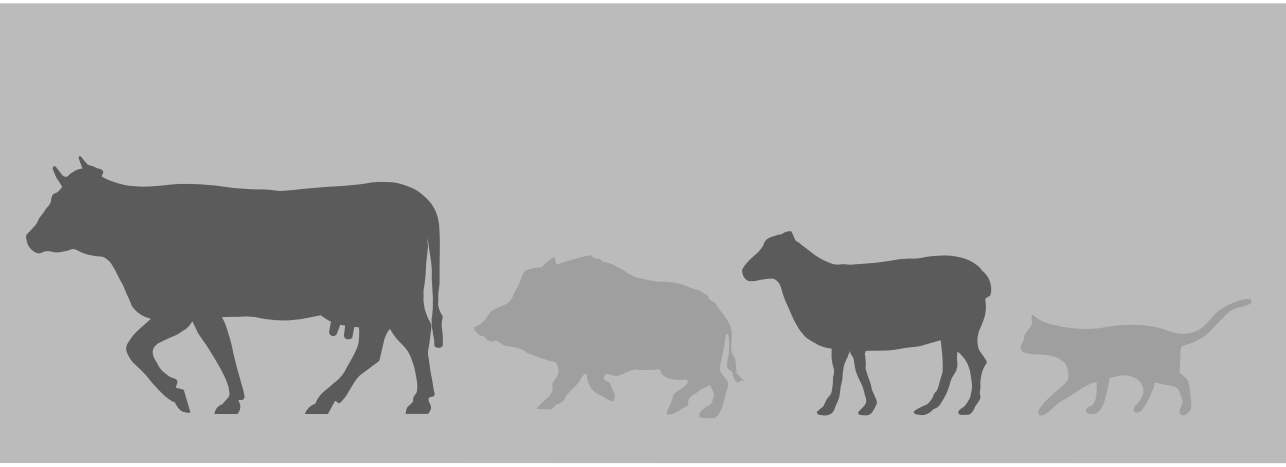

Revised Version

Toxoplasma gondii is an important foodborne pathogen, and the cause of a high disease burden due to congenital toxoplasmosis in The Netherlands. The aim of this study was to quantify the relative contribution of sheep, beef and pork products to human *T. gondii* infections by Quantitative Microbial Risk Assessment (QMRA). Bradyzoite concentration and portion size data were used to estimate the bradyzoite number in infected unprocessed portions for human consumption. The reduction factors for salting, freezing and heating as estimated based on published experiments in mice, were subsequently used to estimate the bradyzoite number in processed portions. A dose-response relation for *T. gondii* infection in mice was used to estimate the human probability of infection due to consumption of these originally infected processed portions. By multiplying these probabilities with the prevalence of *T. gondii* per livestock species and the number of portions consumed per year, the number of infections per year was calculated for the susceptible Dutch population and the subpopulation of susceptible pregnant women. QMRA results predict high numbers of infections per year with beef as the most important source. Although many uncertainties were present in the data and the number of congenital infections predicted by the model was almost twenty times higher than the number estimated based on the incidence in newborns, the usefulness of the advice to thoroughly heat meat is confirmed by our results. Forty percent of all predicted infections is due to the consumption of unheated meat products, and sensitivity analysis indicates that heating temperature has the strongest influence on the predicted number of infections. The results also demonstrate that, even with a low prevalence of infection in cattle, consumption of beef remains an important source of infection. Developing this QMRA model has helped identify important gaps of knowledge and resulted in the following recommendations for future research: collect processing-effect data in line with consumer style processing and acquire product specific heating temperatures, investigate the presence and concentration of viable bradyzoites in cattle, determine the effect of mincing meat on bradyzoite concentrations using actual batch sizes, and obtain an estimate of the fraction of meat that has been frozen prior to purchase. With more accurate data this QMRA model will aid science-based decision-making on intervention strategies to reduce the disease burden from meatborne *T. gondii* infections in The Netherlands.

Introduction

Toxoplasma gondii is a protozoan parasite belonging to the Phylum of Apicomplexa. Its sexual reproduction only occurs in members of the family Felidae, the definitive hosts, and results in shedding of millions of oocysts within 1 to 3 weeks after primary infection. All warm-blooded animals can function as intermediate hosts and allow two stages of parasite multiplication. The first, rapidly multiplying, stage of the parasite is known as tachyzoite. After 1 to 3 weeks tachyzoites transform into bradyzoites: the slowly replicating form that is contained in tissue cysts (Dubey et al., 1998). Ingestion of oocysts or of meat containing tissue cysts can result in infection of both definitive and intermediate hosts. Tachyzoites are considered of limited importance in oral transmission because they are present at high concentrations for a short time only and, if present, only occasionally survive digestion (Dubey, 1998a). Therefore, in humans, postnatally acquired infection with *T. gondii* can essentially occur via two routes: ingestion of oocysts shed by felines, or through the consumption of undercooked meat containing tissue cysts. Infection usually remains asymptomatic, but if a primary infection is acquired during pregnancy *T. gondii* tachyzoites can be transmitted to the foetus (Dunn et al., 1999). This congenitally acquired infection can have serious consequences such as abortion, stillbirth, neonatal death, signs of encephalomyelitis or hydrocephalus at birth, or the development of ocular toxoplasmosis later in life (Tenter et al., 2000). Besides the threats to unborn children, *T. gondii* infection may lead to serious illness in immunocompromised patients such as AIDS and transplant patients (Tenter et al., 2000). In addition, postnatally acquired infections are less harmless than previously perceived: about two thirds of ocular toxoplasmosis cases may have been acquired postnatally (Gilbert, Stanford, 2000), and evidence for a link between *T. gondii* infection and schizophrenia (Yolken et al., 2009) or behavioral changes (Flegr, 2007) is accumulating.

Based on the incidence of 2 per 1,000 live-borns in The Netherlands in 2006 the disease burden of congenital toxoplasmosis alone was estimated at 2,300 disability-adjusted life-years (DALYs) per year which makes *T. gondii* one of the most important foodborne pathogens in The Netherlands (Kortbeek et al., 2009). However, this disease burden still underestimates the total burden of *T. gondii* infections because postnatally acquired infection with *T. gondii*, and thus the risk of ocular toxoplasmosis, is high based on the overall seroprevalence of 26 percent in the Dutch population (Hofhuis et al., 2011). Because of this high disease burden, *T. gondii* has recently been ranked second on a list of 86 emerging zoonotic pathogens in The Netherlands (Havelaar et al., 2010), and it urges the need for intervention. As treatment of (congenital) toxoplasmosis is difficult (the effectiveness of the drugs still needs to be proven (Chene, Thiebaut, 2009; Gilbert, 2009; Thiebaut et al., 2007)), it is of great importance to focus on prevention of infection. In Europe meat is the most important source of infection with *T. gondii*: 30 to 63 percent of new infections in pregnant women could be attributed to consumption of undercooked or cured meat (Cook et al., 2000). Risk factors for *T. gondii* infection for the Dutch population are living in the Northwest, ethnicity, living in urban areas, owning a cat and the consumption of raw pork (Hofhuis et al., 2011).

To develop effective prevention strategies the relative importance of the various sources of infection needs to be known. A suitable tool for this is risk assessment which is defined by Haas et al. (1999) as "the qualitative or quantitative characterization and estimation of potential adverse health effects associated with exposure of individuals or populations to hazards". A qualitative risk assessment to determine the relative importance of various types of meat in

T. gondii exposure has shown that the processing steps that affect the viability of *T. gondii*, including salting, freezing and heating, influence the risk of infection from these products (Mie et al., 2008). Recently, a technique was developed to quantify bradyzoites in tissue samples (Opsteegh et al., 2010b), which enables a comparative quantitative microbial risk assessment (QMRA) approach for meatborne *T. gondii* infection in analogy to comparative QMRAs for other pathogens such as *Campylobacter* and *Escherichia coli* (Evers et al., 2008; Kosmider et al., 2010). These models aim to estimate the exposure to pathogens and compare the importance of each source of infection. In a QMRA, results from various types of research (such as consumption surveys, prevalence studies and pathogen survival experiments) are integrated to add to the understanding of the transmission and thereby support risk-based decision making.

The objective of this study was to quantify the relative contribution of sheep, beef and pork products to human *T. gondii* infections in The Netherlands by comparative QMRA, both in the total Dutch population and in the subpopulation of pregnant women.

Method

Model building

The QMRA model was set up in accordance with guidelines set by the Codex Alimentarius Commission (1999) and involved the following steps (Fig. 6.1): 1. The number of bradyzoites per infected portion was calculated by combining portion sizes with the concentration of bradyzoites in meat. 2. The effect of each step of processing (first salting, followed by freezing and finally heating) on the number of bradyzoites per infected portion was estimated based on literature data. 3. The probability of human infection per infected portion was predicted using a dose-response relation. 4. The probability of infection per portion was obtained by multiplying the outcome of step 3 with the prevalence of *T. gondii* per livestock species. 5. The number of infections per year in the Dutch population was calculated by multiplying the probability of infection per portion with the number of portions consumed per year, and adjusted for prior immunity by multiplication with the prevalence of seronegatives. Variability in bradyzoite concentration, portion size and processing parameters was taken into account in the model, whereas uncertainty of parameters was not. To investigate the effect of input parameters on model outcome a sensitivity analysis was conducted. The effects of the most important model assumptions (i.e. mincing of meat, prevalence of infectious parasites in cattle, estimated initial doses for processing effect experiments, and dose-response parameter for mouse infection) were evaluated using alternative scenarios.

Data sources and calculations

Selection of meat products

Pig, poultry, cattle and sheep are the most consumed livestock species in The Netherlands (PVE, 2010). Other livestock species consumed are for example horse, goat and game. Consumption data on beef, sheep and pork products were obtained from the 1998 Dutch National Food Consumption Survey (DNFCS, 1998) and included in the QMRA. Poultry was left out of the calculations because the prevalence in poultry (chicken and turkey) is expected to be very low (no seropositives were detected among 82 broilers from several farms (van

Knapen et al., 1982)), and poultry products are assumed to be always heated to temperatures that inactivate *T. gondii*. Horse, goat and game were excluded because these species were not sufficiently present or not specified in the consumption survey. Consumption of a total of 98 pork, beef and sheep products was reported, including 6 products of non-muscle origin. These non-muscle products and products that were heated sufficiently to inactivate *T. gondii* prior to purchase (28 products, e.g. corned beef, boiled ham, luncheon meat and various sausages) or at home (14 products, e.g. shin of beef, shoulder of lamb and other products meant for stewing or braising) (van de Ven, van Trigt, 2003; Vlees.nl, 2010), were excluded from the assessment, leaving 50 products in total.

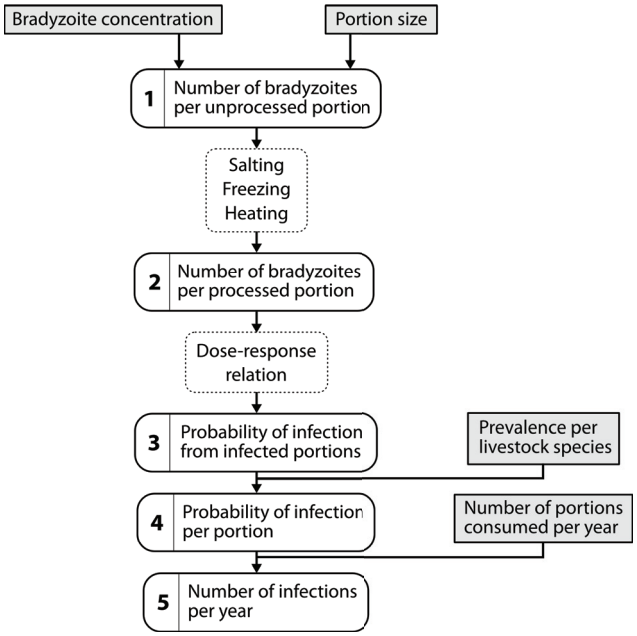

Figure 6.1 Flow diagram representing the QMRA model used to estimate the number of *T. gondii* infections in the Dutch human population.

Number of bradyzoites per unprocessed portion

Portion sizes reported by the 6,250 individuals participating in the DNFCS were used. Only people living in an institution, people with insufficient knowledge of the Dutch language and children less than 1 year old were excluded from participation in the DNFCS. If applicable, reported portion sizes were multiplied with fixed reduction factors for bone weight and weight loss during preparation (Donders-Engelen et al., 1997) because the concentration of bradyzoites used later on applies to unprepared muscle tissue. Gamma distributions were fitted to these corrected portion sizes, resulting in a specific shape (α) and scale (β) parameter per product (Appendix A). For 5 products an insufficient number of portions (<5) was consumed to fit a gamma distribution, therefore α and β parameters from comparable products were used (Appendix A). Although the maximum reported portion size was 800 g (pork unspecified) we chose to set the maximum portion size for all products somewhat

higher at 1,000 g. If a larger portion size was drawn from the gamma distribution it was automatically set at 1,000 g.

An estimate of bradyzoite concentration in meat was obtained from real-time PCR results of 35 sheep heart samples of 100 g (as described by Opsteegh et al. (2010b)). A betageneral distribution with a lower bound of 0 was fitted to the $\log_{10}$-transformed bradyzoite numbers for 100 g samples, resulting in shape parameters α_1 of 6.5, α_2 of 5.7, a minimum of 0 and maximum of 6.8. This distribution has a mean value of 3.6, or $10^{3.6}$ bradyzoites per 100 g.

Bradyzoite concentrations drawn from this distribution and divided by a 100 were multiplied with the drawn portion sizes to calculate the number of bradyzoites per unprocessed portion.

Number of bradyzoites per processed portion

Processing of meat can reduce the probability of infection from a portion by reducing the number of viable bradyzoites. To obtain estimates of the effectiveness of inactivation of *T. gondii* at given parameter values for salting, freezing and heating, logistic regression models were fitted to data from mouse infection experiments described in the literature. Three experiments that had the largest groups of mice and the best defined treatments were selected (Dubey, 1997; Dubey et al., 1990; Kotula et al., 1991). In these experiments, *T. gondii* infected meat was processed at different temperatures, durations and with different salt concentrations. Next, processed and unprocessed meat was bioassayed in groups of mice to determine the probability of *T. gondii* infection (Appendix B, C and D). The fitted logistic regression models can be used to predict the probability of a mouse becoming infected (P_{inf}) from portions processed at any given parameter value using equation 1, but the effect of processing on bradyzoite number rather than the probability of infection for mice was needed for the QMRA model. Because no measured doses were available for the mouse experiments, the reduction factor for bradyzoites was calculated indirectly. First, the combination of the logistic regression model (equation 1) and the inverse dose-response relation (equation 2b (Haas et al., 1999)) was used to calculate the initial doses in unprocessed samples (D_0) in the mouse experiments. This was done for salting, freezing and heating separately by setting the variables in the logistic regression model at control values (duration and salting concentration at zero, and temperature at 4°C for freezing and salting and 25°C for heating). Subsequently, the logistic regression model and inverse dose-response relation were used in the same way to calculate the predicted dose for processed samples (D_p) in each run of the model. The predicted dose for a processed sample (D_p) was then divided by the calculated initial dose in unprocessed samples (D_0), to obtain the reduction factor for the number of bradyzoites ($RF=D_p/D_0$). By multiplying the number of bradyzoites before processing in the portions (step 1 Fig. 1) with the calculated reduction factors, the number of bradyzoites at the end of all processing steps is calculated (step 2 Fig. 6.1). Note that, in contrast to bacteria, *T. gondii* does not grow after death of the host. Therefore only reduction and no growth can occur after slaughter.

$$P_{\text{inf}} = \frac{1}{1+e^{-(\beta_0+\beta_1 x_1+\beta_2 x_2+\beta_3 x_1 x_2)}} \qquad (1)$$

With x_1 and x_2 the variables in the logistic model and β_0–β_3 the regression coefficients of the logistic model. For heating only one variable was included, for salting a third variable and a second interaction term were included.

$$P_{\inf} = 1 - e^{-rD} \qquad (2a) \qquad \Rightarrow \qquad D = -\frac{\ln(1 - P_{\inf})}{r} \qquad (2b)$$

With P_{inf}=probability of infection, D=number of bradyzoites (dose), and r=dose-response parameter which can be interpreted as the probability of one bradyzoite to successfully initiate an infection.

The effect of salting on the probability of a mouse becoming infected was estimated from an experiment described by Dubey (1997). Treatments included temperatures of 4, 10, 15 and 20°C; salt concentrations were 0.85, 2.0, 3.3 and 6.0% and durations of salting were 3, 7, 14, 21, 28, 35, 42, 49 and 56 days (Appendix B). Mice were subcutaneously inoculated with GT1 strain infected rat brains for the 10, 15 and 20°C groups, and with Me49 strain infected mouse brains for the 4°C group. Inoculation was performed without prior artificial digestion. Estimates for the β-coefficients are presented in Table 6.1. The Hosmer and Lemeshow test for this model was not significant (χ^2 (df=8) is 6.289, p=0.615). Therefore, there was no indication of a lack of fit of the model (Dohoo et al., 2003). We used the r-value reported by the French Food Safety Agency (AFSSA, 2005) for the GT1 strain (0.011) to calculate the dose, because this strain was used in most of the treatment groups. Using the output for the logistic regression model at salt concentration 0.0%, duration 0 days, and temperature 4°C, as input for the inverse dose-response model with r=0.011 the initial dose (D_0) was calculated at 1882 bradyzoites. No indication of the dose was given with the original data (Dubey, 1997). Parameter values for salted products included in the QMRA are described in Table 6.2. Temperatures and durations in this table are the highest temperature and the sum of all preparation time given in the reference. Salt concentrations were obtained from the packaging of retail products, with the exception of dry suçuk Turkish sausage for which the salt concentration was obtained from Feiner (2006).

Table 6.1 Estimated β-coefficients for variables in the logistic regression models fitted to data from salting, freezing, and heating experiments.

Variable	Salting	Freezing	Heating
constant (β_0)	22.349	9.536	44.181
temperature (°C)	-0.412	1.269	-0.834
treatment duration[a]	-0.193	-0.021	NA[b]
NaCl conc. (%w/v)	-3.316	NA	NA
temp*duration	-0.022	-0.001	NA
temp*NaCl	NS[c]	NA	NA
duration*NaCl	NS	NA	NA
temp*duration*NaCl	NS	NA	NA

[a] in days for salting, hours for freezing, and minutes for heating; [b] NA: not applicable; [c] NS: not significant

Table 6.2 Parameters settings for salted meat products included in the QMRA.

Product	Species	NaCl (%)	Temp (°C)	Time (days)	Reference
Smoke-dried beef	Beef	3.9	20	21	(Faber et al., 1991)
Dry suçuk Turkish sausage	Beef	2.5	20	21	(Feiner, 2006)
Saveloy salami sausage	Mixed[a]	3	15	30	(Faber et al., 1991)
Salami	Mixed[a]	3.5	15	30	(Faber et al., 1991)
Bacon	Pork	3.4	5	9	(Faber et al., 1991)
Streaky rashers bacon	Pork	5	20	21	(Faber et al., 1991)
Raw smoked ham	Pork	4	20	30	(Faber et al., 1991)
Spiced and smoked sausage	Pork	3	22	5	(Faber et al., 1991)
Lean bacon	Pork	3.4	5	9	(Faber et al., 1991)
Fat bacon	Pork	3.4	20	7	(Faber et al., 1991)

[a] mixed beef and pork product

The effect of freezing on P_{inf} was estimated from an experiment described by Kotula et al. (1991). Treatments included temperatures of 4, -1, -3.9, -6.7, -8.0, -9.4, -12.2 and -15°C and durations of freezing were 64, 128, 256 minutes, 8.5, 17 hours and 1.4, 2.8, 5.6, 11.2, 16.8, 22.4, 33.6, 44.8 and 67.2 days (Appendix C). Treated meat portions were subcutaneously inoculated in mice after artificial digestion. Estimates for the β-coefficients are presented in Table 6.1. The Hosmer and Lemeshow test for this model was significant (χ^2 (df=8) is 47.694, p<0.001), indicating a lack of fit. Plots of the deviance residuals against temperature showed the presence of possible outliers (-1°C after 33.6 and 67.2 hours). Substituting the observed values for these two treatment groups by their expected values significantly reduced the deviance for this model, and resolved the lack of fit (Hosmer and Lemeshow χ^2 (df=8) is 9.718, p=0.285). With these substitutions, the β-coefficients were estimated at 12.550 (β_0), 1.674 (temperature), -0.027 (treatment duration), and -0.002 (interaction between temperature and time). We nevertheless used the β-coefficients estimated using all observed data. For r, we used the geometric mean (0.011) of 4 r-values reported by the AFSSA (2005) for 3 strains of *T. gondii* bradyzoites $(0.001535 \times 0.0023 \times 0.3772 \times 0.011)^{0.25}$, because the mice received a mixture of strains. Although the strain with the largest r-value (0.3772) may actually have the largest influence on the probability of infection, this value was also the most uncertain (95% credibility interval: 0.00067-0.947) and therefore we preferred limiting its influence by taking the geometric mean. Using the output for the logistic regression model at duration 0 days and temperature 4°C in the inverse dose-response model with r=0.011, the initial dose (D_0) was calculated at 1328 bradyzoites. With r=0.3772 the initial dose was 39. In the original data it was reported that depending on the sample the 10^{-2} up to the 10^{-5} dilution was still infective for mice (Kotula et al., 1991). This is more consistent with the initial dose estimate based on the geometric mean r-value than the dose based on the extreme r-value, and supports our decision to take the geometric mean r-value. It was assumed that processed meat products meant for sandwich preparation (9 products) are never frozen (temperature always set at 4°C and time at 0), leaving 41 products that were frozen in 42.3% of the occasions (Appendix A and Table 6.3). The percentage of frozen storage was distributed evenly over the temperatures according to the four star rating system for freezers (Table 6.3). The duration of freezing was set as a uniform distribution ranging from 1 to 30 days and included in the model in hours.

Table 6.3 Frequency distribution of storage temperatures for meat products that are occasionally frozen included in the QMRA.

Temp (°C)	Frequency (%)
4	57.7[a]
-6[b]	10.6
-12[b]	10.6
-18[b]	10.6
-24[b]	10.6

[a] based on GFK Group (Temminghoff, 2004); [b] based on international four star rating system for freezers.

The effect of heating on P_{inf} was estimated from an experiment described by Dubey et al. (1990). Treatments included temperatures of 4, 49, 52, 55, 58, 61, 64 and 67°C and durations of heating were 0.01, 3, 6, 12, 24, 48 and 96 minutes (Appendix D). Treated meat products were subcutaneously inoculated in mice after artificial digestion. Time measurement in the mouse experiments started once the designated core temperature was reached, whereas consumer data (FDA, 1999) contained only core temperatures measured directly after preparation (without indication of preparation time). Because consumer reported temperatures probably represent the maximum temperatures reached, these are most comparable to data for samples taken out of the water bath directly after reaching the designated core temperature in the mouse experiment. For that reason only the experimental data for time 0.01 min were used to estimate the effect of temperature on the probability of infection (β=-0.834, Table 6.1). The Hosmer and Lemeshow test was not significant (χ^2 (df=6) is 5.060, p=0.536). Again we used the geometric mean *r*-value (0.011), because the mice received a mixture of strains. Using the output for the logistic regression model with temperature at 25°C in the inverse dose-response model the initial dose (D_0) was calculated at 2121 bradyzoites with *r*=0.011, and at 62 bradyzoites with *r*=0.3772. In the original data it was reported that depending on the sample the 10^{-3} or the 10^{-4} dilution was still infective for mice (Dubey et al., 1990), which again, is more consistent with the initial dose estimate based on the geometric mean *r*-value. Because no preparation data were available from the DNFCS the temperature distributions for heating were based on data available online (FDA, 1999). These data consist of core temperatures measured post preparation by consumers in either minced beef or beef/pork/lamb products. For all heated minced meat products included in our model (11 products) temperatures were drawn from the minced beef distribution (yes 1, Appendix A) and for all heated non-minced products (30 products) temperatures were drawn from the combined beef/pork/lamb distribution (yes 2, Appendix A). Nine products were not heat-treated or only smoked cold in the processing steps and are consumed raw.

Dose-response relation in humans and probability of infection from infected portions

The numbers of bradyzoites per processed portion were followed by a dose-response relation to predict the probability of infection per infected portion according to equation 2a. As no human dose-response parameter value is available, we used mouse data as a surrogate. In this case *r*=0.001535 is used because this is the *r*-value for *T. gondii* type II in mouse experiments (AFSSA, 2005). Type II is the type most commonly found in humans and livestock in Western

Europe (Sibley et al., 2009) and type II was the only type found in Dutch sheep based on sequencing of the GRA6 gene (Opsteegh et al., 2010a). At the used *r*-value a dose of 452 bradyzoites results in probability of infection of 0.5 (N_{50}= ln *(0.5)* / *-r* (Haas et al., 1999)).

Prevalence per livestock species and probability of infection per portion

Not all animals and therefore not all portions are infected. The probability of infection per infected portion was multiplied by the prevalence for the livestock species to obtain the probability of infection per portion. *T. gondii* prevalence estimates for the Dutch cattle, pig and sheep population were obtained from literature. For cattle the estimated PCR prevalence of 2% was used (Opsteegh et al., 2011b). For pigs the weighted average of the seroprevalence in intensively raised pigs (prevalence 0.38%, weight 0.981) and free-range pigs (prevalence 5.62%, weight 0.019) of 0.5% was used (Bakker, 2007; van der Giessen et al., 2007). For sheep the overall seroprevalence was estimated at 27.8% (Opsteegh et al., 2010c), but we used the original data to calculate the prevalence for lambs and sheep over one year old separately. Seventy-seven out of 380 lambs (sheep<1 year) (20.4%), and 101 out of 188 sheep (>1 year) (53.7%) tested positive. These apparent prevalences were adjusted for sensitivity (0.978) and specificity (0.964) according to the Rogan–Gladen estimator (Rogan, Gladen, 1978), resulting in true prevalences of 17.7% for lambs and of 53.2% for sheep.

Number of consumed portions and number of new infections

The number of portions was estimated from 6,250 individuals that recorded their meat consumption during 2 consecutive days (DNFCS, 1998). The number of portions per product was multiplied with 365/2 to obtain the number of portions consumed in one year, and brought to Dutch population level by multiplication with 16×10^6/6,250 (Appendix A). The total number of portions of all included meat products for the total Dutch population was estimated at 5×10^9 per year. The number of portions consumed by the subpopulation of pregnant women was obtained accordingly but multiplied with 181,336/6,250 (based on the number of live born children in 2007 (181,336) (CBS, 2010)) and adjusted for the duration of pregnancy (by multiplication with 9/12), resulting in a total of 42×10^6 consumed portions per year. The number of portions consumed per year was multiplied with the mean probability of infection per portion for each product and summed for all products to obtain the number of infections for both the total Dutch population and the subpopulation of pregnant women. Because *T. gondii* infection persists in humans, uptake of an infectious dose is not relevant for already infected people (26.0% of the Dutch population (Hofhuis et al., 2011)). To account for this immunity resulting from earlier infection, the calculated number of infections per year for the Dutch population was multiplied with factor 0.74 to estimate the number of new infections per year in the susceptible population. For pregnant women, the number of infections was multiplied with factor 0.815, because the seroprevalence of *T. gondii* in women in reproductive age (15-49 years) is 19.5% (Hofhuis et al., 2011).

Sensitivity analysis

To determine the extent to which input parameters affect the outcome of the model a sensitivity analysis was conducted. Parameter values were one at a time reduced by 10% compared to the value in the base model. The number of infections for the model with the changed parameter value was subsequently compared to the number of infections in the base model (equation 3).

$$\frac{\Delta M / Mb}{\Delta P / Pb} = \frac{(Mb - Ms)/Mb}{(Pb - Ps)/Pb} = \frac{(Mb - Ms)/Mb}{(Pb - 0.9Pb)/Pb} = \frac{(1 - Ms/Mb)}{0.1} \quad (3)$$

With *Mb*=model output base scenario, *Pb*=parameter value in base scenario, *Ms*=model output alternative scenario and *Ps*=parameter value alternative scenario, in this case *Ps=0.9Pb*.

Parameters included in the sensitivity analysis were: bradyzoite concentration, portion size, salting concentration, salting temperature, salting duration, reduction factor for salting, fraction unfrozen, freezing temperature, freezing duration, reduction factor for freezing, heating temperature, reduction factor for heating, human dose-response parameter (*r*), and prevalence in livestock. Parameter values were only changed for products with an applicable parameter value (e.g. for a product that was not frozen, temperature and time of storage were not reduced by 10%). Sensitivity is calculated for both the number of infections due to all products and the number of infections due to products that have a changed parameter value.

Alternative scenarios

To investigate the effect of certain model assumptions four alternative scenarios were evaluated. First, the effect of using several animals to prepare a single meat product was taken into account for minced meat products. These are 11 heated-minced products (yes 1, Appendix A) and 5 unheated products (saveloy salami sausage, salami, filet américain, dry suçuk Turkish sausage, and spiced and smoked sausage). An assumption made in this scenario is that the batch of animals used to prepare the mass of minced meat is thus large that with prevalence >0% all portions become infected and the bradyzoite concentration decreases proportionally to the prevalence of *T. gondii* for the livestock species (e.g. in case the prevalence was 10%, now all portions are infected but with 10 rather than 100 bradyzoites per gram).

In the second scenario the prevalence for cattle was reduced from 2.0% to 0.5%, because the infectiousness of detected parasites in cattle is questioned (Opsteegh et al., 2011b). A value of 0.5% was chosen to determine whether beef or pork is more important at equal prevalence.

In the third scenario, the initial doses given in the processing experiments (D_0) were calculated on the basis of 7°C for freezing, 1°C for salting and 20°C for heating, instead of the temperatures used in the base scenario. This was done to determine the uncertainty in the model due to the estimation of the initial dose via an inverse dose-response model rather than having measured doses available.

Because the meat samples used in the freezing and heating experiments were infected with a mixture of strains, we used the geometric mean of published *r*-values. However, with reasonable doses for all strains the most infectious strain has the largest effect on the probability of infection. To evaluate the effect of choosing the geometric mean *r*-value we used *r*=0.3722 for freezing and heating in our last scenario.

Software

Logistic regression analyses on the processing-effect data were run in SPSS 18.0 (PASW Statistics, Chicago, IL). Simulations for the QMRA model were performed using @Risk 5.0.0 (Palisade Corporation, Ithaca, NY), an add-in for Microsoft Excel 2002 (Microsoft Corporation, Redmond, WA). Model output for base and alternative scenarios was based on 50,000 iterations. For the sensitivity analyses 20,000 iterations were run.

Results

Forty percent of the portions included in this QMRA were of pork origin. Beef contributed 34% of all included portions, leaving 25.2% for mixed products (beef and pork combined in one product) and 0.8% for sheep products.

Base model

According to our base model beef is responsible for 67.6% of the meatborne infections (Table 6.4). In addition, 7.1% of the predicted infections are due to mixed meat products, originating from beef and pork. Pork and sheep are estimated to be responsible for respectively 11.2% and 14.0% of the infections. Forty percent of the total number of predicted infections with *T. gondii* was caused by the consumption of 9 products that were not heated (Appendix A and E). The modelled number of infections per product is highest for filet américain (a raw minced beef product that is usually consumed on sandwiches), whereas the predicted probability of infection per portion is highest from unspecified mutton (Appendix E).

Alternative scenarios

Minced meat

When mincing of meat was included in the analysis, the total number of predicted infections increased almost 7-fold compared to the base model. The relative contribution of beef and mixed meat increased to 85.2% and 9.0% of all modelled infections, while the contributions of pork and sheep decreased to respectively 3.7% and 2.2% (Table 6.4).

Lower prevalence for cattle (0.5%)

When the prevalence for cattle was reduced to 0.5%, the total number of predicted infections halved compared to the base model. Now beef is estimated to account for 34.4% of meatborne *T. gondii* infections (Table 6.4). The estimated contribution of sheep, pork and mixed meat increased to respectively 28.3%, 22.9% and 14.4% of the infections.

Initial dose unprocessed samples

In this scenario the initial doses of bradyzoites in unprocessed samples (D_0) in the processing experiments were calculated using different temperature settings. The estimated relative contributions are similar to those in the base model, and the total number of predicted infections decreased only slightly (Table 6.4).

Dose-response parameter freezing and heating experiments

In this scenario the highest *r*-value reported for the most infectious *T. gondii* strain was used to calculate the reduction factors for freezing and heating. Both numbers of infections as well as relative contributions are highly similar to those estimated using the base model (Table 6.4).

Table 6.4 Predicted number of meatborne *T. gondii* infections in the Dutch population and the population of pregnant women per livestock species using QMRA base model and alternative scenarios.

Model	Species	No. of infections/year in total population		No. of infections/year in pregnant women		Relative contribution
		unadj.[a]	adj.[b]	unadj.[a]	adj.[b]	
Base	Beef	2,573,248	1,904,204	21,873	17,826	67.6%
	Mixed[c]	270,194	199,944	2,297	1,872	7.1%
	Pork	427,661	316,469	3,635	2,963	11.2%
	Sheep	533,878	395,070	4,538	3,698	14.0%
	Total	3,804,981	2,815,686	32,343	26,359	100.0%
Mincing	Beef	22,167,616	16,404,036	188,428	153,568	85.2%
	Mixed[c]	2,335,621	1,728,359	19,853	16,180	9.0%
	Pork	961,705	711,662	8,175	6,662	3.7%
	Sheep	568,143	420,426	4,829	3,936	2.2%
	Total	26,033,085	19,264,483	221,284	180,347	100.0%
Cattle 0.5%	Beef	642,544	475,483	5,462	4,451	34.4%
	Mixed[c]	269,889	199,718	2,294	1,870	14.4%
	Pork	427,181	316,114	3,631	2,959	22.9%
	Sheep	529,007	391,465	4,497	3,665	28.3%
	Total	1,868,622	1,382,780	15,884	12,945	100.0%
Initial dose	Beef	2,316,694	1,714,354	19,692	16,049	68.7%
	Mixed[c]	228,508	169,096	1,942	1,583	6.8%
	Pork	369,618	273,517	3,142	2,561	11.0%
	Sheep	455,848	337,328	3,875	3,158	13.5%
	Total	3,370,668	2,494,295	28,651	23,351	100.0%
Dose-response parameter mice	Beef	2,554,421	1,890,271	21,713	17,696	67.3%
	Mixed[c]	269,526	199,449	2,291	1,867	7.1%
	Pork	430,160	318,319	3,656	2,980	11.3%
	Sheep	539,812	399,416	4,588	3,740	14.2%
	Total	3,793,919	2,807,500	32,249	26,283	100.0%

[a] unadj: not adjusted for immunity; [b] adj.: adjusted for immunity; [c] mixed beef and pork product

Sensitivity analysis

Sensitivity analysis indicates heating temperature as the most influential parameter: if heating temperature is reduced by 10%, the number of infections from heated products increases by 91% and for all products by 54% (Table 6.5). A 10% reduction of the parameter values for prevalence in livestock, salting concentration and fraction unfrozen, leads to changes of approximately 10% in the model output for treated products. The difference between the sensitivity for treated products and all products depends on the number of products that have a changed parameter value. For example, there are fewer salted than heated products and therefore the decrease in sensitivity from including all products is larger for the salting parameters than for the heating parameters.

Table 6.5 Sensitivity of the estimated number of infections from products with a changed parameter-setting (treated) or all products (all) to changes in parameter-settings in the QMRA model. Sensitivity is expressed as the percentage change in number of infections per year per percentage change in the parameter setting. Negative outcomes indicate an increase of the model output with a decrease in parameter setting.

Parameter	Sensitivity	
	treated	all
Bradyzoite concentration	0.4	0.4
Portion size	0.4	0.4
Salting: NaCl concentration	-0.9	-0.1
Salting: temperature	-0.6	0.1
Salting: duration (days)	-0.3	0.0
Salting: reduction factor	0.4	0.0
Freezing: fraction unfrozen	1.0	0.6
Freezing: temperature	0.3	0.2
Freezing: duration (hours)	0.2	0.1
Freezing: reduction factor	0.1	0.1
Heating: temperature	-9.1	-5.4
Heating: reduction factor	0.2	0.1
Human dose-response parameter	0.5	0.5
Prevalence in livestock	1.1	1.1

Discussion

The aim of the study was to develop a QMRA model for the estimation of the number of meatborne *T. gondii* infections in humans and compare the predicted relative importance of meat from different livestock species. To build the model, data on consumed quantities of the various meat products and their infection level, depending on the treatments during pre-consumption processing, were extracted from several sources. Final output of the model consists of a predicted number of *T. gondii* infections in the total Dutch population and the subpopulation of pregnant women.

Our QMRA model estimates the number of new infections in the susceptible Dutch population at 2.8×10^6 per year and the number of new infections in susceptible pregnant women at 2.6×10^4 per year, with beef as the most important source of infection. With a transmission rate from mother to unborn child of 29% (Dunn et al., 1999; Thiebaut et al., 2007), this number of infected pregnant women would result in approximately 7.6×10^3 congenital infections per year. This number is almost 20-fold higher than the expected 388 congenital infections per year based on the incidence of *T. gondii*-specific IgM antibodies in newborns (Kortbeek et al., 2009). However, the overestimation by our model is even higher because not all congenital infections are due to the consumption of infected meat by the mother: in the study by Cook et al. (2000) 30 to 63% of the seroconversions in pregnant women could be attributed to meat consumption, but 6 to 17% was attributed to soil contact, and 14 to 49% remained unexplained. The high relative contribution of beef is also unexpected because raw pork was the only meat product identified as a risk factor for *T. gondii* infection (Hofhuis et al., 2011). Uncertainties around the estimates due to a lack of sufficient data are a common problem in exposure and risk assessments (Pires et al., 2009). In our case the discrepancy can originate from several sources.

First, data on the concentration of bradyzoites determined in 100 g of sheep heart was used for beef, pork and sheep products because insufficient species or product specific data were available. The concentration of bradyzoites in heart is higher than in skeletal tissue (Dubey, 1988; Esteban-Redondo, Innes, 1998), resulting in an overestimation of the bradyzoite concentration for the meat products. In addition, the two cattle hearts that tested positive by MC-PCR had a low concentration of parasites (Opsteegh et al., 2011b), indicating that the overestimation may be worse for beef products. However, the sensitivity analysis indicates that the effect of a 10% reduction of this parameter is limited in comparison to other parameters. For portions sizes that deviate much from 100 g, and especially smaller portions, there may be another issue: The distribution for the number of bradyzoites was determined in 100 g of heart, but the number of tissue cysts they derived from remained unknown (Opsteegh et al., 2010b). Because bradyzoites are present in tissue cysts and not distributed homogeneously, using the concentration that was determined in 100 g samples may underestimate the concentration in infected smaller portions (i.e. those that contain at least one tissue cyst). On the other hand using the seroprevalence that was comparable to PCR detection in 100 g samples (Opsteegh et al., 2010b) as estimate for the probability of infection per portion may lead to an overestimation of this probability for smaller portions. Based on the results for the minced meat scenario (with mincing modelled as an increase in the probability of infection per portion but a decrease in the concentration per portion) the effect of an overestimated probability of infection per portion is expected to be more influential. Therefore the predicted number of infections from products that are consumed in small portions may have been overestimated by our QMRA.

Second, the fraction of bradyzoites inactivated by pre-consumption processing (salting, freezing, and heating) was determined by fitting logistic regression models to data from mouse infection experiments with subsequent estimation of inoculated numbers of bradyzoites in processed and unprocessed meat using an inverse dose-response relation. This indirect calculation rather than having measured doses available leads to uncertainty in our outcomes. However, the scenarios with the alternative dose for unprocessed samples and the extreme dose-response parameter for the mice showed only small differences with the base model indicating that the model outcome is not very sensitive to these uncertainties. Nevertheless,

it is preferable to measure the bradyzoite dose in unprocessed meat using quantitative PCR in future processing-effect experiments. Additionally, establishing the dose-response relation based on dilutions of infected meat products within the same experiment would improve the estimation of the processing effect.

Possibly, the lower number of mice per measurement in the salting experiment compared to freezing and heating, and the lack of fit for the freezing experiment added to the uncertainty in our outcomes. With fewer mice per treatment the β-coefficients are estimated with less precision. Nevertheless, the Hosmer and Lemeshow test statistic of the logistic regression model for salting (p=0.615) indicates that the model fits properly. Sensitivity analysis indicates a relative impact of 0.4 for the reduction factor for salting. The lack of fit for the logistic regression model for freezing (Hosmer and Lemeshow test statistic p<0.001) probably resulted from the presence of outliers. Because these are not our experiments, we could not verify this by going back to the original data, and therefore we did not exclude these values from the analysis. Without the outliers, parameter estimates would have been slightly lower, which would have lead to slightly higher probabilities of infection for the mice. Sensitivity for the reduction factor of freezing is 0.1 concerning treated products.

Further, the processing treatments in the mouse bioassay experiments were not entirely comparable to processing of meat products for human consumption. In the salting experiments salted rodent brains rather than meat samples were used. In the experiments for measuring the effect of heating, samples were put in a water bath with a fixed temperature and time measurement was started when the desired core temperature was reached. In contrast, consumers often heat their meat for a number of minutes, not aware of core temperature but only regarding experience or the preparation advice on the label of the product. In addition, in the experiments mice were inoculated subcutaneously which might considerably overestimate the probability of infection from oral intake. These discrepancies between laboratory data and kitchen practices induce uncertainty in the QMRA output. To reduce this uncertainty, bioassay experiments should be repeated better mimicking consumer processing and consumption of meat products.

The discrepancy might also be due to the parameter values used for processing which all have some uncertainty. Sensitivity analysis indicated heating temperature as by far the most important parameter in the QMRA model with a relative impact of –9.1. Heating temperature is based on core temperature distributions for either beef/pork/lamb or minced beef obtained from American consumers' data and is not product-specific. However, the heating of meat by consumers is highly product dependent. For example, we think that roast beef, rump steak and lamb chops are frequently consumed undercooked. By using the same temperature distribution for all products, the number of infections from products often consumed undercooked might be underestimated, whereas the number of infections from products that are mainly eaten well done is overestimated. Including product specific core temperatures for heating would result in more reliable estimates. A similar uncertainty is present in the freezing distribution: Some products might be more likely to be stored frozen than others, whereas we applied the same frequency (42.3%) to all products, except meat products used for sandwich preparation. In addition, salting concentration depends on regional differences in the production process (Feiner, 2006), but this variation has not been taken into account in the model. Because most salted products are pork products, and two out of three mixed products are salted, uncertainties in salting will mostly affect the predicted numbers of infections for

those species. Sensitivity analysis indicates that the percentage of salt is important with a relative change of –0.9 in the number of susceptible people infected from salted products, but only with –0.1 if infections from all products are considered.

Next to salting, freezing and heating, there are other types of meat processing (e.g. fermenting, drying, smoking) that may reduce the number viable of bradyzoites. However, because no quantitative data for the effect of these processes were available they were not included in the model. This may have led to an overestimation for some products included in our model (e.g. salami, dry suçuk Turkish sausage, and raw smoked ham).

Another source of uncertainty is that a dose-response parameter determined in mice was used for humans, because no dose-response data for humans are available. Humans might be more or less susceptible to infection with *T. gondii* than mice, and thus using the mouse dose-response relation might lead to an over- or underestimation of the number of infections in our model. Although the same *r*-value was used for all products, this can still influence the relative contributions. Furthermore, the dose-response parameter for type II *T. gondii* was used, because this was the only type identified in Dutch sheep (Opsteegh et al., 2010a) and is generally considered most common in humans and livestock in Western Europe (Sibley et al., 2009). Nevertheless, it is possible that cattle carry a different type, especially because recently (incomplete) typing results for *T. gondii* isolated from cattle in Switzerland demonstrated the presence of types different from those isolated from sheep and cats (Berger-Schoch et al., 2010). Sensitivity analysis indicated that the results are not very sensitive (0.5) to the human dose-response parameter.

Furthermore, only freezing by consumers was included in our model, but some of the products stored in the fridge by the consumer may have been bought frozen. On top of that, meat imported from overseas frequently arrives frozen in The Netherlands, and part of this is sold refrigerated or used for processed meat products. Therefore, the actual fraction of frozen products is probably underestimated in our model and this may lead to a substantial overestimation as sensitivity analysis shows that the fraction stored unfrozen has a relative impact of 1.0 or 0.6 depending on the products considered. Import of meat and meat products can also affect the number of infections because the prevalence of *T. gondii* in livestock can differ from the prevalence in The Netherlands. Although prevalences are available for cattle, sheep and pigs from many countries (Tenter et al., 2000), specified import data were hard to obtain, especially concerning the temperature condition of the meat during transport. This lack of information results in the implicit assumption that all meat consumed in The Netherlands is of Dutch origin and this probably leads to an overestimation of the number of infections because, although the sensitivity of the fraction unfrozen and the prevalence is similar, the error in the fraction unfrozen is expected to be larger and therefore more influential.

For sheep and pigs the seroprevalence was used to estimate the number of infected portions. This is reasonable because for these species seropositivity is correlated to the presence of tissue cysts (Dubey et al., 2008; Gamble et al., 2005). In cattle, there is no correlation between seropositivity and detection of parasites (Opsteegh et al., 2011b); therefore the prevalence of parasitic DNA detected with PCR was used instead. However, the PCR prevalence of 2% does not necessarily indicate the presence of viable bradyzoites: DNA from inactivated parasites or tachyzoites (which are less infective) may have been detected (Opsteegh et al., 2011b). To take the possibility into account that only a fraction of the detected DNA in cattle originates

from viable bradyzoites, an alternative scenario model was set up with the prevalence in cattle decreased from 2.0% to 0.5%. Even with this prevalence set at 0.5%, beef remained the major source with a relative contribution of 34.4%. Risk-factor analyses not often indicate undercooked beef as a source of meatborne *T. gondii* infections, and only consumption of raw pork was identified as a risk factor for the Dutch population (Hofhuis et al., 2011). This may, however, be because in the cited risk-factor study the presence of IgG (and thus chronic infection) was studied, whereas the QMRA uses recent prevalence data for the sources of infection, and it is known that the prevalence in pigs has decreased substantially with the upcoming of industrialized breeding (van Knapen et al., 1982; van Knapen et al., 1995). A European risk-factor study based on recent infections did indicate consumption of raw/undercooked beef as an important source of infection (Cook et al., 2000). Nevertheless, the reduced prevalence of 0.5% was chosen rather arbitrarily and may still be an overestimation of the true prevalence of viable bradyzoites in cattle. It is essential to further investigate the presence of infectious bradyzoites in beef to reliably estimate the number of infections due to the consumption of raw beef, because our results indicate that even with a low prevalence, beef can be an important source of infections due to the high consumption of filet américain.

A point of improvement for the consumption data would be to distinguish between users and not-users of certain meat products. In the present consumption survey consumption of products on 2 consecutive days is recorded. From these numbers, the number of consumed portions per year is calculated and averaged over the total population with a seroprevalence of 26%. It is however, likely that a proportion of consumers frequently consume (partially) raw meat, while another group hardly ever consumes raw types of meat. Infected portions are thus more likely to be consumed by people frequently consuming raw meat products. Because these consumers of high risk products probably already have a higher seroprevalence, consumption of infected portions is less likely to result in a primary infection. To illustrate, 67% of sheep meat is consumed by a small fraction of the Dutch population (immigrants) (Paul Westra, PVE, personal communication). Therefore the absolute number of infections from sheep and relative contribution of sheep might be overestimated for the total Dutch population. The lack of specified consumption data also means that, in our model, the average consumption pattern was applied to pregnant women; whereas their actual consumption of risk products might be lower thanks to counselling offered.

Because game was not specified in the food consumption survey, it could not be included in our QMRA. In future, it is important to collect these data as game is often consumed undercooked and the seroprevalence in wild boar (Opsteegh et al., 2011a) and possibly other wildlife is high.

The alternative scenario that takes mincing of meat into account results in a number of infections per year that exceeds the total population size. Because mincing is assumed to have effects in two directions (all portions become infected, but the concentration of bradyzoites decreases) we did not anticipate the increase to be this large. Probably the concentration for intact portions (based on real-time PCR results) is often this high that the decreased concentration still results in a high probability of infection. Despite of the impossibly high number of infections, taking into account the mixing of several different animals is a realistic refinement that should be considered. For future improvements of similar risk assessments it is necessary to collect information on batch sizes as this influences the chance of including a positive animal in the batch. In this case the batch sizes were assumed infinitely large. That the bradyzoite concentration will be homogenous in all portions, as assumed in this scenario,

is probably not very realistic either because bradyzoites are not distributed homogenously through the meat, but are clustered in tissue cysts. Because this scenario shows an enormous effect on the outcome and risk-factor analysis has also shown high odds-ratios for minced meat products (Kapperud et al., 1996), obtaining data on batch sizes, and generating experimental data to estimate the effect of mincing meat on the variability of the number of bradyzoites per portion is crucial.

In general, it is difficult to obtain scientific data on consumer behaviour, and a better connection between consumer data and data from scientific literature is necessary to improve the reliability of QMRA-based conclusions on the number of meatborne *T. gondii* infections in The Netherlands. Our results confirm the usefulness of the advice to pregnant women to thoroughly heat meat: A large proportion (40%) of infections was caused by unheated products and the sensitivity analysis showed that heating had the highest relative impact on the model output. But both the predicted absolute numbers of infections and the relative importance of the species contradict results from prevalence and risk-factor studies. This discrepancy calls for further research, and with the development of this QMRA the areas in meatborne transmission that require more or better data have been identified. The most important points for improvement are related to those parameters that show a large effect on the total number of infections in sensitivity or scenario analysis and those for which the uncertainty is perceived to be large. Therefore, acquiring more precise information on processing of meat in consumer style and product specific heating temperatures are key elements in obtaining better estimates for future risk analysis of *T. gondii* infection. In addition, investigating the presence and concentration of viable bradyzoites in cattle will reduce uncertainty in the estimates. Regarding consumption data, it would be very useful to get information on a larger time span than 2 days, or accompany the consumption survey with a questionnaire to distinguish between users and non-users of product categories. Furthermore obtaining data on batch sizes and the effect of mincing meat on bradyzoite concentrations is essential. It is also necessary to better know the fraction of meat that is frozen prior to purchase. More accurate data will improve the reliability of the QMRA results and thereby aid decision-making on prevention strategies.

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Appendix A. Products included in QMRA, specified by livestock species. Consumption parameters are α and β parameters for fitted gamma distribution per product, mean portion size in grams, and number of portions consumed in the population per year. Processing parameters indicate whether products were included as salted, frozen or heated in QMRA model.

Species	Product	Consumption				Processing		
		α	β	mean (g)	portions	Salting	Freezing	Heating[a]
Beef	Filet américain	2.5	15.5	39	1.05E+08	no	no	no
	Smoke-dried beef	4.5	3	13.7	2.46E+08	yes	no	no
	Dry suçuk Turkish sausage	9.5	4.2	40	4.20E+06	yes	no	no
	Veal olive	6.1	16.4	100.2	1.12E+07	no	yes	yes 1
	Hamburger	5.9	16.3	95.9	2.27E+08	no	yes	yes 1
	Köfte Turkish minced beef	115	0.7	85.1	2.34E+06	no	yes	yes 1
	Minced beef	2.8	36.7	102.5	5.59E+08	no	yes	yes 1
	Beef steak tartare	5	20.1	100.9	1.06E+08	no	yes	yes 1
	Veal prime ribc	5.5	24.8	135.7	9.34E+05	no	yes	yes 2
	Veal fricandeau	2.5	37.5	93.6	9.34E+06	no	yes	yes 2
	Veal unspecified	4.9	21.4	105.4	1.03E+07	no	yes	yes 2
	Beef frying steak	3.7	30.9	115.5	1.21E+08	no	yes	yes 2
	Beef rump steak	5.6	23.6	132.2	1.14E+08	no	yes	yes 2
	Beef prime rib	5.5	24.8	135.7	1.64E+07	no	yes	yes 2
	Rolled beef	1.4	43.7	60.4	1.31E+07	no	yes	yes 2
	Roast beef	2.1	22.4	47.8	1.13E+08	no	yes	yes 2
	Beef unspecified	2.2	61.5	133.9	4.81E+07	no	yes	yes 2
Mixed[b]	Saveloy salami sausage	3.1	7.7	23.6	5.63E+08	yes	no	no
	Salami	3.2	7.1	23	1.42E+08	yes	no	no
	Minced beef/pork	2.5	40	100.7	5.57E+08	no	yes	yes 1
Pork	Bacon	2.3	12.1	27.4	4.39E+07	yes	no	no
	Streaky rashers bacon	1.9	16.9	31.6	1.98E+08	yes	no	no
	Raw smoked ham	3.5	6	21.2	2.04E+08	yes	no	no
	Spiced and smoked sausage	2.5	10.1	25.3	9.34E+06	yes	no	no
	Filled Kromesky meat	6.2	17.7	110.1	6.35E+07	no	yes	yes 1
	Pork frying sausage	4.1	26.3	107.5	1.79E+08	no	yes	yes 1
	Minced pork	2.7	43	116.3	8.88E+06	no	yes	yes 1
	Pork schnitzel (not breaded)	5.9	22.3	131.4	1.85E+08	no	yes	yes 2
	Rasher of bacon with rind	5.5	25.5	141	8.88E+06	no	yes	yes 2
	Rasher of bacon without rind	1.8	50	91.5	2.45E+08	no	yes	yes 2
	Pork fillet	2.3	43.8	100.6	8.74E+07	no	yes	yes 2
	Pork fricandeau	0.8	60.2	48.8	6.31E+07	no	yes	yes 2
	Pork tenderloin	5	27	135	3.13E+07	no	yes	yes 2
	Pork loin chop	7.5	18.1	134.9	3.97E+07	no	yes	yes 2
	Pork steak	5.7	22.7	128.4	1.01E+08	no	yes	yes 2

	Pork spare rib	2	53.3	107.6	1.96E+07	no	yes	yes 2
	Pork chopped stewing meat	2.9	39.8	114.5	6.87E+07	no	yes	yes 2
	Pork tenderloin medaillon[d]	5.7	22.7	128.4	4.67E+05	no	yes	yes 2
	Pork rib chop	4.1	21.1	86	7.99E+07	no	yes	yes 2
	Pork shoulder chop	3.6	30.8	109.5	1.76E+08	no	yes	yes 2
	Pork unspecified	2.4	56	133.3	8.97E+07	no	yes	yes 2
	Lean bacon	2.3	36.7	84.7	5.93E+07	yes	yes	yes 2
	Fat bacon	0.7	105.5	77.1	4.25E+07	yes	yes	yes 2
Sheep	Köfte Turkish minced mutton[e]	115	0.7	85.1	1.40E+06	no	yes	yes 1
	Minced lamb[f]	2.8	36.7	102.5	1.40E+06	no	yes	yes 1
	Lamb leg	1.7	102.7	174.9	4.20E+06	no	yes	yes 2
	Lamb chop	3.9	21.5	83.7	1.35E+07	no	yes	yes 2
	Lamb unspecified	2	40.2	78.9	7.48E+06	no	yes	yes 2
	Lamb saddle[g]	2.2	61.5	133.9	1.87E+06	no	yes	yes 2
	Mutton unspecified	3.3	25.2	82.2	1.21E+07	no	yes	yes 2

[a] For yes 1 temperature data for minced beef were used, for yes 2 temperature data for beef/pork/sheep were used; [b] mixed beef and pork product; [c] α and β-parameter from beef prime rib; [d] α and β-parameter from pork steak; [e] α and β-parameter from köfte Turkish minced beef; [f] α and β-parameter from minced beef; [g] α and β-parameter from unspecified beef.

Appendix B. Data of mouse bioassay experiment (Dubey, 1997) used to fit a logistic regression model to predict the inactivation of *Toxoplasma gondii* due to the salting of meat. For four treatment temperatures the numbers of mice positive for the presence of *T. gondii* per total number of inoculated mice at each time-salt concentration combination are given.

Temp (°C)	Time (days)	%NaCl			
		0.85	2	3.3	6
4	7	2/2	2/2	2/2	0/2
	14	2/2	2/2	2/2	1/2
	21	2/2	2/2	2/2	0/2
	28	2/2	2/2	0/2	0/2
	35	2/2	2/2	0/2	0/2
	42	2/2	2/2	0/2	0/2
	49	2/2	2/2	0/2	0/2
	56	2/2	0/2	0/2	0/2
10	3	6/6	6/6	6/6	0/4
	7	4/4	4/4	4/4	0/4
	14	6/6	6/6	4/6	0/4
	21	4/4	4/4	2/4	NA[a]
	28	2/2	2/2	0/2	NA
	35	2/2	0/2	0/2	NA
15	3	2/2	2/2	2/2	NA
	7	2/2	2/2	2/2	NA
	14	4/4	4/4	2/4	NA
	21	4/4	0/4	0/4	NA
	28	0/2	0/2	0/2	NA
	35	0/2	0/2	0/2	NA
20	3	2/2	2/2	2/2	NA
	7	2/2	2/2	0/2	NA
	14	3/4	0/4	0/4	NA
	21	0/2	0/2	0/2	NA
	28	0/2	0/2	0/2	NA
	35	0/2	0/2	0/2	NA

[a] NA: not available

Appendix C. Data of a mouse bioassay experiment (Kotula et al., 1991) used to fit a logistic regression model to predict the inactivation of *Toxoplasma gondii* due to the freezing of meat. Per time-temperature combination the number of mice positive for the presence of *T. gondii* per total number of inoculated mice is given.

Time (hrs)	Temp (°C)							
	4[a]	-1	-3.9	-6.7	-8	-9.4[b]	-12.2[b]	-15[b]
1.1	15/15	15/15	15/15	14/15	NA[c]	0/15	1/15	0/15
2.1	15/15	15/15	15/15	10/15	NA	0/15	0/15	0/15
4.3	15/15	15/15	15/15	15/15	NA	0/15	0/15	0/15
8.5	15/15	15/15	15/15	14/15	NA	0/15	1/15	0/15
17	15/15	15/15	15/15	15/15	NA	0/15	0/15	0/15
33.6	15/15	27/30	30/30	26/30	1/15	0/15	0/15	0/15
67.2	15/15	41/45	42/45	20/45	0/15	0/15	0/15	0/15
134.4	15/15	45/45	45/45	19/45	0/15	0/15	0/15	0/15
268.8	15/15	45/45	33/45	4/45	0/15	0/15	0/15	0/15
403.2		12/30	10/30	0/30	0/15	0/15	0/15	0/15
545.8		3/30	2/30	0/30	0/15	0/15	0/15	0/15
806.4		0/30	0/30	0/30	0/15	0/15	0/15	0/15
1075.2[d]		0/15	0/15	0/15	0/15	0/15	0/15	0/15
1612.8[d]		0/15	0/15	0/15	0/15	0/15	0/15	0/15

[a] Samples at 4°C were stored for 2-30 days in three trials, in trial 2, 3 and 4 no mice were positive from samples stored for 26, 27 and 25 days respectively, since the exact number is not known for the days prior to that we limited these data to 268.8 as may be expected that all mice were still infected using these samples; [b] data for -9.4, -12.2 and -15°C are not included in the table 2 but are given in text (Kotula et al., 1991). It was assumed that at least the smallest reported group size was used for these groups. [c] NA: not available; [d] Results at times 1075.2 and 1612.8 hours were not included in table 2 in (Kotula et al., 1991) but there is a footer that all mice in these groups were negative. It was assumed that at least 15 mice were used for these groups.

Appendix D. Data of the mouse bioassay experiment (Dubey et al., 1990) used to fit a logistic regression model to predict the inactivation of *Toxoplasma gondii* due to the heating of meat. Per time-temperature combination the number of mice positive for the presence of *T. gondii* per total number of inoculated mice is given.

Time (min)	Temp (°C)							
	4	49	52	55	58	61	64	67
0.01	45/45[a]	45/45[a]	29/45	6/45	2/45	0/45	0/45	0/45
3	45/45[a]	42/45	7/45	0/45	5/45	0/45	1/45	0/45
6	45/45[a]	37/45	0/45	0/45	0/45	0/45	0/45	0/45
12	45/45[a]	21/45	1/45	0/45	0/45	0/45	0/45	0/45
24	45/45[a]	0/45	0/45	0/45	0/45	0/45	0/45	0/45
48	45/45[a]	0/45[a]	0/45[a]	0/45	0/45	0/45	0/45	0/45
96	45/45[a]	0/45[a]	0/45[a]	0/45	0/45	0/45	0/45	0/45

[a] Results for these groups are not mentioned in article (Dubey et al., 1990) but confirmed by J.P. Dubey (personal communication).

Appendix E. Product-specific outcome of QMRA base model: Predicted mean probability of infection per portion (P_{inf}), and immunity-adjusted predicted numbers of meatborne *Toxoplasma gondii* infections per year in the Dutch total population (Total) and the subpopulation of pregnant women (Pregnant).

Species	Products	P_{inf}	Total (inf/yr)	Pregnant (inf/yr)
Beef	Filet américain	1.4E-02	1,063,783	9,959
	Smoke-dried beef	3.2E-08	6	0
	Dry suçuk Turkish sausage	9.7E-06	30	0
	Veal olive	8.2E-04	6,798	64
	Hamburger	8.1E-04	136,022	1,273
	Köfte Turkish minced beef	8.0E-04	1,376	13
	Minced beef	8.0E-04	332,466	3,112
	Beef steak tartare	8.2E-04	64,024	599
	Veal prime rib	1.0E-03	714	7
	Veal fricandeau	8.9E-04	6,138	57
	Veal unspecified	9.5E-04	7,231	68
	Beef frying steak	9.7E-04	87,069	815
	Beef rump steak	1.0E-03	85,729	803
	Beef prime rib	1.0E-03	12,443	116
	Rolled beef	7.4E-04	7,126	67
	Roast beef	7.0E-04	58,187	545
	Beef unspecified	9.8E-04	35,063	328
Mixed[a]	Saveloy salami sausage	4.8E-07	201	2
	Salami	9.1E-08	10	0
	Minced beef/pork	4.8E-04	199,733	1,870
Pork	Bacon	2.2E-03	70,517	660
	Streaky rashers bacon	4.7E-10	0	0
	Raw smoked ham	2.9E-11	0	0
	Spiced and smoked sausage	7.7E-04	5,295	50
	Filled Kromesky meat	2.0E-04	9,331	87
	Pork frying sausage	2.0E-04	25,838	242
	Minced pork	2.0E-04	1,286	12
	Pork schnitzel (not breaded)	2.4E-04	33,404	313
	Rasher of bacon with rind	2.5E-04	1,640	15
	Rasher of bacon without rind	2.1E-04	37,563	352
	Pork fillet	2.2E-04	14,143	132
	Pork fricandeau	1.5E-04	6,901	65
	Pork tenderloin	2.5E-04	5,719	54
	Pork loin chop	2.5E-04	7,308	68
	Pork steak	2.4E-04	18,293	171
	Pork spare rib	2.2E-04	3,219	30
	Pork chopped stewing meat	2.3E-04	11,614	109

	Pork tenderloin medaillon	2.4E-04	84	1
	Pork rib chop	2.1E-04	12,616	118
	Pork shoulder chop	2.3E-04	29,691	278
	Pork unspecified	2.4E-04	15,713	147
	Lean bacon	1.3E-04	5,868	55
	Fat bacon	1.4E-05	425	4
Sheep	Köfte Turkish minced mutton	2.1E-02	22,152	207
	Minced lamb	7.1E-03	7,398	69
	Lamb leg	9.2E-03	28,713	269
	Lamb chop	7.8E-03	77,910	729
	Lamb unspecified	7.4E-03	40,674	381
	Lamb saddle	8.7E-03	12,037	113
	Mutton unspecified	2.3E-02	206,186	1,930

[a] mixed beef and pork product

Chapter 7

Seroprevalence and risk factors for *Toxoplasma gondii* infection in domestic cats in The Netherlands

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Revised Version

Cats, as definitive hosts, play an important role in the transmission of *Toxoplasma gondii*. To determine the seroprevalence and risk factors for *T. gondii* infection in Dutch domestic cats, serum samples of 450 cats were tested for *T. gondii* antibodies by indirect ELISA. Binary mixture analysis was used to estimate the seroprevalence (18.2%, 95% CI: 16.6-20.0%), the optimal cut-off value and the probability of being positive for each cat. Hunting (OR 2.8), presence of a dog in the household (OR 2.4), former stray cat (OR 3.1) and feeding of raw meat (OR 2.8) were identified as risk factors by multivariable logistic regression analysis on dichotomized test results. Prevalence differences were estimated by linear regression on the probabilities of being positive and used to calculate the population attributable fractions for each risk factor. Hunting contributed most to the *T. gondii* seroprevalence in the sampled population (26%).

Introduction

Infection with the protozoan *Toxoplasma gondii* is one of the most common parasitic infections of man and warm-blooded animals all over the world. Nearly all species of felids, for example domestic cats, are definitive hosts. Sexual reproduction of *T. gondii* takes place in their intestines and results in shedding of environmentally resistant oocysts. In addition, *T. gondii* has an exceptionally wide range of intermediate hosts: it can reproduce asexually and form tissue cysts in all warm-blooded animals including humans. For both definitive and intermediate hosts infection occurs via one of the following routes: 1) horizontal by oral ingestion of infectious cat-shed oocysts via contaminated soil, food or water, 2) horizontal by oral ingestion of tissue cysts with bradyzoites contained in raw or undercooked meat, 3) vertical by transplacental transmission of tachyzoites (Tenter et al., 2000).

Specific antibodies to *T. gondii* have been detected in up to 74% of adult cats in some populations (Tenter et al., 2000). Both tissue cysts and antibodies persist, while in general, a cat sheds oocysts for up to three weeks after a primary infection only (Dabritz, Conrad, 2010). The prevalence of oocyst excretion in faeces is therefore much lower than the seroprevalence: large scale screening demonstrated *Toxoplasma*-like oocysts (this includes *Hammondia hammondi* oocysts) in 0.31% of faecal samples from German and other European cats, 0.11% was confirmed as *T. gondii* (Schares et al., 2008). The quantity of oocysts produced after primary infection varies from 3 to 810 million oocysts (Dabritz, Conrad, 2010). In addition to the considerable amount of oocysts shed, the oocysts are very resistant and can remain viable for up to 18 months in soil depending on humidity, temperature and exposure to direct sunlight (Frenkel et al., 1975; Yilmaz, Hopkins, 1972), and for 6 up to 54 months in water (Dubey, 1998b) and seawater (Lindsay, Dubey, 2009). Contamination of the environment with *T. gondii* oocysts by cats plays an important role in the epidemiology of toxoplasmosis of animals and humans, which is illustrated by the low prevalence of *T. gondii* infections in areas without cats (Dubey et al., 1997b; Munday, 1972; Wallace et al., 1972).

The overall seroprevalence of *T. gondii* for the Dutch human population has recently been estimated at 26.0% (Hofhuis et al., 2011) and the incidence of congenital toxoplasmosis was estimated at 2 per 1000 live-born children leading to a disease burden similar to that of the major foodborne pathogen *Campylobacter* (Kortbeek et al., 2009). Because of the high disease burden in humans there is a need for intervention. The effectiveness of prenatal and postnatal treatment to prevent congenital toxoplasmosis is debated (Gilbert, 2009); therefore the focus is on prevention of infection. As contamination of the environment with *T. gondii* oocysts only occurs by felines and all intermediate host infections have a cat shedding oocysts as starting point of the cycle, prevention of cat infection is likely to be highly effective in reducing both oocyst- and tissue cyst-acquired infections in humans.

To prevent infection of cats and thereby reduce oocyst shedding by cats, the risk factors for infection need to be well understood. Depending on prey composition and the prevalence of *T. gondii* in these animals, prey intake from hunting is important for the prevalence of the infection (Afonso et al., 2007). Other risk factors for infection in cats identified by multivariable analyses are: age (Afonso et al., 2006; De Craeye et al., 2008; Lopes et al., 2008), outdoor access (Afonso et al., 2009; Lopes et al., 2008), raw meat in their diet (Lopes et al., 2008), number of kittens in groups of farm cats (Afonso et al., 2009), and precipitation (Afonso et al., 2006; Afonso et al., 2009). However, in The Netherlands the risk factors for *T. gondii* infection in cats and levels of exposure of cats to these risk factors are unknown. It

was our aim to determine the seroprevalence of *T. gondii* in cats in The Netherlands, identify the risk factors and estimate the proportion of cat infections that could be prevented if the exposure was removed from the population.

Methods

Study population

Cats are popular pets in The Netherlands and from 1997 the population of domestic cats increased by 50% to an estimated 3.3 million in 2006 (Raad voor Dierenaangelegenheden, 2006). The number of stray cats is unknown. Based on a limited sample (n=150) the seroprevalence of *T. gondii* for the Dutch cat population was estimated at 49% in 1993 (personal communication Prof. Dr. F. van Knapen, Utrecht University). Because this estimate is imprecise but close to 50% the sample size was calculated based on a seroprevalence representing half of the sample. With an expected seroprevalence of 50% (P), an accepted deviation of the true prevalence of 5% (d) and a confidence level of 95% (z=1.96) the sample size necessary to estimate the seroprevalence was calculated at 384 (according to $n=P(1-P)\ z^2/d^2$).

Samples were collected between January and August 2010 at 26 veterinary clinics recruited by a call in the journal of the Royal Dutch Veterinary Association (Opsteegh et al., 2010a). Clinics in the provinces of North Holland, South Holland and Utrecht were overrepresented, while no clinics from Zeeland, Friesland and Overijssel participated. All cats presented at the cooperating clinics were eligible to take part in the study. The cat owners, who volunteered in the project, gave their permission to collect a blood sample and filled out a questionnaire. A total of 342 cat serum samples was collected. In addition, 108 cat serum samples collected at various veterinary clinics between May 2005 and August 2007 as part of a vaccination status project (Dr. H. Egberink, Utrecht University, Faculty of Veterinary Medicine, Department of Virology) were tested.

To enable proper fitting of the binary mixture model, 203 available domestic cat sera from Romania were tested additionally. Inclusion of these sera generates a higher proportion of positive sera (seroprevalence 56%, Györke et al., in preparation) which facilitates fitting of the positive component of the mixture. These sera were not included in the seroprevalence estimation or risk-factor analyses as risk factors may vary per country.

Serological assay

An in-house developed indirect ELISA was used to detect antibodies against *T. gondii*. The protocol for this in-house ELISA was, in brief: 96-well microtitre plates were successively incubated (1h at 37°C) with 100µl/well of 4.3 µg/ml *T. gondii*-RH-antigen in 0.1M Na_2CO_3 solution (pH 9.6), 125µl 1%BSA in PBS, 1:100 diluted serum in 1%BSA in PBS-T (PBS-T: 0.07M PBS/0.05%Tween 20), and 100 µl 1:36000 diluted conjugate (goat anti-feline IgG: HRP, AbD Serotec, Düsseldorf, Germany) in 1%BSA in PBS-T. Plates were washed four times with PBS-T after each incubation. A 100µl of substrate (Sure Blue™ TMB Microwell Peroxidase Substrate, KPL, Gaithersburg, MD, USA) was added to each well and incubated for 10 min

at room temperature. The reaction was stopped by the addition of 100µl of 2M H_2SO_4. The optical density (OD) at 450nm was read using a microplate reader (EL808, Ultra Microplate Reader, Bio-Tek Instruments, Bad Friedrichshall, Germany).

Six control sera with different OD-values and two blank controls were tested in duplicate on each plate. OD-values were corrected for blank measurement and plate-to-plate variation as described previously (Opsteegh et al., 2010c) thereby obtaining ODc-values.

Questionnaires

To identify the risk factors for infection with *T. gondii* in cats, a self-administered questionnaire with 32 multiple-choice and 8 open-ended questions for the cat owners was formulated. Questions related to potential risk factors such as housing, feeding of raw meat and hunting behavior. Other data gathered included the personal data of the cat, such as age, sex, and neutering and vaccination status (usually against panleukopenia-, herpes- and calicivirus). In addition, we were interested in owner habits regarding the litter tray and the maximum price cat owners are willing to pay for vaccination against *T. gondii* of their own cat or produced kittens would such a vaccine come available. To answer this last question, owners were informed that *T. gondii* vaccination of cats would only reduce toxoplasmosis in humans if many cats are vaccinated and that vaccination does not affect the health of the cat: it does not harm, but the cat does not benefit from vaccination either. For the cats from the vaccination status survey, only personal data and data on outdoor access and the presence of a dog or more cats in the household were available.

Data analyses

Descriptive statistics

Frequency distributions for exposure to risk factors, owner habits concerning the litter tray, and willingness to pay for *T. gondii* vaccination are presented because these are interesting for future risk assessment purposes and not included in the results from the bivariable analyses presented later on in this paper.

Binary mixture model to estimate the seroprevalence, establish a cut-off value and calculate the probability of positive for each cat

The seroprevalence was estimated directly from observed ODc-values using a binary mixture model (Opsteegh et al., 2010c) with the following modification: Usually both OD and $\log_{10}$-transformed OD-values for positive sera show a normal distribution (Jacobson, 1998; Thrusfield, 2005). However, in this case OD-values for positive cats tended to be very high and outside of the linear relation between OD-value and antibody concentration. To account for this negative skew, a mirrored gamma distribution, rather than a normal distribution, was fitted for the positive component. The maximum for the mirrored gamma distribution was set at a logODc of 0.7, which corresponds to the maximum OD-value measurable by the ELISA reader (5.0). Thus, a binary mixture model, in which a mirrored gamma distribution described the positive and a normal distribution the negative component, was fitted to the frequency distribution of log-transformed ODc-values. Subsequently, seroprevalence, cut-off value, and accompanying test sensitivity and specificity were estimated as described previously (Opsteegh et al., 2010c). The cut-off was used to classify all cats as positive or negative based on their logODc-value.

In addition to scoring the animals positive and negative based on the cut-off value, the probability of being positive was calculated for each animal using:

$$P(pos|X = x) = cf_2(x)/(cf_2(x) + (1-c)f_1(x))$$

With X the random variable indicating the logODc of an individual cat, $f_1(x)$ the probability density function for the negative component (in this case *N(logODc;-0.574;0.248)*), $f_2(x)$ the probability density function for the positive component (in this case *Gamma(-logODc+0.7;20.336;0.023)*), and c the mixing parameter, i.e. the prevalence (in this case 0.182) estimated from the binary mixture model.

This is a more informative result than a simple negative or positive based on a cut-off value, because it takes into account the uncertainty in the test results.

Seroprevalence by age

With the parameters for the two distributions (except prevalence c) as obtained above fixed, the binary mixture model was fitted to each group of 50 to 100 cats categorized by age (as described previously (Opsteegh et al., 2011a)). That way, for each age group, a direct estimate of the true seroprevalence c_i per age category i was obtained, and the mean age a_i was calculated.

Logistic regression analysis to identify predictors of seropositivity

For most variables the original answers from the questionnaires were categorized or combined into fewer categories because seroprevalences were similar or numbers per category were low. Age was divided into three categories consisting of equal numbers of cats *(≤1.5 years*, *1.5-10 years*, *>10 years*). Outdoor access was aggregated into two categories with *no* including indoor cats as well as cats with restricted outdoor access (e.g. balcony, area without contact with other cats) and *yes* only those with free outdoor access. Vaccination status categories were limited to *yes* (including *annually* and *once*) and *no*. The use of the litter tray was re-categorized by combining *seldom* and *never* into *no* and *always* and *often* into *yes*. The amount of cats around the house was re-categorized from *none*, *one or two*, *three to five*, and *more than five* into *no* and *yes*. Although owners that fed their cat raw meat were asked to answer whether this was done a few times a year (22 cats), monthly (nine cats) or weekly (five cats) these categories were combined into *yes*. The two categories for hunting are *no* and *yes* with *yes* including only cats that bring home prey or have been observed eating prey animals. Residential environment was divided into *urban* and *not urban* (including *rural*, *nature*, *industrial area* and *other*). Missing values in the analysis were coded with an indicator variable.

To identify predictors of seropositivity for *T. gondii* infection in cats, first bivariable logistic regression analyses were performed to test which of the variables were age-adjusted predictors of seropositivity. Age was always included because with a persistent infection such as *T. gondii*, the prevalence of antibodies is known to accumulate with age. Next, all variables which reached a significance level of $p \leq 0.20$ (by likelihood ratio test) in bivariable analysis were included in a multivariable logistic regression model. The model was reduced by backwards elimination of non-significant variables ($p > 0.05$ based on likelihood ratio test), however confounders (as indicated by a more than 10% change in the odds-ratio (OR) for another variable compared to the OR in the full model) were kept in the final model. All logistic regression analyses were performed in SPSS 18.0 (PASW Statistics, Chicago, IL, USA).

Linear regression on probability of positive to calculate population attributable fractions

Seroprevalence was additionally checked for differences between groups using linear regression on the probabilities of being positive *P(pos|X=x)*. The coefficients in the linear regression model for the probability of being positive represent prevalence differences (*RD*) between cats exposed and not exposed to a certain risk factor. By multiplication of these coefficients with the prevalence of exposure (*p(E+)*) the population attributable risk for each factor is calculated (*PAR=RD* × *p(E+)*) (Dohoo et al., 2003). Next the population attributable fraction is calculated by dividing the PAR by the prevalence of disease (*AFp=PAR/p(D+)*) to get an indication of the proportion of disease in the population that would be avoided if the exposure was removed from the population (Dohoo et al., 2003). All factors included in the final logistic regression model were included in the linear regression model. Risk differences, population attributable risks, and population attributable fractions were calculated for all (borderline) statistically significant factors in the model except age because this is a confounder and not a causal factor.

Results

Descriptive statistics

Of the 450 cats tested, 91 (20.2%, 95% CI: 16.5-23.9%) tested positive for *T. gondii* antibodies. The apparent seroprevalences did not differ significantly for the newly collected samples (68/341) and those from the vaccination status study (23/109) (Pearson's χ^2 (df=1), $p = 0.79$). The total of 341 cat serum samples was collected at 26 cooperating veterinary clinics with 1 to 25 samples per clinic. For 443 cats (98.4%) age was reported and it varied between 0.25 and 20.0 years. Many young cats were sampled (Fig. 7.1). The cats originated from 408 separate cat owners (314 in this study, 94 from the vaccination status project) participating with one or more cats. For three pairs of cats it was not clear whether the cats belonged to the same owner and these were excluded from the analysis of owner characteristics. Cat owner characteristics are presented in Table 7.1, information concerning the litter tray is provided in Table 7.2, and the maximum price owners are willing to pay for *T. gondii* vaccination is provided in Table 7.3.

Seroprevalence and cut-off value by binary mixture model

The frequency distribution of the 450 measured logODc-values showed two components (Fig. 7.2). Using the parameters of the distributions fitted to all cats including those from Romania (*N(logODc;-0.574;0.248)* and *Gamma(-logODc+0.7;20.336;0.023)*), the seroprevalence for the Dutch cats was estimated at 18.2% (95% CI: 16.6-20.0% based on 500 bootstrap samples). The ROC-curve analysis showed that the test had a good discriminatory power with an area under the curve (AUC) of 0.998. The optimum cut-off was estimated at a logODc-value of -0.058, with sensitivity (*Se*) at 99.0% and specificity (*Sp*) at 98.1%. With these test characteristics and an apparent prevalence (*AP*) of 20.2% (estimated using the cut-off value) the true prevalence (*TP*) is estimated at 18.8% (according to *TP=(AP+Sp-1)/(Se+Sp-1)*) (Rogan, Gladen, 1978)), which is similar to the direct estimate of the true prevalence from the binary mixture model (18.2%).

Table 7.1 Distribution of 408 cat owners by their characteristics. Totals per variable may vary because of missing data.

Variable		*n*	Frequency (%)
Number of cats participating per owner	1	377	92.4
	2	27	6.6
	3	3	0.7
	4	1	0.2
Vaccinated (first cat from owner)	No	62	16.5
	Yes	313	83.5
Cat stays away from home during vacation	No	282	91
	Yes	28	9
Residential environment	Urban	264	85.4
	Other	45	14.6
Dog in the household	No	306	76.3
	Yes	95	23.7
Extra cats in the household	0	146	37.1
	1 or 2	198	50.3
	3 or more	50	12.7
Outdoor access (first cat from owner)	No	91	23.6
	Yes	294	76.4
Garden available	No	45	14.7
	Yes	262	85.3
Breeding kittens	Never	11	3.5
	Once	260	83.3
	More than once	39	12.6
Feeding of raw meat (first cat from owner)	No	275	89.3
	Yes	33	10.7
Litter tray available	No	33	10.6
	Yes	277	89.4

Seroprevalence by age

The seroprevalence by mean age per age group showed a decrease from 6 months up to 1 year (Fig. 7.3). After the decline during the first year of age, the seroprevalence rose up to 25% around 4 years and ranged between approximately 20 and 30% in cats over 4 years old.

Table 7.2 Distribution of 277 cat owners with a litter tray by their characteristics concerning the litter tray. Totals per variable may vary because of missing data.

Variable		*n*	Frequency (%)
Use of litter tray by cat	No, seldom to never	20	6.7
	Yes, sometimes	179	59.7
	Yes, often	63	21
	Yes, always	38	12.7
Filling litter tray	Clay or mineral-based	214	77.5
	Other (e.g. biodegradable, sand, paper)	62	22.5
Cleaning litter tray	Immediately after defecating	17	6.2
	Daily	112	41.0
	Every 2 or 3 days	87	31.9
	Every 4 to 7 days	47	17.2
	Less than once a week	10	3.7
Disposal of litter tray filling	Household waste	197	71.4
	Organic waste	67	24.3
	Compost	3	1.1
	Household waste or organic waste	4	1.4
	Household waste or toilet	3	1.1

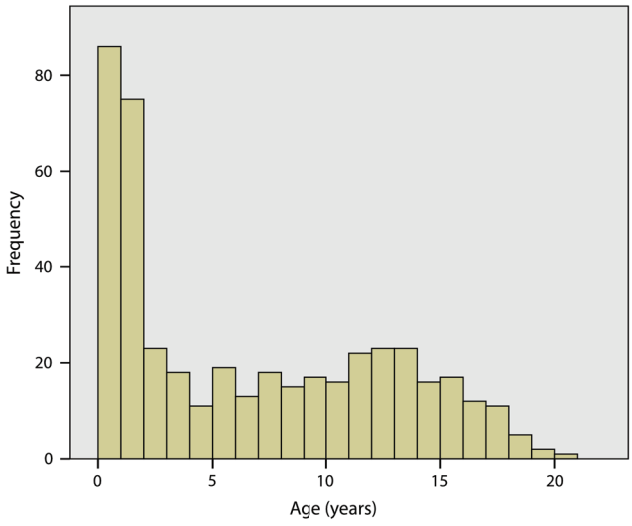

Figure 7.1 Frequency distribution of age (in years) for 443 cats.

Table 7.3 Distribution of 314 cat owners regarding the maximum price they are willing to pay for vaccination against *T. gondii* of their own cat or of bred kittens before delivery to the new owner. Totals per variable may vary because of missing data.

Cat	Maximum price	*n*	Frequency (%)
Own cat	not willing	18	6.4
	free	24	8.5
	€ 0-2	18	6.4
	€2-5	53	18.8
	€5-10	75	26.6
	€10-20	44	15.6
	> €20	50	17.7
Kittens	not willing	20	7.6
	free	27	10.2
	€ 0-2	21	8
	€2-5	49	18.6
	€5-10	58	22
	€10-20	33	12.5
	> €20	56	21.2

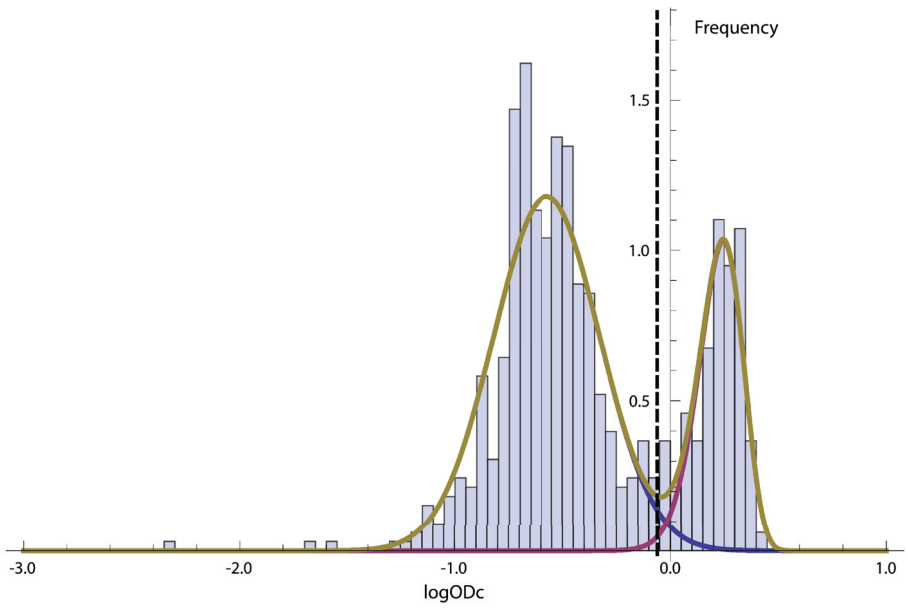

Figure 7.2 Frequency distribution of log10-transformed ODc values for 653 cats in *T. gondii* ELISA (bars), fitted mixure of normal (left) and gamma (right) distribution (lines), and cut-off value (logODc = -0.058) (vertical dashed line)

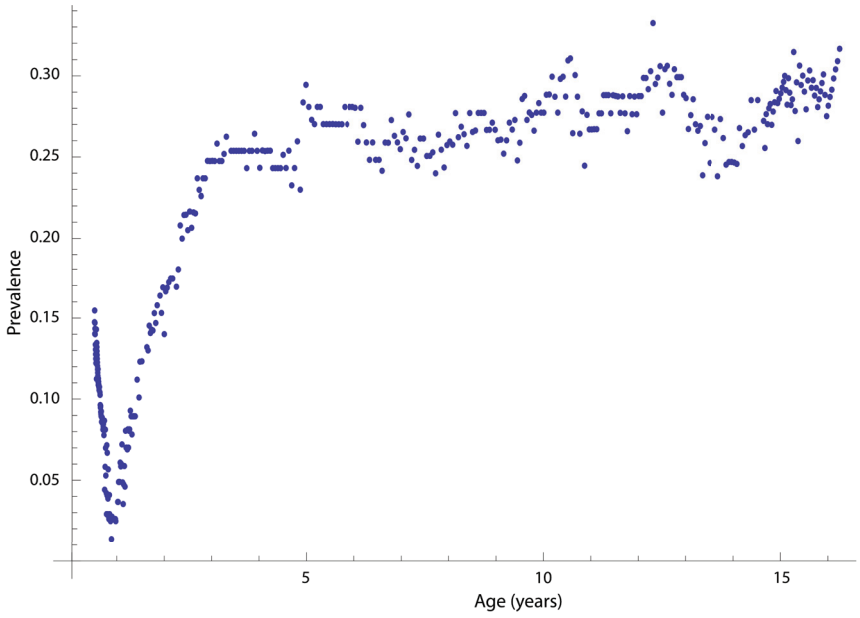

Figure 7.3 *T. gondii* seroprevalence by age group in cats. Seroprevalence was estimated by fitting the binary mixture model to age groups including 50-100 cats and plotted at the mean age of the group in years.

Predictors of seropositivity identified by logistic regression analysis

The bivariable analyses showed significant differences ($p < 0.05$, based on likelihood ratio test) in seroprevalence for former stray cats, feeding of raw meat, residential environment, outdoor access, hours outside per day, hunting, and the use of a litter tray; and additional differences at the $p < 0.20$ level for sex, presence of a dog in the household, more cats in the household, and contact with young cats/kittens (Table 7.4). The final model for the multivariable logistic regression analysis (Hosmer and Lemeshow goodness-of-fit test $p = 0.682$) is presented in Table 7.5. Age, former stray cat, presence of a dog in the household, and hunting behavior remained significant ($p < 0.05$) predictors of seropositivity (Table 7.5). Feeding of raw meat was borderline significant ($p = 0.066$) and was retained because of our interest in this factor. Outdoor access ($p = 0.161$) and extra cats in the household ($p = 0.184$) were retained for their effects on the OR for hunting and feeding raw meat respectively. If outdoor access was excluded from the model, the OR for hunting increased to 3.67 (95% CI: 1.89-7.15), while changes for the ORs of other variables were less than 10%.

Table 7.4 Cat serum samples positive for antibodies against *T. gondii* and age-adjusted OR with 95% confidence interval in bivariable logistic regression analysis.

Variables		*n*	Prevalence (%)	OR
Sex	Male	236	24.6 (19.1-30.1)	ref
	Female	201	15.9 (10.9-21.0)	0.63 (0.38-1.04)
Former stray	No	261	18.0 (13.3-22.7)	ref
	Yes	44	36.4 (22.1-50.6)	2.58 (1.24-5.37)
Dog in the household	No	334	17.7 (13.6-21.8)	ref
	Yes	109	28.4 (20.0-36.9)	1.92 (1.13-3.26)
More cats in the household	No	152	26.3 (19.3-33.3)	ref
	1 or 2	222	19.4 (14.2-24.6)	0.74 (0.44-1.25)
	3 or more	62	9.7 (2.3-17.0)	0.30 (0.12-0.77)
Raw meat	No	297	17.2 (12.9-21.5)	ref
	Yes	36	38.9 (23.0-54.8)	2.65 (1.22-5.73)
Cats around the house	No	30	10.0 (0.0-20.7)	ref
	Yes	306	21.2 (16.7-25.8)	2.54 (0.73-8.86)
Residential environment	Urban	284	18.0 (13.5-22.4)	ref
	Not Urban	51	31.4 (18.6-44.1)	2.55 (1.24-5.21)
Outdoor access	No	176	9.7 (5.3-14.0)	ref
	Yes	188	28.7 (22.3-35.2)	3.47 (1.89-6.39)
Hours outside per day	Not outside	104	9.6 (3.9-15.3)	ref
	< 1 hr	69	15.9 (7.3-24.6)	1.41 (0.54-3.68)
	1 - 5 hr	127	22.0 (14.8-29.3)	2.17 (0.98-4.84)
	> 5 hr	64	34.4 (22.7-46.0)	4.29 (1.81-10.16)
Hunting	No	196	11.2 (6.8-15.6)	ref
	Yes	104	36.5 (27.3-45.8)	4.49 (2.39-8.42)
Neutered	No	86	10.5 (4.0-16.9)	ref
	Yes	337	23.4 (18.9-28.0)	1.10 (0.48-2.52)
Vaccinated	No	70	25.7 (15.5-36.0)	ref
	Yes	344	19.5 (15.3-23.7)	0.56 (0.30-1.07)
Contact with young cats or kittens	No	194	16.5 (11.3-21.7)	ref
	Yes	56	23.2 (12.2-34.3)	1.62 (0.76-3.47)
Use of litter tray	No	20	30.0 (9.9-50.1)	ref
	Yes	280	15.7 (11.5-20.0)	0.54 (0.19-1.53)

Population attributable fractions estimated using linear regression

Linear regression on the probabilities of being positive showed similar risk differences for feeding of raw meat and hunting. However, taking the prevalence of exposure into account, it was shown that the largest fraction of *T. gondii* infections in the whole population (*AFp*) could be attributed to hunting (Table 7.6).

Table 7.5 Odds-ratios (OR) with 95% confidence interval and *p*-values based on likelihood ratio test for variables associated with *T. gondii* seropositivity in cats in multivariable logistic regression analysis.

Variable		*n*	OR (95% CI)	*p*-value
Age (years)	< 1.5	158	ref	<0.0005
	1.5 - 10	152	5.26 (2.35-11.80)	
	> 10	133	6.71 (2.98-15.11)	
Former stray	No	261	ref	0.02
	Yes	44	3.05 (1.34-6.95)	
Dog in the household	No	334	ref	0.014
	Yes	109	2.44 (1.34-4.43)	
More cats in the household	No	152	ref	0.184
	1 or 2	222	0.83 (0.47-1.47)	
	3 or more	62	0.35 (0.13-0.97)	
Raw meat	No	297	ref	0.066
	Yes	36	2.76 (1.16-6.58)	
Outdoor access	No	176	ref	0.161
	Yes	188	1.79 (0.83-3.85)	
Hunting	No	196	ref	0.025
	Yes	104	2.75 (1.28-5.89)	

Table 7.6 Risk difference (*RD*), prevalence of exposure (*p(E+)*), population attributable risk (*PAR*) and population attributable fraction (*AFp*) for identified risk factors for *T. gondii* infection in Dutch domestic cats.

Variable	*RD* (95% CI)	*p(E+)*	*PAR*	*AFp*
Former stray	0.138 (0.028;0.247)	0.144	0.020	10%
Dog in the household	0.106 (0.03;0.182)	0.246	0.026	13%
Raw meat	0.151 (0.031;0.270)	0.108	0.016	8%
Hunting	0.153 (0.054;0.251)	0.347	0.053	26%

Discussion

To determine the seroprevalence of *T. gondii* in cats in The Netherlands and investigate the risk factors for infection, we used an in-house ELISA to test cat serum samples and determined a cut-off value by binary mixture analysis. Using the binary mixture model the seroprevalence of *T. gondii* was estimated at 18.2% (95% CI: 16.6-20.0%). This prevalence is lower than the 49% that was found in 1993, but because no details are available for the population sampled in the nineties (this population may for example have been older on average) and the use of a different test, this difference does not necessarily represent an actual decrease in prevalence. The estimated seroprevalence is comparable to seroprevalences in other European countries, for example 18.6 % in an urban cat population in France (Afonso et al., 2006) and 25.0% in house cats in Belgium (De Craeye et al., 2008).

The seroprevalence increased with age for cats from 1 year up to almost 4 years of age. After this rise, the seroprevalence up to 12 years old ranged between 20 and 30%. In correspondence with our observations in wild boar (Opsteegh et al., 2011a), this relatively stable seroprevalence could be explained by a decline of IgG titre to a level below cut-off (i.e. reversion to seronegative). However, this type of pattern can also be expected if 60 to 70% of the cats is either not exposed or resistant to infection. Review of the literature on persistence of antibodies does not clarify much: Although IgG titres are known to stay high for months up to 6 years in cats (Dubey, 1995; Dubey, Thulliez, 1989), it has been described that 4 out of 9 cats that had shed oocysts earlier, re-excreted oocysts after infection with tissue cysts six years after primary infection (Dubey, 1995) which may indicate a loss of immunity, even though all nine cats were still seropositive at re-infection (titre ≥ 1:10,000 in modified agglutination test). Whether antibodies decay to a level that would be scored negative in our ELISA needs further investigation.

In addition, we observed a decreasing seroprevalence by age in cats less than one year old. Kittens can develop detectable antibody titres upon infection at a very young age, but can also obtain high IgG antibody titres via colostrum. Maternal antibodies will decrease to non-detectable levels at 8-12 weeks after birth (Omata et al., 1994), and a decrease in seroprevalence for very young cats has been observed previously (Afonso et al., 2006). Although the youngest cats in our study were all over 3 months old, part of the observed decrease in prevalence might still be due to declining maternal antibody titres if natural variation in decline is larger than was observed experimentally (Omata et al., 1994).

Next, risk factors for *T. gondii* infection were identified by logistic regression analysis. Before interpreting the identified risk factors, it is important to realize that there is potential participation bias: the owners visited the veterinarian with their cat and volunteered in the study. Therefore both owners and sampled cats in this study are probably not representative of the total Dutch cat population. The cats are relatively young (mean age 6.5 years) compared to the age distribution reported by the Council for Animal Affairs (Raad voor Dierenaangelegenheden, 2006). From the seroprevalence by age curve, it can be understood that an overrepresentation of young cats means that the overall prevalence for the Dutch cat population is probably somewhat higher than 18.2%. It is also likely that the willingness to pay for *T. gondii* vaccination is overestimated in our study, because the vaccination coverage in our study (83% of the cats was vaccinated at least once) is much higher than the vaccination coverage for the total Dutch cat population (overall cat vaccination coverage in The Netherlands is estimated at 30-35%, personal communication, Jacqueline Poot, Intervet). Sampling at veterinary clinics may also have limited the number of farm cats kept for rodent control included in our study, therefore the prevalence of outdoor access and hunting behavior are likely underestimated. The effects on the frequency of other characteristics or possible predictors (e.g. feeding of raw meat) are less obvious, but can be present. In general, this means that the calculated population attributable fractions (which are calculated based on the observed prevalences of exposure) apply to the sampled population and would not necessarily be the same for the total population.

The population attributable fractions are based on risk differences estimated by linear regression on the probability of being positive for each cat. Because these probabilities are limited to values between 0 and 1, and often close to 0 or 1, they are not normally distributed. Neither are the residuals from the model and this is a violation of an important assumption in linear regression analysis. The effects of this violation need to be assessed but may be limited,

as the average probability of being positive for most groups of cats is not too extreme. To our opinion, linear regression on these probabilities is a useful method to estimate risk differences because it allows controlling for confounding factors while taking the uncertainty in the test outcome into account.

The risk factors identified were: former stray cat, presence of a dog in the household, hunting behavior and (at $p = 0.066$) feeding of raw meat. Feeding of raw meat was borderline statistically significant but this is probably due to the small number of cat owners that feed their cat raw meat present in the study. Eating raw meat was previously identified as a risk factor for *T. gondii* seropositivity in cats by others (Lopes et al., 2008). The small fraction of owners feeding their cat raw meat also means that a total ban would reduce the seroprevalence by 8% only. Nevertheless, it would still be useful to advise cat owners against feeding of raw meat as this is a simple measure that can be expected to be effective because, from the life cycle of *T. gondii,* it can be understood that this risk factor is directly causal.

Hunting is another risk factor with a clear mechanism: eating infected prey animals will cause infection. It is also one of the strongest risk factors identified, and because many cats hunt (34.7% of the cats in our study), prevention could reduce the seroprevalence by 26%. However, preventing cats from hunting may be more complicated than banning feeding of raw meat. As 93% of the cats showing hunting behavior had free outdoor access, keeping the cat inside will be very effective. However, keeping the cat indoors may not be preferred by the owner. Because outdoor access itself is not a significant predictor after controlling for hunting ($p = 0.161$), measures aimed at preventing hunting without completely limiting outdoor access may be just as effective in reducing the risk of *T. gondii* infection. Possible measures include equipping the cat with a bell (Ruxton et al., 2002; Woods et al., 2003), keeping the cat indoors at night (Robertson, 1998; Woods et al., 2003) and including raw meat in the diet (N.B. after freezing to prevent the risk of infection with raw meat) (Robertson, 1998). That outdoor access is not a significant predictor in the model that includes hunting, may mean that the effect of exposure to environmental oocysts (the effect of outdoor access that is not due to hunting) is not strong. This is in line with the fact that cats are not very susceptible to oocyst infection in comparison to tissue cyst infection (Dubey, 1996a).

Besides feeding of raw meat and hunting, being a former stray cat and presence of a dog in the household were identified as independent predictors for *T. gondii* infection. These factors are unlikely to have a direct effect. Probably the effect of formerly being a stray cat is due to high levels of hunting in the past. The presence of a dog in the household has also been identified as a risk factor for *T. gondii* infection in humans, and especially children (Etheredge et al., 2004; Frenkel et al., 1995; Sroka et al., 2010). In that case dogs may facilitate human exposure to environmental oocysts by rolling in contaminated cat faeces or soil, or passing oocysts in faeces after eating cat faeces (Frenkel et al., 1995; Frenkel et al., 2003; Lindsay et al., 1997). Whether dogs fulfill the same role for their feline housemates which, in contrast to humans, are not very susceptible to oocyst-infection is questionable. Possibly the presence of a dog in the household indicates the presence of an unidentified risk factor: it could, for example, be that dog owners are more likely to live in an area with a dense rodent population. Because the causal mechanism for former stray cat and the presence of a dog is unlikely to be direct, it cannot be assumed that removal of exposure to these risk factors will have the effect as estimated by the population attributable fractions. Nevertheless, preventing cats from straying is expected to give a reduction of the overall seroprevalence.

We were interested in the risk factors for *T. gondii* infection in cats because these risk factors indicate potential targets for measures to reduce infections and thereby oocyst shedding into the environment. However, the amount of oocysts shed into the environment also depends on the cat population size (including stray cats), the fraction of cats defaecating outside, and the amount of oocysts shed by an infected cat (Dabritz, Conrad, 2010). Therefore reduction of the cat population (for example by controlling stray cat populations and advocating timely neutering of domestic cats) and increasing the use of the litter tray could be effective intervention measures too. From the questionnaire, it was clear that almost 90% of the cat owners have a litter tray for their cat, but less than 40% of the cats use it often. That the total amount of oocysts shed influences the environmental contamination with oocysts is an important consideration in vaccine development: a cat vaccine that does not induce sterile immunity but reduces the number of oocysts shed could still be a useful part of a strategy to reduce the exposure of humans and animals to *T. gondii*. Most (85%) of the owners participating in this study were willing to pay some amount for *T. gondii* vaccination of their cat.

In conclusion, the overall prevalence in domestic cats in The Netherlands was estimated at 18.2% (95% CI: 16.6-20.0%) and hunting behavior and feeding of raw meat were identified as risk factors that could be potential targets for intervention measures in order to prevent future human infections.

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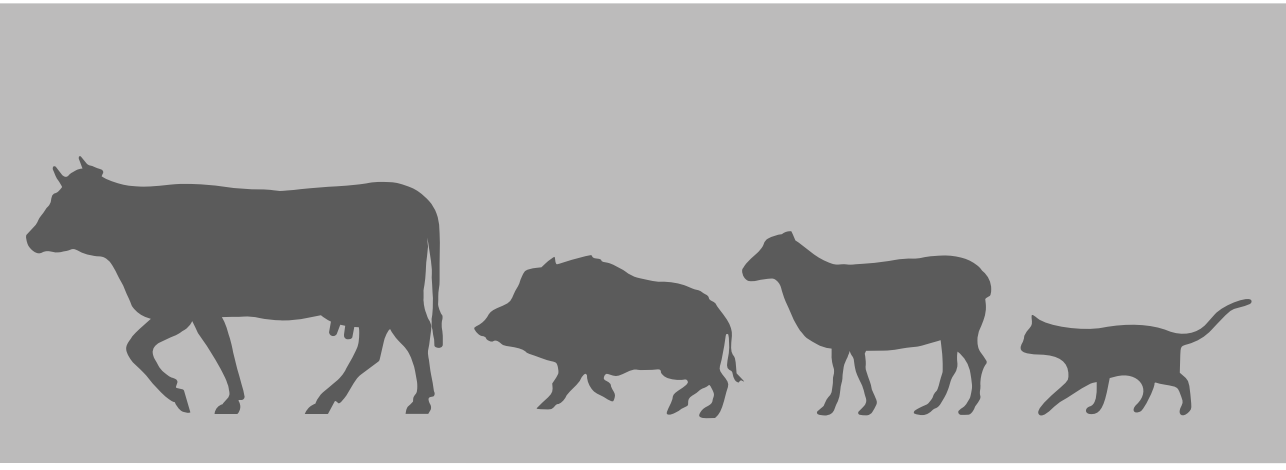
Chapter 8

Discussion



Toxoplasma gondii is an important zoonotic pathogen and the cause of a high disease burden in humans. Because the effectiveness of treatment is debated and no human vaccine is currently available, prevention of infection is considered the most effective strategy to reduce the disease burden. Humans acquire infection through ingestion of cat shed oocysts or consumption of undercooked meat containing tissue cysts. The goal of the work presented in this thesis was to determine the most important sources of *T. gondii* infection for the Dutch population. This knowledge should guide evidence-based decision making on prevention strategies. To investigate and compare the different sources of infections six studies were conducted. The aim of the first study was to develop a sensitive and quantitative method that could replace bioassay for detection of *T. gondii* in meat samples. This study also showed a strong correlation between detection of *T. gondii* by PCR and seropositivity in sheep, and therefore, in the next study, the role of sheep was studied based on serology alone (Chapter 3). For cattle, there was no such relation between the presence of antibodies and of detectable parasitic DNA (Chapter 4), and although the numbers were small, it seemed that seronegative cattle are more likely to carry parasites detectable by PCR. For that reason, we hypothesized that only recently infected cattle have a parasite (possibly tachyzoite) load high enough to be detectable by direct assays. Later, more data were published by Berger-Schoch and colleagues (2010) that support this hypothesis: They found a much higher PCR prevalence in calves than in adult cattle, which is also not consistent with persistence of a detectable infection (Berger-Schoch et al., 2010). Whether PCR detected parasites are infective for humans remains to be studied, but certainly, in cattle the PCR prevalence is more indicative of the risk for human infection than the seroprevalence. For this reason, the PCR prevalence rather than the seroprevalence was used as input for cattle in the QMRA model that combines prevalence data with consumption and preparation data (Chapter 6). The QMRA showed that, even at this low prevalence, beef may be an important source of infection, and that heating practice substantially influences the predicted number of infections (Chapter 6). Unfortunately, no specific consumption data were available for wild boar, which is why the results from the fourth study could not be integrated into this QMRA model. The age-seroprevalence pattern in wild boar suggests that reversion to seronegative may occur, and future studies including PCR-based detection and longitudinal follow-up are required, although the high seroprevalence suggests that consumption of wild boar can pose an important risk of infection (Chapter 5). The stable seroprevalence over the years indicates a comparable infection pressure from the environment. Because direct detection of oocysts from soil and water is difficult, we chose to study *T. gondii* infection in the domestic cat (the almost exclusive definitive host in The Netherlands) to get an indication of the environmental contamination and identify options to prevent shedding of oocysts. Seroprevalence and risk factors for infection with *T. gondii* in cats were studied (Chapter 7). The risk factor that contributed most to the prevalence of cat infection in The Netherlands was hunting. This means that measures that limit hunting behavior of cats, such as limiting outdoor access and wearing a bell, can reduce oocyst excretion in the environment.

In the following sections three subjects will be discussed in more detail. First, the value of the newly developed MC-PCR will be discussed. Next, the conclusions from the use of binary mixture models in four different serological studies (sheep, cattle, wild boar, and cats) will be addressed. Then we will integrate the results from the QMRA and cat study to arrive at our conclusions regarding the most important sources of *T. gondii* infection for the Dutch population, and discuss the implications these findings may have for prevention strategies.

Magnetic capture and PCR-based detection of *Toxoplasma gondii*

The development of a sensitive and quantitative method for PCR-based detection of *Toxoplasma gondii* in tissue samples is an important advancement of the state of the art. The method provides an alternative to the use of mice or cats in bioassay experiments, and has the advantage of providing quantitative results. These quantitative data are necessary as input for a quantitative risk assessment as described in this thesis, but other applications for the method are conceivable. It could be a useful method to determine the outcome in vaccination experiments aimed at prevention or reduction of tissue cyst development in consumption animals. At the moment, the lack of standardized outcome measures in vaccine development hampers comparison between studies and by that slows down the process of finding the best vaccine candidate (Jongert et al., 2009). Quantification of parasite load can also be useful to gain further insight in parasite biology in different hosts. It can for example be used to study predilection sites in naturally infected animals or to study the effects of infectious dose or time after infection on tissue cyst burden in experimental infection studies. Use of MC-PCR in combination with serology would shed more light on the reversion to seronegative that seems possible in wild boar based on the seroprevalence by age curve (Chapter 5).

The disadvantage of MC-PCR is, like with all PCR-based detection, that a positive reaction does not necessarily mean infectious parasites were present. This issue has been discussed extensively in chapter 4, because in cattle it is likely that not all detected parasites were viable, and this would have important implications for the role of beef in human infection with *T. gondii*. In species such as sheep and pigs, the detection of parasitic DNA represents the presence of viable tissue cysts in most cases, as the persistence of *T. gondii* in those species has been shown experimentally by bioassays (Dubey et al., 2008; Gamble et al., 2005). For cattle however, a comparison between PCR and bioassay results should still be made. This would require many bioassays, because it will be difficult to select samples for bioassay based on their PCR results as fresh samples are required for bioassay. This, in combination with a low prevalence of *T. gondii* by PCR, means that many cattle would need to be tested by both assays to obtain a reasonable number of PCR-positive samples for comparison. A step towards elucidating this problem, while limiting the need for bioassays, would be the development of mRNA based detection to indicate the presence of live parasites. This could then be used in combination with the detection of the genomic 529bp repeat region which is likely to be more sensitive. For this comparison between bioassay and PCR, it would also be helpful to focus on calves, because it was recently shown that the prevalence of PCR-positivity in calves is much higher than in older cattle (Berger-Schoch et al., 2010).

Another limitation of the MC-PCR is that selective extraction of the DNA fragment restricts the possibilities for genotyping. In the work presented, this has been solved by additional capture of the GRA6 gene, but as explained in the introduction multilocus typing is preferred. In future, this could be achieved by multiplexing the DNA isolation for several typable fragments at the same time.

Binary mixture models to analyze *Toxoplasma gondii* ELISA results

In the described studies binary mixture models have been used to analyze the serological results for *T. gondii* in sheep, cattle, wild boar and cats (Chapters 3, 4, 5, and 7). The use of this method has been discussed at least briefly in all of these chapters. There are however, some additional lessons that can be learned from examining the results from the four different studies together.

The method has worked especially well with the ELISA results for *T. gondii* in sheep. The results for sheep seem to fulfill all the requirements for the binary mixture analysis to work properly: the seroprevalence was quite high (almost 30%) which allowed both distributions to be fitted based on a reasonable number of sera, and the distributions of log-transformed OD-values for the negative and positive population were well separated and both fitted nicely to a normal distribution. For the wild boar there seemed to be more overlap between the positive and negative distribution, and the analysis did not work if all wild boar were included; it had to be limited to those less than 20 months of age. That the SIS (Susceptible-Infected-Susceptible) compartmental infection model fitted these data best possibly has the same underlying cause: Reversion to seronegative would mean that animals gradually move from the positive population back to the negative population which inevitably leads to overlap of the distributions. Because this reversion is estimated to occur at on average 9 months after infection, there will be more reverting animals among the older wild boar, and exclusion of these animals will help fitting the model. This same reversion to seronegative and the development of low titers if infected later in life, in combination with a low seroprevalence, explain why the situation with cattle sera was even worse. In addition to that, some cross-reactivity with highly prevalent parasites was shown for bovine sera. In cats, the difficulty to fit two normal distributions was of a different nature. The ELISA had been set-up and gave the expected results consistently using sera from before and after experimental infection in cats. However, after testing the sera from the sampled population, it was shown that OD-values were high on average, and the distribution for the positive population looked asymmetrical, i.e. negatively skewed. This was probably due to the high OD-values that reached the limit of the measuring range of the ELISA reader. For that reason, in this case a mirrored gamma distribution was fitted to the positive population. The use of a gamma and a normal distribution to analyze the serological results for cats was also our reason to rename the binormal mixture analysis (Chapters 3, 4, and 5) to binary mixture analysis in chapter 7.

Although the binary mixture model analysis does not work in all situations, it is a valuable method in analyzing serological results. As outlined in chapter 4 it is important to evaluate ELISA results using sera that are representative for the sampled population, because the use of control sera from SPF and experimentally infected animals is likely to overestimate the discriminative power of the assay. In addition, many control sera are required to reliably estimate sensitivity and specificity by receiver-operating characteristic (ROC) curve analysis, and these are usually difficult to obtain. The binary mixture analysis is perfectly suited to evaluate assays using the sera from the sampled population itself. The only prior assumptions relate to the number of distributions to be fitted and their shape. Actually, in case the analysis does not work this provides valuable information that may have been missed if other test validation methods would have been used. For example, a low discriminative power due to reversion to seronegative is likely to affect all serological assays, but may not become apparent

if a cut-off has been established using sera from known negative and experimentally infected animals as control sera. If the lack of fit is due to a too low seroprevalence this is different as it does not indicate a lack of discriminative power. Fortunately, this problem can be solved by collecting additional samples from animals originating from farms, regions or countries where the prevalence is expected to be higher (as has been described for cats (Chapter 7) and previously for *Trichinella spiralis* in pigs (Teunis et al., 2009)). Thus far, fitting the model has worked reasonably well up to a prevalence as low as 11.4% for *Coxiellla burnetti* in rats (Reusken et al., submitted), but this probably also depends on the discriminative power of the assay and the absolute number of positive sera in the sample.

In the studies on wild boar and cats we used an extension of the binary mixture model to estimate the seroprevalence by age. As shown for the wild boar, these seroprevalence by age curves can provide useful information on infection dynamics by fitting compartmental infection models. Such compartmental infection models were not fitted to the cat data, because for cats there is the possibility that some are never exposed to infection (meaning that it would be incorrect to assume that the seroprevalence can go up to 100%) and there is the possibility of age cohort effects due to a changing infection pressure over time (for wild boar these were excluded by demonstrating that the overall prevalence did not differ significantly between two sampling years). The use of the binary mixture model rather than the cut-off to estimate the prevalence per age category makes correction for sensitivity and specificity unnecessary, the true prevalence is estimated directly. This characteristic of the method was also applied differently: The fitted distributions were used to calculate the probability of belonging to the positive distribution for each individual cat in chapter 7. This is a more informative test result than simply scoring the animal positive or negative based on the cut-off value. In statistical analysis, the use of the probability of being positive instead of the binary test outcome means that the uncertainty in the test results (sensitivity and specificity are not 100%) is taken into account and should therefore lead to less misclassification bias. The coefficients from a linear regression on these probabilities are estimates of the adjusted prevalence differences rather than odds-ratios. This means that the effects of exposure to a risk factor are assessed on an additive rather than multiplicative scale, which is considered more plausible biologically. These extensions thus provide advantages of the use of binary mixture models that add up to the usefulness of the method for test evaluation and prevalence estimation.

Relative importance of the sources of *Toxoplasma gondii* infection and implications for prevention

The complicated life cycle of *T. gondii* that includes many different routes leading to human infection, provides several options for intervention. However, before discussing and weighing the different options based on the presented information on the different sources, the preferred target population, either pregnant women or the total population, should be clear.

Target population

To answer the question of the preferred target population, it is important to realize that acquired infection in immune-competent non-pregnant individuals is not harmless. Especially ocular toxoplasmosis, but possibly also mental alterations can contribute substantially to the total disease burden. In fact, it has been suggested that the disease burden from ocular

toxoplasmosis is higher than from congenital toxoplasmosis (Gilbert, Stanford, 2000). However, for pathogens that have more severe consequences in pregnant women or elderly people, interventions aimed at reducing overall transmission in the population may lead to a paradoxal increase in incidence of problems in these specific risk groups: The reduced force of infection leads to an increased average age at infection. The classic example is the increase in congenital rubella syndrome that can be observed after implementation of a vaccination program with low coverage (Knox, 1980; Panagiotopoulos et al., 1999). *T. gondii* is considered especially harmful in pregnant women and prevention strategies are unlikely to completely eliminate transmission, therefore prevention aimed at the total population could potentially increase the incidence of congenital toxoplasmosis. As authors often focus on the decreasing seroprevalence and increasing numbers of seronegative pregnant women at risk for infection (Berger et al., 2009; Hofhuis et al., 2011; Jones et al., 2001) this paradoxal increase seems implicitly assumed. However, because the seroprevalence in women at reproductive age in The Netherlands is low (18.5%), the average age at infection already lies above reproductive age and a further increase from aiming prevention strategies at the total population is unlikely to result in an increase in the incidence of congenital toxoplasmosis. Moreover, prevention measures aimed at the total population still also aim at pregnant women, and probably pregnant women should still be addressed separately just like other risk groups. Targeting a toxoplasmosis prevention program at the total population is not comparable to rubella vaccination of children only.

Turning it around, the same reasoning could lead to the conclusion that we should not prevent infections with *T. gondii*, but need to increase exposure at young age to decrease the incidence of congenital toxoplasmosis. However, although a 100% seroprevalence in women of reproductive age is likely to prevent all congenital toxoplasmosis, it would lead to a substantial increase in ocular toxoplasmosis and in *T. gondii* related complications in immune-compromised individuals. Moreover, if *T. gondii* is indeed causal in the relation observed between *T. gondii* positivity and schizophrenia advocating exposure prior to pregnancy is out of the question. Besides that, it should not be forgotten that many infectious diseases share transmission routes. Increasing raw meat consumption by children to increase the *T. gondii* seroprevalence at reproductive age would lead to more cases of other meatborne illnesses such as salmonellosis, campylobacteriosis and haemolytic-uremic syndrome. Moreover, in a country with a low prevalence, increasing exposure will result in a temporary or, if a high enough prevalence is not reached, constantly higher incidence of congenital toxoplasmosis.

In conclusion, because there is no risk of a paradoxal increase in congenital toxoplasmosis from targeting at the total population, while there is the additional benefit of reducing the disease burden from acquired toxoplasmosis, prevention strategies should target the total population.

Intervention options

At the moment, *T. gondii* prevention in The Netherlands focuses only on congenital toxoplasmosis and is based on informing and educating pregnant women about the risks using a brochure (NVOG et al., 2010). This brochure includes information on prevention of the various common routes of *T. gondii* transmission: proper heating of all meat, hygiene around gardening or other soil contact, delegate cleaning of the cat litter box or do it daily wearing gloves followed by proper hand washing, and proper washing of vegetables and fruit consumed raw. Providing this information to pregnant women should probably always be

part of any prevention program, whatever strategy is the main focus of the program, if only because pregnant women are a specific risk group that are seen by professionals who have the responsibility to inform them. However, the effectiveness of providing this information is debated: On the one hand gynecologists, physicians, obstetricians and midwifes may not provide all the necessary information, on the other hand women may not adhere to the advice given (Jones et al., 2010; Jones et al., 2003; Wallon et al., 1994). There is a lack of studies looking at the effect of *T. gondii*-related health education. The few studies that have been published show no clear effect of health education on *T. gondii*-related knowledge, behavior, and risk of seroconversion in pregnancy (Di Mario et al., 2009; Gollub et al., 2008). Only one small intervention study that includes a randomized control group has been described: This Canadian study shows that pregnant women with a 10 minute session on congenital toxoplasmosis included in their standard prenatal class, improved their pet hygiene behavior and cooking methods for hamburgers and roast beef (Carter et al., 1989).

Because the effectiveness of providing information to pregnant women might be limited, and because prevention should focus on the total population, it may be more effective to include other measures. Education about the risks can be extended to the total population, but it is probably even more difficult to change their behavior than that of pregnant women. Instead of putting the responsibility with the consumer alone, measures to prevent meatborne transmission could also be implemented at the farm (pre-harvest) or processing level (post-harvest).

Post-harvest freezing of meat for at least 2 days at less than -12°C prior to sale, either for all meat or, after implementation of a monitoring program, for meat from animals that tested positive, could be very effective (Kijlstra, Jongert, 2008b). Other options for effective decontamination of meat are irradiation (with effective doses reported varying between 0.3 and 0.7 kGy, (e.g. Dubey, Thayer, 1994; Song et al., 1993)) and high-pressure pasteurization at 300MPa or more (Lindsay et al., 2006). However, because of the presumed effects on color, texture and taste there may be difficulties with consumer acceptance of these options and producer willingness to do so. Moreover, irradiation and high pressure pasteurization are limited by law: Under Framework Directive 1999/2/EC and Implementing Directive 1999/3/EC irradiation of food is restricted to products on the positive list, or those with member-state specific clearance. High pressure pasteurized products need to be authorized as novel food under EC 258/97. For these reasons, freezing of meat prior to sale seems the most feasible option for post-harvest reduction of the concentration of infectious *T. gondii*.

Pre-harvest prevention of infection in livestock animals could be attained by limiting the exposure to *T. gondii* by, for example, limiting outdoor access and use of sterilized feed and bedding and clean drinking water (as is the case in conventional pig and broiler breeding in The Netherlands), removing cats from the farm, and strict rodent control (e.g. Garcia-Bocanegra et al., 2010; Meerburg et al., 2006). However, many of these options are in contradiction with the tendency to provide outdoor access to livestock for welfare reasons. Future pre-harvest options may include vaccination of cats (all cats or only those on and around farms) to prevent oocyst excretion, or vaccination of production animals to prevent tissue cyst formation. As has been mentioned in the introduction, there is a live-vaccine available for sheep to prevent abortion (Toxovax®). The vaccine strain is not able to form tissue cysts (Buxton, 1993; Wastling et al., 1993), but it is unknown whether use of this vaccine also prevents tissue cyst formation from natural exposure after vaccination. Development of new vaccines for livestock is ongoing (e.g. Hiszczynska-Sawicka et al., 2010; Jongert et al., 2008).

To prevent oocyst-acquired infections by measures other than educating the public about hygiene around cleaning of the cat litter box, gardening and other soil contact, measures need to aim at reduction of oocyst shedding by cats into the environment. Aiming at oocyst shedding by cats has the benefit of additionally reducing meatborne infections, because all infections in intermediate hosts have a cat shedding oocysts at the starting point of the cycle. Reduction of oocyst deposition in the environment could be achieved by limiting the number of cats, by reducing the incidence of infection in cats, by reducing the number of oocysts shed by infected cats, and by preventing faecal deposition by cats in the environment (Dabritz, Conrad, 2010). Our study of the risk factors for *T. gondii* infection of cats demonstrated that limiting hunting and feeding of raw meat can reduce the risk of infection. It was also shown that former stray cats were more likely to be seropositive, which means that preventing cats from straying will also reduce the total number of oocysts shed in the environment. To avoid oocysts of ending up in the environment, owners should be made aware that it is important that cats use their litter box, and that the litter box filling is best disposed of with household waste (as this will certainly be heated enough to inactivate any oocysts present, whereas this is less certain for organic waste). There are several options to control the cat population size. First of all, because there are already many unwanted cats (almost 50,000 cats are admitted to animal shelters yearly (Raad voor Dierenaangelegenheden, 2006)), it is important that cat owners are discouraged of breeding kittens and stimulated to have their cats neutered. Secondly, although the size of the stray cat population in The Netherlands is unknown it is perceived to be large. Knowledge on the population dynamics is needed to identify the best control options. With respect to that, it is important to realize that, in general, cats only shed oocysts upon primary infection with *T. gondii*, and stray cats are often already infected in their first year of life (in the nineties 47% of 30 stray cats less 1 year old had antibodies against *T. gondii*, personal communication Prof. Dr. Frans van Knapen). Therefore, in terms *T. gondii* contamination of the environment, a stable older stray cat population might be less harmful than a disturbed population with continuous inflow of young cats.

Vaccination of cats could be an interesting option in future. For cats there is an experimental vaccine available (Frenkel et al., 1991) and in a farm-based experiment, this vaccine has been shown to decrease the exposure of pigs to *T. gondii* (Mateus-Pinilla et al., 1999). The vaccine is based on a mutant strain of *T. gondii* (named T-263) that has lost the ability to complete sexual development. However, because this is a live vaccine produced in mice, there are limitations to the scale of production and the shelf life. Moreover, the vaccine requires frozen storage until administration, and could be hazardous to the people administering it (Innes et al., 2009b). A different type of vaccine would be favored, but vaccine development for cats seems relatively slow. In addition, high vaccination coverage in cats might be difficult to obtain. Although 85% of the owners participating in our cat study were willing to pay some amount for cat vaccination, vaccination coverage against feline diseases of clinical importance is already low (20% or 30-35%, personal communication with Herman Egberink (Utrecht University) and Jacqueline Poot (Intervet)), and uptake of a vaccine that is not of benefit to the cats themselves may be even lower. The actual uptake will, however, largely depend on the communication of the campaign and the financing (subsidized, or paid fully by the cat-owner). For vaccine development, it is important to realize that sterile immunity does not necessarily need to be attained: Reduction of the number of oocysts shed by infected cats may already give an effective reduction of the environmental oocyst burden.

Depending on the type of intervention to prevent *T. gondii* transmission chosen, there may be effects on the transmission of other pathogens. If, for example, the focus is on educating the public to properly heat all meat this will also decrease the incidence of other meatborne pathogens that are sensitive to heat treatment, such as *Campylobacter* spp, *Salmonella* spp, *E. coli* O157, *Staphylococcus aureus*, *Listeria monocytogenes*, norovirus, coronavirus, hepatitis E virus, *Cryptosporidium parvum*, and *Trichinella spiralis*. However, especially viral contamination of food usually occurs by an infected food handler. This may occur at any stage in processing and preparation, including after preparation. For this reason, transmission of these infections is not expected to be eliminated by educating people to properly heat meat. Freezing of all meat prior to retail will reduce transmission of the parasites and may have minor effects on the bacteria, but has no influence on the pathogens introduced after preparation. Limiting outdoor access for farm animals to reduce exposure to *T. gondii* oocysts will also reduce exposure to *Trichinella* and possibly *Campylobacter*, but on the other hand organic farming with limited use of antibiotics has been shown to reduce the prevalence of antibiotic resistance in bacteria (Graveland et al., 2010; Hoogenboom et al., 2008; Kijlstra et al., 2009; Wagenaar, van de Giessen, 2009). Because in The Netherlands the disease burdens of especially *Campylobacter* spp, norovirus, *Salmonella* spp, rotavirus, *Staphylococcus aureus*, and *Listeria monocytogenes* are considerable (Haagsma et al., 2009), the effects of intervention strategies on the transmission of especially those pathogens should be taken into account when comparing the effectiveness of different options.

QMRA and prevention strategies

Now that the preferred target population has been addressed and the options for intervention have been summarized, this section will outline how the QMRA model and the knowledge obtained on the sources of *T. gondii* infection can be used to choose between the different options.

The QMRA, despite of the data gaps, demonstrates that the prevalence of *T. gondii* is not the most important determinant of a species' relative contribution. Preparation practice, especially heating, and consumed volumes substantially influence the number of infections per livestock species predicted by the QMRA. The results of our QMRA also point out that, if the PCR prevalence in cattle represents the actual presence of infectious parasites in cattle, beef is an important source of infection for the human population. Mutton and pork were found more or less equally important.

The relative contribution of different livestock species is important when the prevention strategy is aimed at reduction of the infection levels in meat. In that case, the QMRA model can be used to compare the decrease in number of potential infections by, for example, reducing the prevalence in sheep to 5% (by vaccination or limiting the exposure to *T. gondii)* with the decrease resulting from freezing of all beef prior to retail. The information on the relative contribution of meat from different species should probably not be used to make any distinctions in the advice to individual pregnant women. Making distinctions per livestock species is likely to complicate the advice and thereby reduce its effectiveness. In addition, the relative contribution of livestock species may differ for other pathogens transmitted via raw meat. Instead, all raw and undercooked meat should be stressed as a potential risk, because the QMRA pointed out that 40% of all predicted infections were due to the consumption of unheated products. Clear examples of raw meat products should be provided, because it is often not realized which meat products are raw: Only 74.7%, 58.1%, and 36.3% of German

consumers realized that Mettwurst, salami, and Teewurst were raw meat products (Bremer et al., 2005). The same seems to hold for Dutch consumers of filet américain: Only 5 out of 11 Dutch *E. coli* STEC O157 patients indicated eating filet américain when asked for consumed raw products. The other 6 first reported consuming filet américain when asked about this product specifically (Friesema et al., 2008). Furthermore, to improve *T. gondii* related health education, it is more important to specifically address common misconceptions. For instance, a dangerous idea that seems common is that if you've had cats all your life, you're probably already infected with *T. gondii* and therefore not at risk for a primary infection during pregnancy. Although having a cat is sometimes associated with seropositivity in risk factor studies (e.g. Hofhuis et al., 2011; Kapperud et al., 1996; Pereira et al., 1992), this does not mean that 100% of cat owners is infected.

In the work presented, study of the environmental component was limited to the seroprevalence in cats and species that acquire their infections through the environment (sheep and wild boar). As a consequence, the environmental route could not be incorporated into the QMRA model. Although the importance of cats is clear from the life cycle, direct soilborne transmission to humans might be limited (Cook et al., 2000). This would influence the choice between prevention strategies as it could, for example, mean that the extra costs from vaccinating all cats rather than only cats on livestock breeding farms outweigh the additionally prevented infections. Therefore inclusion of the environmental route into the QMRA model is highly desired. To obtain these data future work should focus on the development of sensitive and quantitative detection methods for oocysts in soil and water. Alternatively, oocyst density estimations could be based on the numbers of oocyst shed by cats and oocyst survival data. Furthermore, quantitative data on human contacts that can lead to oocyst ingestion need to be gathered.

Besides extending the QMRA model with the environmental route it is necessary to obtain more reliable data regarding meatborne transmission. This has been described in detail in chapter 6 and the most important points identified were: collect data on the effects of processing in line with consumer-style processing and acquire product-specific heating temperatures, investigate the presence of viable bradyzoites in cattle, determine the effect of mincing meat on bradyzoite concentration using actual batch sizes, and obtain an estimate of the fraction of meat that has been frozen prior to purchase. Once these data have been included in the QMRA model, the model can be used to calculate the effectiveness (reduction of numbers of infections) of different prevention strategies, provided that the effect of implementing a measure on the input data for the QMRA is known. This means that, for example, the effect of compulsory vaccination on the prevalence and bradyzoite concentration in sheep needs to be known. Or, once the environmental route has been included, the number of cats vaccinated and the effect of vaccination on oocyst excretion is needed to estimate the effect of cat vaccination. Then, to change the outcome of the QMRA from the reduction of the number of infections to the reduction in disease burden (DALYs), further studies on the incidence of different outcomes of especially acquired but also congenital toxoplasmosis are needed. Subsequently, these need to be systematically evaluated regarding severity and duration of symptoms as has been described for congenital toxoplasmosis (Havelaar et al., 2007). Cost calculation for the different prevention strategies is another aspect that needs more attention. This also includes investigating the most effective implementation strategy: for example, will the government pay for cat vaccination or is it the responsibility of the owners? The costs and feasibility of implementation are factors that will strongly influence the optimal choice.

Furthermore, it is important to realize that transmission of *T. gondii* is a dynamic process. Previous shifts in transmission are assumed: The seroprevalence in pigs and poultry has decreased substantially with the upcoming of industrialized breeding (van Knapen et al., 1982; van Knapen et al., 1995) and this, in combination with an increase in frozen storage, is considered responsible for the decrease in seroprevalence for the Dutch human population as was observed between 1995/1996 and 2006/2007. Likewise, the QMRA pointed out that pork is not the most important source of meatborne infection anymore, the role of sheep is similar and beef may be more important. However, with the increasing attention for animal welfare, organic farming with outdoor access is on the rise, and pigs may regain their role as most important source of infection. In addition to changes in the exposure of Dutch consumption animals to *T. gondii*, shifts in importing countries may lead to changes in the overall prevalences for the meat-producing species consumed in The Netherlands. Lastly, changing eating habits may influence the relative importance of the different meat sources of infection. It is, for example, known that the consumption of lamb and mutton in The Netherlands is increasing, although still low at the moment (approximately 1.4 kg/head/year, PVE 2009). Consumption of game and more exotic types of meat (such as ostrich) is also increasing, and there may be changes in the level of heating and proportion freezing prior to consumption by consumers or during import or processing.

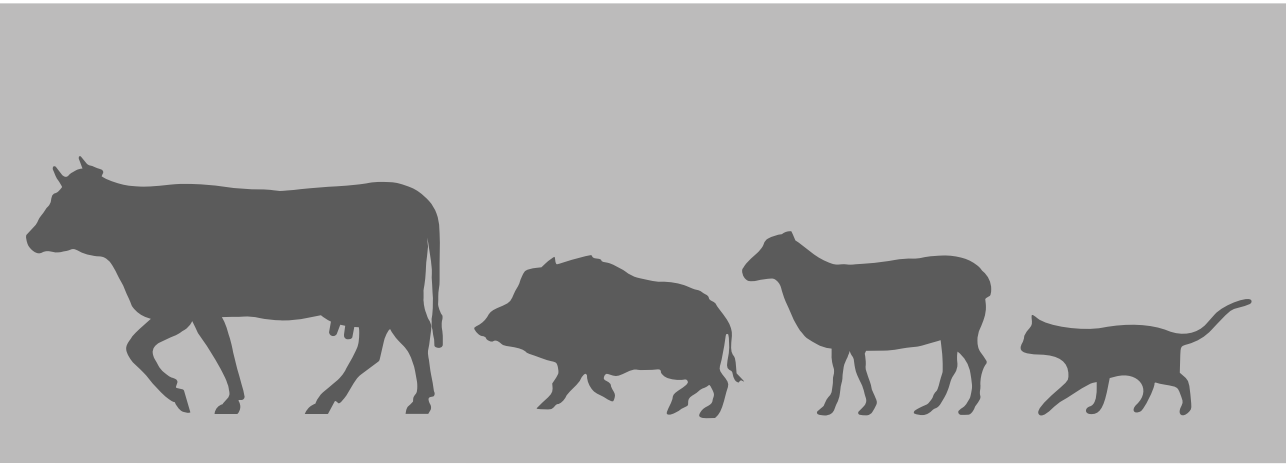
Human exposure to oocysts may change in relation to the number of cats present outdoors or to the incidence of *T. gondii* infection in cats due to changes in cat housing (restriction of outdoor access will give less hunting opportunity) and feeding (more raw meat will increase the risk of infection). The high seroprevalences for sheep, wild boar and cats suggest that the infection pressure from the environment is unchanged, or at least not substantially lower. Although the infection pressure may have increased in especially urban areas, because the number of cats kept as pet has increased from 2.2 million to 3.3 million between 1997 and 2006 (Raad voor Dierenaangelegenheden, 2006). Because shellfish are a potential source of human infection, increased raw shellfish consumption can also lead to higher numbers of oocyst transmitted infections. Even climate change may influence the exposure to oocysts as oocyst survival depends on temperature, humidity and UV irradiation (Meerburg, Kijlstra, 2009). Travel can lead to more diversity in *T. gondii* strains found to infect people, which, as has been pointed out in the introduction, may influence the clinical outcome.

Because the most important sources of infection are likely to shift due to changing prevalences, changing eating habits or a changing environment, monitoring of the presence of *T. gondii* in the various sources as well as human contact with these sources should be repeated to enable adjustment of the prevention strategy. The QMRA model can easily be used to reassess the importance of the different routes when new input data become available.

The work presented in this thesis focuses on the different sources of *T. gondii* infection for humans. Much insight in the transmission of *T. gondii* in The Netherlands has been gained and many options for intervention in *T. gondii* transmission have been outlined. The disease burden from *T. gondii* infection in humans can be reduced by incorporating measures aimed at the total population, in addition to educating pregnant women, into the prevention strategy. Now that the options are clear, the time has come to initiate projects to study the effect of different intervention measures. As argued, these interventions should target the total population, and could besides education, also include interventions aimed at cats or pre- and post-harvest reduction of the prevalence of *T. gondii* in the meatborne sources of human infection. For these studies we would like to stress the importance of an integrated

microbiological approach: The effect on the incidence of pathogens that share the same transmission routes should be included in the cost-benefit analyses. When more data become available to improve the reliability of the QMRA results for meatborne transmission and the model is extended with the environmental route, the developed QMRA model will be a useful tool to compare the effectiveness of different intervention options now and in the future.

Summary
Samenvatting



Summary

The protozoon *Toxoplasma gondii* was discovered in 1908, and is recognized as a human pathogen since 1939. Its life cycle includes sexual multiplication in felines (the definitive hosts) and asexual multiplication in virtually all warm-blooded animals, including humans. Primary infection of a cat results in shedding of millions of environmentally resistant oocysts, whereas infection in intermediate hosts results in the development of tissue cysts. Oocysts and tissue cysts are infective for both definitive and intermediate hosts. In addition, there is the possibility of transplacental transmission which is the main cause of clinical problems in both humans and animals. If a woman is primary infected during pregnancy *T. gondii* may be transmitted to the foetus which can result in death, central nervous system abnormalities or eye disease. Besides that, a new *T. gondii* infection or recrudescence of a latent infection can lead to severe disease in immunocompromised individuals. In addition, *T. gondii* is an important cause of chorioretinitis and, because of its predilection for nervous tissue, the subject of study for a relation with various types of mental alterations.

Humans can be infected by ingestion of tissue cysts in unfrozen and undercooked meat or by ingestion of oocysts while cleaning the cat litter box, from consuming unwashed vegetables or fruit, from contact with soil, and from drinking untreated water. Raw milk is another potential source of infection. In The Netherlands the overall seroprevalence for the Dutch human population has decreased from 40.5% in 1996 to 26.0% in 2006. The incidence of congenital toxoplasmosis in 2006 was estimated at 2 per 1000 live-born children. This is high, and based on this estimate the disease burden of congenital toxoplasmosis alone was estimated at 2300 DALYs, making *T. gondii* one of the most important foodborne pathogens. Including the disease burden of ocular toxoplasmosis from acquired infection is expected to add substantially to the total burden from *T. gondii* infection. Nevertheless, screening for infection during pregnancy is not implemented because prevention of infection is considered more cost-effective in The Netherlands. At the moment prevention only involves educating pregnant women about the risks of infection, but inclusion of other measures may be more effective. Knowledge on the relative importance of the different sources will guide the development of effective prevention strategies. In The Netherlands it has been shown that the seroprevalence in pigs is low, and highly dependent on the housing system. For other livestock species and cats no recent data are available. The goal of the research presented in this thesis was to answer the question: What are the most important sources of *T. gondii* infection for the Dutch population? To answer this question the prevalence of infection in various animals was studied.

In chapter 2 the development of a quantitative PCR-based detection method for *T. gondii* in tissue samples is described. Sensitivity of PCR-based assays so far, was hampered by the small sample size in combination with a low concentration and inhomogeneous distribution of tissue cysts. These drawbacks were overcome by DNA isolation from 100g samples followed by selective extraction of the target DNA (529-bp repeat element of *T. gondii*) using magnetic capture. The detection limit was approximately 230 tachyzoites added to a 100g sample, which means that one tissue cyst should be detectable in most cases. The results were comparable to mice bioassay results for 4 experimentally infected pigs, and to serological findings for 73 naturally infected sheep. In addition, capture of the GRA6 gene was incorporated to allow genotyping. *T. gondii* GRA6 type II was identified in sixteen sheep samples. The development

of this method provides an alternative to bioassay and has the additional benefit of quantitative results.

Because the ELISA results and detection by MC-PCR corresponded well for sheep and a similar correlation had previously been found for modified agglutination test and bioassay, we studied the prevalence of antibodies against *T. gondii* in sheep as a proxy for the presence of *T. gondii* parasites in chapter 3. The overall prevalence was high (27.8%), and significantly higher in sheep over one year old, indicating that consumption of undercooked lamb and especially mutton poses an important risk of infection. The significantly higher seroprevalence in the central provinces indicates that there may be regional differences in environmental contamination, although an effect of regional differences in farm management cannot be excluded. Because of a lack of appropriate reference sera, we analysed the results by binary mixture analysis. This proved to work very well and estimated test characteristics were similar to those obtained from Bayesian analysis including three commercial tests.

In chapter 4, the same type of study is described for cattle. However, for this species the correlation between seropositivity and detection of tissue cysts was less clear, therefore our aim was to first develop a serological assay that accurately detects antibodies against *T. gondii* in cattle, and subsequently investigate the correlation between seropositivity and detection of tissue cysts by the sensitive MC-PCR method described in chapter 2. Although longitudinal follow-up of 27 calves showed seroconversion in 24, the ELISA results for the cross-sectional study were difficult to interpret. Fitting the binary mixture model did not work as easily as for sheep. This became even worse when the ELISA protocol was changed to work with a higher serum dilution, even though this gave better results with the control sera. Some cross-reactivity and aspecific binding was observed, and the results analysed using the binary mixture model did not correspond well with the results based on the cut-off obtained from longitudinal follow-up in calves. Even agreement with the modified agglutination test was poor. But since our main goal was to get an indication of the role of beef in human infection with *T. gondii*, the PCR prevalence of *T. gondii* and its correlation with seropositivity was our main interest. These were found completely discordant: Only two cattle negative in ELISA and MAT were found positive by PCR. We hypothesized that maybe only recently infected animals, which have not yet developed antibodies, have a parasite load high enough to be detectable by PCR. It had previously been suggested that cattle are able to clear an infection with *T. gondii* and this alleged capability can explain both the difficulty to develop a sufficiently discriminatory serological assay (because titers will decrease) and the fact that only seronegative cattle have PCR detectable parasites present (because clearance is likely to occur in line with seroconversion). In conclusion, our results show that in cattle the seroprevalence cannot be used as an indicator of the number of cattle carrying infectious parasites. Whether meat from PCR positive cattle is infectious requires further investigation, as non-viable or tachyzoite stages may have been detected.

In chapter 5, the seroprevalence in wild boar was studied to get an indication of the role of their meat in human infection and to investigate the environmental contamination with *T. gondii*. The seroprevalence was high (24.4%) and similar over regions and sampling years, indicating a stable and homogeneous infection pressure from the environment. In this chapter we also describe an extension of the binary mixture model to calculate the seroprevalence by age: A direct estimate of the true prevalence per each age group was obtained by fitting the model to animals categorized by age using the previously obtained parameters, but leaving the mixing parameter to be estimated. Thus, these seroprevalences do not need correction for sensitivity

and specificity of the test. The plot of these seroprevalences against the mean ages for the groups revealed a steep increase until 10 months of age and stabilization of the seroprevalence at approximately 35% thereafter. Fitting of several types of compartmental infection models confirmed that the observed pattern is not consistent with a constant infection pressure and lifelong persistence of antibodies. The SIS-model, which includes reversion to seronegative, fitted the data much better than an SI-model. If the stabilization of the seroprevalence is indeed due to seroreversion this means that a negative serological result does not exclude prior exposure. If this loss is preceded or accompanied by a loss of tissue cysts it means that wild boar can clear their infection, but on the other hand, if it is not, it means that seronegative wild boar can carry tissue cysts. Because of these important implications of seroreversion, future studies should focus on longitudinal follow-up of infected wild boar regarding the presence of antibodies and tissue cysts.

In chapter 6, the results from chapters 2, 3 and 4 together with previously published data on the seroprevalence in pigs, consumption and preparation data, and data from *T. gondii* survival experiments are combined into a Quantitative Microbial Risk Assessment (QMRA) model to compare the relative contribution of the different livestock species to meatborne *T. gondii* infections in The Netherlands. Unfortunately consumption of game was not specified by species in the available consumption data, which is why the data on wild boar (Chapter 5) could not be included. Although many uncertainties existed in the data used to build the model and the total number of congenital infections predicted by the model was at least 20 times higher than the number estimated based on the incidence of congenital toxoplasmosis in newborns, the results underline the usefulness of the advice to properly heat meat: Forty percent of the predicted infections was caused by unheated products, and sensitivity analysis indicated heating as the most influential parameter. The results also demonstrate that beef, even at a low prevalence of *T. gondii* for cattle, can contribute substantially to the total number of predicted infections, because beef is often consumed (partially) raw. Therefore, further investigation of the presence of viable and infective tissue cysts in beef is one of the most important recommendations for future research that arose from this study. Other recommendations to improve the QMRA model for meatborne *T. gondii* infection are: collect processing-effect data in line with consumer style processing and acquire product specific heating temperatures, determine the effect of mincing meat on bradyzoites concentration using actual batch sizes, and obtain an estimate of the fraction of meat that has been frozen prior to purchase. Once more accurate data become available, the QMRA will provide a useful tool to aid science-based decision-making on prevention strategies which is further discussed in chapter 8.

Chapter 7 addresses the seroprevalence and risk factors for *T. gondii* infection in the definitive host, the cat. As definitive hosts, cats play an important role in the epidemiology of *T. gondii* and therefore measures aimed at preventing cat infection could be very effective in decreasing both oocyst- and tissue cyst acquired infections in humans. Sera from 450 cats were tested and the seroprevalence was estimated at 18.2% by binary mixture analysis. Hunting and feeding of raw meat were risk factors identified by logistic regression analysis that are also possible targets for prevention measures. It was shown that outdoor access is not a significant risk factor when hunting is included in the model, indicating that if hunting can be prevented without limiting outdoor access this may be just as effective in reducing the incidence of infection as limiting outdoor access. Other factors that influence contamination of the environment by cats are summarized: These include limiting the cat population, and stimulating use of the

cat litter box. In this chapter, another extension of binary mixture analysis is described: The fitted distributions were used to estimate the probability of being positive for each individual cat. This is a more informative test result than a score based on a cut-off value because it takes into account the sensitivity and specificity of the test. This may have useful applications in epidemiological studies because it limits misclassification of the outcome. In this case the results were used to estimate the risk differences corrected for confounding by other factors in a linear regression model.

In the general discussion (Chapter 8) three topics, which are described in this thesis, are addressed in a broader perspective. First the usefulness of the MC-PCR both in and outside of the scope of this thesis is discussed. Because this method limits the need of experimental animals and provides the desired quantitative test results, it can be seen as a true advancement of the state of the art. In our opinion, the same is true for the binary mixture analysis. At first, it was mainly used because it allows test evaluation when no large number of reference sera or a gold standard test is available, and it proved very useful for this purpose. Later, we extended the model for estimation of the age-related seroprevalence (Chapter 5) and fitting compartmental infection models to this curve led us to the interesting hypothesis that antibodies to *T. gondii*, and possibly the infection itself, do not persist lifelong in wild boar. In chapter 7, another extension is described: The binary mixture model was used to estimate the probability of being positive for each cat. The binary mixture analysis thus offers additional advantages over test comparison using reference sera, even if plenty of those are available.

In the final part of the last chapter the implications of the findings in the previous chapters for prevention strategies are discussed. First it is argued that prevention strategies should focus on the total population rather than pregnant women alone, mainly because the disease burden from acquired toxoplasmosis contributes substantially to the total disease burden from *T. gondii*. Next, the possible intervention options are listed. At the moment prevention in The Netherlands focuses on educating pregnant women about the risks of infection. This could be extended by aiming education at the total population, but the effectiveness of educating pregnant women is questioned, and influencing the behavior of the total population is probably even more difficult. Therefore it might be more useful to include pre-harvest (decontamination) or post-harvest (prevention of infection in livestock) options to decrease the concentration and prevalence of infectious *T. gondii* in meat, and measures aimed at reducing oocyst shedding by cats. Vaccination of livestock or cats would be an interesting future option. Depending on the type of intervention measure chosen, there may be effects on the transmission of other foodborne pathogens, and it is preferred that these effects are taken into account when comparing the cost-effectiveness of different intervention options. After further development, the QMRA will be a useful tool for comparison of the effectiveness of the different strategies. As described in chapter 6, more data are needed to improve the model and obtain a more reliable outcome, but other options for further development are described in this last chapter: the environmental route should be included and, for comparison purposes, the outcome would best be measured as the decrease in DALYs rather than predicted infections. Furthermore, in order to calculate the cost-effectiveness of certain options, the effects of these options on the input data for the QMRA model (e.g. the prevalence of *T. gondii* in sheep) need to be studied and the costs for the most feasible implementation method need to be estimated. All of these extensions require further study, and a focus on intervention studies for future work is recommended. It is also important to realize that the importance of different *T. gondii* transmission routes may shift due to changing prevalences in consumption

animals, changing human behavior or a changing environment. For that reason, monitoring should be repeated, and with new input data, the QMRA model can be used to compare the effectiveness of different intervention options now and in the future.

Samenvatting

De protozoaire parasiet *Toxoplasma gondii* werd in 1908 ontdekt en sinds 1939 is bekend dat *T. gondii* ziekte kan veroorzaken bij mensen. De levenscyclus van *T. gondii* omvat seksuele vermeerdering in de darm van katachtigen (de eindgastheren) en aseksuele vermeerdering in vrijwel alle warmbloedige dieren inclusief mensen (de tussengastheren). Na een eerste infectie gaat een kat miljoenen oöcysten uitscheiden in het milieu, terwijl zich bij tussengastheren weefselcysten vormen in voornamelijk spier- en zenuwweefsel. Oöcysten en weefselcysten zijn beide infectieus voor zowel katten als tussengastheren. Passage via de placenta is de derde mogelijkheid van overdracht op de volgende gastheer (in dit geval de ongeboren vrucht) en is tevens de belangrijkste oorzaak van ziekteverschijnselen bij zowel mens als dier. Als een vrouw voor het eerst een infectie met *T. gondii* oploopt tijdens de zwangerschap kan de parasiet worden overgedragen op het ongeboren kind, met mogelijk een miskraam of een kind geboren met afwijkingen aan het zenuwstelsel of de ogen tot gevolg. Daarnaast kan een nieuwe infectie, maar ook het reactiveren van een reeds aanwezige infectie, leiden tot ernstige ziekteverschijnselen bij mensen met een immuundeficiëntie. Bovendien is *T. gondii* een belangrijke oorzaak van chorioretinitis (ontsteking van het vaat- en netvlies van het oog) en is, in verband met de voorkeur van *T. gondii* voor zenuwweefsel, de relatie tussen *T. gondii* infectie en verschillende mentale afwijkingen regelmatig onderwerp van onderzoek.

Mensen kunnen met *T. gondii* geïnfecteerd raken door opname van weefselcysten in onvoldoende verhit vlees dat niet ingevroren is geweest, of door opname van oöcysten bij het schoonmaken van de kattenbak meer dan 1 dag na gebruik, bij consumptie van ongewassen groenten of fruit, door contact met grond, en door het drinken van ongezuiverd water. Ook rauwe melk is een potentiële bron van infectie. In 1996 had 40.5% van de Nederlandse bevolking antilichamen tegen *T. gondii* wat betekent dat zij er ooit mee zijn geïnfecteerd. In 2006 was dit percentage afgenomen naar 26.0%, maar werd het voorkomen van *T. gondii* bij pasgeboren baby's geschat op 2 per 1000. Dit is vaker dan werd verwacht, en op basis van deze schatting van het voorkomen van aangeboren toxoplasmose behoort *T. gondii* tot de belangrijkste voedseloverdraagbare ziekteverwekkers in Nederland. Dat dit een onderschatting is van het totale belang wordt duidelijk wanneer men bedenkt dat de ziektelast van de door *T. gondii* veroorzaakte oogafwijkingen na verworven infectie (dat wil zeggen, na infectie door opname van weefselcysten of oöcysten) hierbij nog niet is meegenomen. Toch worden zwangere vrouwen in Nederland niet routinematig getest op *T. gondii*, omdat preventie van infectie meer kosteneffectief wordt geacht. De belangrijkste reden hiervoor is dat de effectiviteit van de behandeling die wordt gestart wanneer een infectie wordt aangetoond niet goed bekend en mogelijk beperkt is. Op dit moment is de preventie van infectie bij zwangere vrouwen volledig gebaseerd op voorlichting, maar mogelijk is het effectiever ook andere maatregelen in het preventieprogramma op te nemen. Om gerichte preventiemaatregelen op te kunnen stellen is het van belang te weten wat de belangrijkste bronnen van infectie zijn voor de Nederlandse bevolking. Wat betreft de bronnen van infectie in Nederland is het bekend dat *T. gondii* bij varkens nauwelijks voorkomt, maar wel wat meer bij varkens met buitenuitloop. Voor andere productiedieren en katten zijn slechts beperkte en vaak verouderde gegevens beschikbaar. Daarom richtte het in dit proefschrift beschreven onderzoek zich op het beantwoorden van de vraag: "Wat zijn de belangrijkste bronnen van *T. gondii* infectie voor de Nederlandse bevolking?" Om deze vraag te beantwoorden werd het voorkomen van *T. gondii* infectie bij verschillende diersoorten bestudeerd.

In hoofdstuk 2 wordt de ontwikkeling van een nieuwe kwantitatieve techniek voor het aantonen van weefselcysten beschreven. De methode is gebaseerd op het aantonen van het erfelijk materiaal (DNA) van de parasiet met behulp van PCR (een kettingreactie waarbij een specifiek stuk DNA zo vaak wordt vermeerderd dat het aantoonbaar wordt). Voorheen was het moeilijk om met dergelijke methoden *T. gondii* aan te tonen omdat slechts een klein stukje vlees kan worden getest (ca. 0,1 gram) terwijl er vaak slechts 1 of 2 weefselcysten per 100 gram aanwezig zijn. De kans dat een stukje vlees wordt getest waar geen weefselcyste (en dus geen *T. gondii* DNA) in zit, terwijl het dier wel geïnfecteerd is, is op die manier groot. De eerste stap was dus het nemen van grotere stukken, in dit geval 100 gram. Maar DNA extractie van een dergelijk stuk resulteert in een DNA isolaat wat voornamelijk DNA van de gastheer en weinig DNA van *T. gondii* bevat en dit gastheer DNA verstoort de PCR-reactie die bedoeld is om *T. gondii* aan te tonen. Daarom is een volgende stap toegevoegd waarbij het DNA van *T. gondii* op basis van de unieke nucleotiden-volgorde met magnetische bolletjes eerst selectief uit het mengsel van DNA wordt gehaald. Op deze manier kan *T. gondii* nu worden aangetoond wanneer minimaal 230 parasieten per 100 gram vlees aanwezig zijn en, aangezien oudere weefselcysten vaak honderden tot duizenden parasieten bevatten, is de aanwezigheid van één weefselcyste meestal voldoende. In een vergelijkingsonderzoek met vlees van 4 experimenteel geïnfecteerde varkens bleek de gevoeligheid van de nieuwe methode vergelijkbaar met die van de muizenproef, de tot nu toe meest gebruikte methode om *T. gondii* aan te tonen. Daarnaast kon in de meeste op natuurlijke wijze geïnfecteerde schapen (wat werd onderzocht door ze te testen op de aanwezigheid van antilichamen tegen *T. gondii*) met de nieuwe methode ook het DNA van de parasiet worden aangetoond. De nieuwe techniek kan muizenproeven vervangen, en geeft als bijkomend voordeel inzicht in de concentratie van *T. gondii* in het vlees.

Omdat de detectie van *T. gondii* met de nieuwe PCR methode goed overeenkwam met het aantonen van antilichamen tegen *T. gondii* bij schapen, werd in hoofdstuk 2 het voorkomen van *T. gondii* bij schapen onderzocht met behulp van een serologische test, in dit geval een ELISA. De seroprevalentie was hoog (27.8%) en significant hoger bij schapen ouder dan 1 jaar dus vormt de consumptie van onvoldoende verhit lams- en met name schapenvlees mogelijk een belangrijke bron van infectie voor de mens. De seroprevalentie was ook significant hoger voor schapen uit de provincies Utrecht en Gelderland. Dit suggereert dat er regionale verschillen zijn in besmetting van de omgeving, maar regionale verschillen in bedrijfsmanagement (waardoor de schapen in deze provincies bijvoorbeeld meer buiten komen) konden niet worden uitgesloten. Omdat er slechts enkele sera beschikbaar waren van bekend positieve en negatieve schapen kon de beste afkapwaarde voor de ELISA niet op de gangbare manier worden vastgesteld. In plaats daarvan werden de met ELISA verkregen OD-waarden log-getransformeerd en uitgezet in een frequentie-distributie. Vervolgens werd mathematisch het best passende mengsel van twee normaalverdelingen (één voor de *T. gondii* positieve en één voor de *T. gondii* negatieve schapen) gezocht en de prevalentie direct geschat uit de verhouding tussen deze twee verdelingen. Daarnaast kan op basis van de verkregen verdelingen, voor iedere mogelijke afkapwaarde worden bepaald welk de deel van de positieve dieren ook positief uit de test zou komen (de sensitiviteit van de test) en welk deel van de negatieve dieren ook als negatief uit de test zou komen (de specificiteit van de test). De op deze manier verkregen sensitiviteit en specificiteit werden vergeleken met schattingen van de sensitiviteit en specificiteit verkregen door de test te vergelijken met drie commerciële testen (met behulp van Bayesiaanse statistiek). De geschatte testeigenschappen bleken goed overeen te komen en het binaire mengsel-model is dus geschikt voor dit gebruik.

In hoofdstuk 4 wordt een vergelijkbare studie bij runderen beschreven. Voor runderen was vooraf duidelijk dat de relatie tussen aanwezigheid van antilichamen en parasieten niet sterk is. Daarom werd in deze studie eerst veel aandacht besteed aan het ontwikkelen van een betrouwbare ELISA voor het aantonen van antilichamen en vervolgens een vergelijking gemaakt tussen de resulaten verkregen met ELISA en de nieuwe gevoelige PCR methode uit hoofdstuk 2. Hoewel het verloop in de tijd liet zien dat 24 van de 27 kalveren antilichamen tegen *T. gondii* ontwikkelden, waren de resultaten voor de steekproef van de Nederlandse runderpopulatie moeilijk te interpreteren. Het mengsel-model paste niet goed op de frequentie-distributie van de log-getransformeerde OD-waarden, en dit ging nog slechter na aanpassing van de serumverdunning in de ELISA, hoewel dit voor de controlesera betere resultaten gaf. Daarnaast werden kruisreactiviteit en aspecifieke binding waargenomen, en kwamen de prevalenties verkregen met het mengsel-model en met een afkapwaarde gebaseerd op het verloop van de OD-waarden bij de kalveren niet met elkaar overeen. De resultaten waren ook slecht te vergelijken met die van een andere serologische test, de MAT (een agglutinatie test). Maar aangezien het verkrijgen van inzicht in de rol van rundvlees bij *T. gondii* infecties bij de mens ons belangrijkste doel was, waren wij voornamelijk geïnteresseerd in de overeenkomst tussen de serologische (ELISA en MAT) en de PCR resultaten. Deze kwamen totaal niet overeen: alleen twee seronegatieve runderen testten positief in de PCR. Mogelijk is alleen bij recent geïnfecteerde dieren de concentratie parasieten hoog genoeg voor detectie met behulp van PCR. Er werd eerder door anderen gesuggereerd dat runderen hun infectie met *T. gondii* kunnen klaren en dit zou zowel de problemen met het ontwikkelen van een voldoende onderscheidende serologische test (antilichaam-titers nemen geleidelijk weer af) als het feit dat alleen seronegatieve dieren positief werden bevonden in de PCR (het klaren gebeurt waarschijnlijk tegelijkertijd of dankzij het ontwikkelen van de antilichaamrespons) kunnen verklaren. In ieder geval kan worden geconcludeerd dat de seroprevalentie bij runderen geen goede indicatie geeft van het aantal dieren dat infectieuze parasieten bij zich draagt. Of dit wel geldt voor de PCR prevalentie moet nog blijken, omdat mogelijk dode of niet-infectieuze parasieten werden aangetoond.

Het onderzoek beschreven in hoofdstuk 5 was erop gericht de seroprevalentie bij wilde zwijnen in Nederland te bepalen. De seroprevalentie bij wilde zwijnen geeft een indicatie van de omgevingscontaminatie met *T. gondii* en ook wild zwijn is een mogelijke bron van infectie voor de mens. De seroprevalentie bleek hoog (24.4%) en gelijk voor de twee gebieden (de Roerstreek en de Veluwe) en de twee perioden (2002/2003 en 2007) wat suggereert dat de omgevingsbesmetting homogeen en stabiel is. Verder wordt in dit hoofdstuk een uitbreiding van het binaire mengsel-model beschreven, die het mogelijk maakt de seroprevalentie per leeftijdscategorie te schatten. Door de parameters (gemiddelde en standaard deviatie) behorende bij de twee verdelingen die passen aan de frequentie-distributie van de OD-waarden voor alle dieren vast te houden, en alleen de mengparameter (welke de verhouding tussen de twee verdelingen aangeeft) per leeftijdsgroep opnieuw te schatten, wordt een directe schatting van de werkelijke seroprevalentie voor die groep verkregen (correctie voor de sensitiviteit en specificiteit van de test is op die manier overbodig). Voor de wilde zwijnen vertoonde de seroprevalentie een zeer snelle stijging tot 10 maanden oud, waarna deze stabiliseerde op ongeveer 35%. Aan dit verloop werden vervolgens verschillende compartimentele infectiemodellen (waarbij dieren bijvoorbeeld een transitie van gevoelig voor infectie, naar geïnfecteerd, naar resistent kunnen doormaken) gepast. Een model waarin een dier alleen van gevoelig voor infectie naar geïnfecteerd kan, en dus vervolgens levenslang geïnfecteerd blijft (zoals voor *T. gondii* infecties in veel dieren wordt aangenomen) paste niet goed aan

dit verloop. Een model waarin een dier weer opnieuw gevoelig voor infectie kan worden (in het model betekent dit dat het dier de opgebouwde antilichamen kwijtraakt) paste veel beter. Als het stabiliseren van de seroprevalentie inderdaad komt doordat dieren weer seronegatief worden heeft dit belangrijke gevolgen. Het betekent dat de afwezigheid van antilichamen niet uitsluit dat het dier ooit geïnfecteerd is geweest, en als het verlies van antilichamen gepaard gaat met verlies van de weefselcysten betekent het dat wilde zwijnen in staat zijn hun infectie te klaren. Aan de andere kant, als dieren seronegatief kunnen worden zonder dat de weefselcysten verdwijnen, betekent het dat ook seronegatieve zwijnen infectieus kunnen zijn. Vervolgstudies waarin wilde zwijnen op verschillende tijden na infectie worden getest met behulp van serologie en PCR moeten uitwijzen welk mechanisme daadwerkelijk aan het stabiliseren van de seroprevalentie ten grondslag ligt.

De resultaten uit hoofdstuk 2, 3 en 4, eerder verschenen gegevens over de prevalentie bij varkens, vleesconsumptie- en bereidingsgegevens, en data uit overlevingsexperimenten met *T. gondii* worden in hoofdstuk 6 gebruikt voor een kwantitatieve microbiële risicoschatting (QMRA). Het model wordt gebruikt om de relatieve bijdrage van varkens-, rund- en schapenvlees aan *T. gondii* infecties bij de Nederlandse bevolking te schatten. Helaas waren geen gespecificeerde gegevens over de consumptie van wild beschikbaar dus kon de prevalentie voor wilde zwijnen (hoofdstuk 5) niet mee worden genomen. Er waren onduidelijkheden in de data die werden gebruikt om het model op te zetten en het aantal voorspelde infecties bij mensen lag minimaal 20 keer hoger dan de schatting op basis van het aantal gevallen van aangeboren toxoplasmose bij pasgeborenen. Desondanks onderschrijven de resultaten het belang van het advies om vlees voldoende te verhitten: 40% van de voorspelde infecties was toe te schrijven aan onverhitte produkten en de gevoeligheidsanalyse liet zien dat vooral de verhittingstemperatuur effect had op het aantal voorspelde infecties. Ook blijkt dat rundvlees, zelfs wanneer *T. gondii* slechts bij een beperkt deel van de runderen voorkomt, een belangrijke bron van infectie kan vormen. Dit komt omdat rundvlees vaak onvoldoende verhit of onverhit wordt gegeten. Om die reden is het extra belangrijk uit te zoeken of de met PCR-aangetoonde *T. gondii* bij runderen (hoofdstuk 4) inderdaad infectieus is. Andere aanbevelingen om het model te verbeteren zijn: verzamel de gegevens over de overleving van *T. gondii* gedurende de bereiding door de bereiding door consumenten beter na te bootsen en verzamel produkt-specifieke verhittingsgegevens, onderzoek het effect van het mengen van vlees van verschillende dieren (voor bijvoorbeeld gehakt) aan de hand van gebruikte partijgroottes, en verzamel een schatting van het aandeel producten dat ingevroren was voordat het verkocht werd. Wanneer deze data beschikbaar komen en het model daarmee is aangepast, kan het model de beslissingen wat betreft *T. gondii* preventiestrategieën ondersteunen zoals verder wordt beschreven in hoofdstuk 8.

In hoofdstuk 7 worden de seroprevalentie van en risicofactoren voor *T. gondii* infectie bij de eindgastheer, de kat, beschreven. Als eindgastheer, en dus de enige diersoort die *T. gondii* uitscheidt in het milieu, spelen katten een belangrijke rol in de verspreiding van *T. gondii*. Daarom zouden maatregelen gericht op het voorkómen van infectie bij de kat erg effectief kunnen zijn in het verminderen van zowel het aantal oöcyste- als het aantal weefselcyste-gebonden infecties bij de mens. Van 450 katten werd een serummonster getest en 18.2% bleek positief voor *T. gondii* op basis van analyse van de resultaten met behulp van het binaire mengsel-model. Jagen en het voeren van rauw vlees werden geïdentificeerd als risicofactoren die een mogelijk aangrijpingspunt zijn voor preventiemaatregelen. Toegang naar buiten was geen significante risicofactor wanneer het geobserveerde jaaggedrag al was opgenomen in het

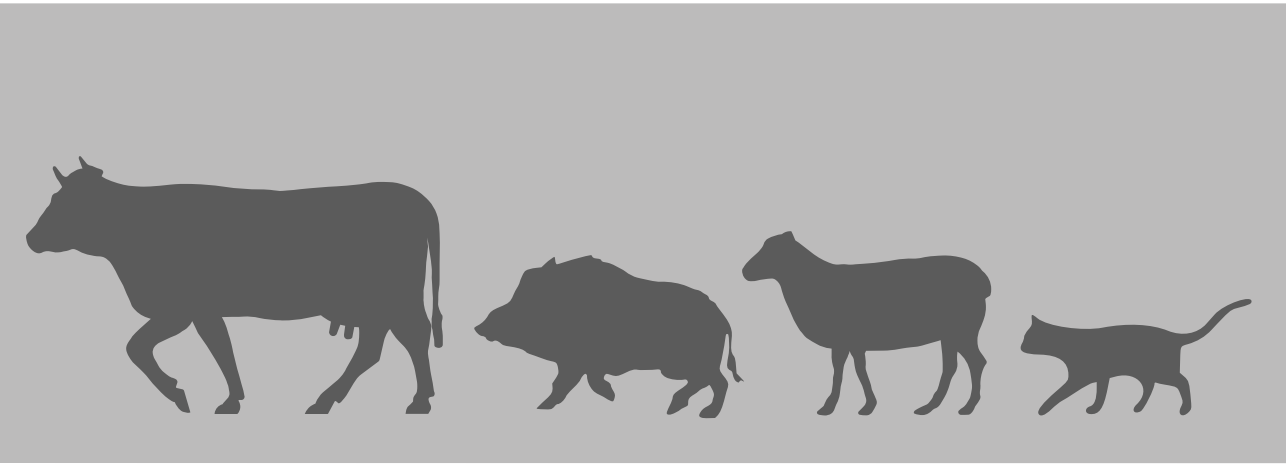
model. Dit betekent dat, als het mogelijk is het jaaggedrag te beperken zonder de kat binnen op te sluiten, dit mogelijk even effectief zou zijn ter voorkoming van *T. gondii* infectie. Andere maatregelen die de omgevingsbesmetting door katten kunnen beperken zijn het verminderen van het aantal katten en het stimuleren van het gebruik van de kattenbak. In dit hoofdstuk wordt nog een uitbreiding van binaire mengsel-model beschreven: De verdelingen worden gebruikt om voor iedere individuele kat een 'kans op positief' te berekenen. Deze testuitslag is informatiever dan positief of negatief op basis van een afkapwaarde, omdat rekening wordt gehouden met de sensitiviteit en specificiteit van de test. In het geval van de kattenstudie zijn deze data gebruikt om de verschillen in de prevalentie per risicofactor te berekenen met behulp van een lineair regressie model zodat gecorrigeerd werd voor de andere risicofactoren.

In hoofdstuk 8 worden drie onderwerpen uitvoeriger bediscussierd. Eerst worden de voordelen van de nieuwe detectie-methode op basis van selectieve extractie en PCR ook buiten de lijn van het in dit proefschrift gepresenteerde onderzoek besproken. Omdat deze techniek het gebruik van proefdieren kan beperken en daarnaast de kwantitatieve resultaten geeft die voor veel analyses noodzakelijk zijn, beschouwen wij dit als een belangrijke vooruitgang. Hetzelfde geldt voor het binaire mengsel-model. In eerste instantie gebruikten wij deze methode vooral omdat het testevaluatie mogelijk maakt wanneer onvoldoende referentiesera en geen 'gouden standaard' test voorhanden zijn. Later werd het model uitgebreid met de mogelijkheid om de prevalentie per leeftijdsgroep te schatten (hoofdstuk 5) en door vervolgens compartimentele infectiemodellen te passen aan deze curve, kwamen we tot de interessante hypothese dat antilichamen en mogelijk ook de infectie met *T. gondii* niet levenslang persisteren in wilde zwijnen. In hoofdstuk 7 wordt een verdere uitbreiding beschreven: De verdelingen werden gebruikt om de 'kans op positief' voor iedere individuele kat te berekenen. Het binaire mengsel-model biedt dus voordelen ten opzichte van het gebruik van referentiesera, zelfs wanneer deze voldoende beschikbaar zijn.

In het laatste deel van hoofdstuk 8 worden de implicaties van de bevindingen in de voorgaande hoofdstukken voor de preventie van *T. gondii* infectie bij de mens besproken. Eerst wordt beargumenteerd dat maatregelen zich op preventie van infectie bij de gehele bevolking in plaats van alleen bij zwangere vrouwen zouden moeten richten, voornamelijk omdat de ziektelast ten gevolge van verworven toxoplasmose een belangrijke bijdrage levert aan de totale ziektelast ten gevolge van *T. gondii* infecties. Op dit moment bestaat de preventiestrategie uit voorlichting aan zwangere vrouwen wat betreft de risico's op infectie. Deze voorlichting zou kunnen worden uitgebreid naar de gehele bevolking, maar de effectiviteit van voorlichting aan zwangeren is twijfelachtig en beïnvloeding van het gedrag van anderen is waarschijnlijk nog moeilijker. Daarom zou het effectiever zijn ook maatregelen gericht op het voorkómen van infectie bij onze consumptiedieren, op decontaminatie van geïnfecteerd vlees, of op vermindering van oöcysten-uitscheiding door katten toe te passen. Vaccinatie van vee of katten is mogelijk een interessante optie voor de toekomst. Afhankelijk van het type maatregel zijn er effecten op de transmissie van andere voedseloverdraagbare ziekteverwekkers. Het heeft de voorkeur deze effecten mee te nemen bij het vergelijken van de kosteneffectiviteit van de verschillende opties. Na uitbreiding en verbetering van het model voor kwantitatieve risicoschatting is het geschikt voor het vergelijken van de verschillende opties. In eerste instantie moeten, zoals beschreven in hoofdstuk 6, meer data verzameld worden om het model te verbeteren zodat een meer betrouwbaar resultaat wordt verkregen. In dit hoofdstuk worden andere mogelijkheden voor verdere ontwikkeling beschreven. Zo zou de omgevingsroute (infectie via oöcysten) ook moeten worden opgenomen in het model en zou, om vergelijking

met de preventie van andere aandoeningen mogelijk te maken, de uitkomst beter als afname van het aantal DALYs (maat voor de ziektelast) dan als het aantal voorkómen infecties kunnen worden berekend. Daarnaast is het, om de kosteneffectiviteit van de verschillende opties te kunnen bepalen, noodzakelijk dat bekend is wat het effect van de interventiemaatregelen is op de in het model in te voeren data (bijvoorbeeld op de prevalentie van infectie bij schapen) en moeten per interventiemaatregel de kosten van de best uitvoerbare implementatiewijze worden berekend. Voor deze uitbreidingen is vervolgonderzoek noodzakelijk en het zou goed zijn als het onderzoek zich in de toekomst dan ook meer zou richten op de effecten van interventies. Daarnaast is het belangrijk zich te realiseren dat door veranderingen in het voorkomen van *T. gondii* bij de verschillende dieren, veranderingen in menselijk gedrag en mogelijk zelfs klimaatsveranderingen het belang van de verschillende transmissieroutes kan verschuiven. Om die reden zal vergelijkbaar monitoringsonderzoek nodig blijven, en kan wanneer nieuwe gegevens beschikbaar komen, het risicoschattingsmodel steeds opnieuw gebruikt worden om de effectiviteit van verschillende interventiemaatregelen te vergelijken.

Curriculum Vitae
List of Publications
Dankwoord



Curriculum Vitae

Marieke Opsteegh was born July 16th 1981 in Nijmegen with the seemingly innate wish to become a veterinarian. She attended primary and secondary school in Nijmegen and graduated *cum laude* from the Kandinsky College in 1999. Luckily, that year a law enabling those graduates to surpass the *numerus fixus* was passed, allowing her direct admission to the Faculty of Veterinary Medicine, Utrecht University.

Upon finishing her Master's in July 2003 Marieke was asked to join the Excellent tracé (the faculty's honours research program). She found a project that suited her interest in tropical infectious diseases in the lab of Prof. Frans Jongejan, and worked on the *'Etiology of Bovine Cerebral Theileriosis in Northern Tanzania'* from September 2003 to September 2004. During this project Marieke acquired technical skills in the lab, attended the Laboratory Animal Science course giving her legal permission to design and perform animal experiments, and performed a *Theileria* infection experiment with four calves. The project also included a 3-month stay with drs. Lieve Lynen in Tanzania. There she collected ticks and clinical samples from cattle with cerebral theileriosis, interviewed the Maasai about the results of an East Coast Fever (*Theileria parva*) immunization trial, and tested some of the collected ticks at Sokoine University of Agriculture in Morogoro (in the lab of Dr. Paul Gwakisa). In addition, she set up the RLB for detection of tickborne pathogens at the Veterinary Investigation Centre in Arusha. This project made her realize how much she liked research which is why she applied for the 2005 Cornell Leadership Program.

The Leadership Program is a 10-week summer program aimed at veterinary students who seek a science-based career. The program combines faculty-guided research with various workshops. Marieke worked in Prof. Ton Schat's lab on the project *'Mutation of E box like element in chicken anemia virus'*. The objective was to study the regulatory function of the E box like element in viral replication by site-directed mutagenesis.

Coming back from Cornell, Frans Jongejan offered Marieke a part-time job as a student-assistant to provide technical support on the project *'Autochthonous canine babesiosis in The Netherlands'* (September 2005-September 2006). The aim was to investigate the prevalence of different ticks and tick-borne pathogens in The Netherlands after a few unexpected autochthonous cases of canine babesiosis were observed. An established population of *Dermacentor reticulatus* was identified.

During the final year of clinical rotations Marieke focused on Ruminant Health with elective courses (e.g. Introductory Course Biostatistics for Researchers, Tropical Veterinary Medicine) and internships focused on research. From mid April to June 2006 she stayed at the Food and Agriculture Organization of the United Nations in Rome to work under supervision of Dr. Katinka DeBalogh at the Emergency Centre for Transboundary Animal Diseases (ECTAD) on various projects related to highly pathogenic H5N1 Avian Influenza. Afterwards, she applied for an internship at the Laboratory for Zoonoses and Environmental Microbiology at the Dutch National Institute for Public Health and the Environment (RIVM).

Marieke started her 10-week internship with Dr. Joke van der Giessen and Dr. Merel Langelaar at the RIVM on July 31st 2006. She set-up an ELISA for *Toxoplasma gondii* in cattle and studied the incidence of infection in a group of calves that was followed longitudinally. In the end this turned out more complicated as is described in this thesis. Marieke obtained her Doctor of Veterinary Medicine degree October 31st 2006 and was given the opportunity to

extend the RIVM internship into the four year PhD project that led to the publication of this thesis. Marieke has combined her PhD studies with a Master's in Veterinary Epidemiology and Economics at Utrecht University, which she completed *cum laude* on February 23rd 2010.

In 2011, Marieke still works at the RIVM, and has broadened her horizon from *T. gondii* to *Echinococcus multilocularis* in foxes, Q-fever in rats, and hantavirus in rodents. At the time of printing, she was still awaiting a final decision on her proposed work on a *T. gondii* sheep vaccine at Moredun Research Institute (Edinburgh) regarding Marie Curie (reserve list) and Rubicon funding.

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Submitted

Dankwoord

De laatste loodjes wegen het zwaarst. Onzin natuurlijk om dat van het dankwoord te zeggen; de koeien, die wogen het zwaarst. Toch is het lastig. De directe invloed van veel mensen is duidelijk maar eigenlijk horen hier misschien wel eerst de mensen te staan die mij hebben aangemoedigd en geholpen deze richting in te gaan, of andere keuzes hebben ontmoedigd. Er is tenslotte toch een weg van basisscholen, een middelbare school, een studie diergeneeskunde, een excellent tracé onderzoek, coschappen, Cornell leadership program, een bijbaan als student-assistent, een stage bij de FAO, en een stage bij het RIVM aan vooraf gegaan. En dat ogenschijnlijk kleine dingen kunnen van grote invloed zijn is duidelijk: Frans van Knapen verwees mij voor een stage bij het RIVM naar Joke van der Giessen.

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Het opzetten van de MC-PCR is begonnen met het opzetten van een nieuwe *Toxoplasma* PCR. Dit was voornamelijk het werk van Jeroen Roelfsema en Sietze Brandes en daarvoor mijn dank. Carla Nijhuis en Denise Hoek hebben ervoor gezorgd dat het kweken van *Toxoplasma* en het maken van antigeen lukte.

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Adriana (first Titilincu, now Györke) came to our institute after an FMD outbreak around the lab where she was supposed to go. This last minute change of plans has boosted my work, as together we tested the first infected instead of spiked tissue samples by MC-PCR with results better than we had hoped for, and within those same two weeks time we also adjusted the ELISA for use with sheep sera. Afterwards we have collaborated again on the cat study. It has been a great pleasure to have you at our lab, and meet you again in Brazil. I hope and expect we will meet again in the future.

Martin Köthe, Bretislav Koudela and Ruth Zadoks' interest in the MC-PCR has been rewarding. It has lead to the stay of Martin and Jana Jurankova in our lab to learn the technique. Both very pleasant people to work with and I've enjoyed those weeks. Ruth's interest has lead to writing a proposal on a sheep vaccine with Moredun Research Institute and more in future?

Dennis Kunkel kindly provided the *Toxoplasma* image on the cover, and David Ferguson the life cycle illustration. In addition, I would like to thank all anonymous reviewers that helped improve the published and submitted chapters in this thesis.

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Gertjan, de allerliefste! Zonder jou was het nooit zo'n mooi boekje geworden en dat is natuurlijk niet alleen vanwege de praktische hulp met de omslag, de plaatjes, computers in het algemeen en het doorlezen van sommige stukken. Er is zoveel meer. Voorlopig allebei wat rustiger?

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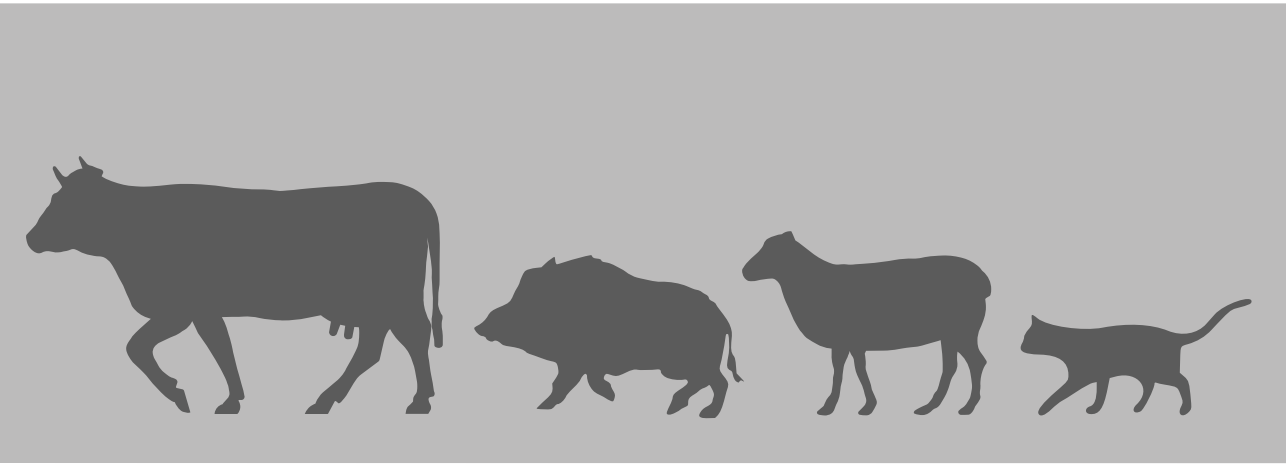

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