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TITOLO TESI

Toxoplasma gondii in animals and the environment

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HISTORY OF THE ETIOLOGICAL AGENT

It's 1908 when Nicolle and Manceux, two scientists of the laboratory of Charles Nicolle at the Pasteur Institute in Tunis, found a protozoan in spleen, liver and blood of a North-African rodent, the gundi (Ctenodactylus gundi), which was being used for leishmaniasis research. Even if they initially believed the parasite to be a piroplasm and then a *Leishmania*, soon they realized that they had been discovered a new organism. They named the parasite *Toxoplasma gondii* based on its morphology and the original host: *toxon*= bow or arc, *plasma*= life and *gondii* may have resulted from a misspelling of the original host, the gundi (Nicolle and Manceaux 2009). In the same year, Splendore discovered the same parasite in a rabbit in Brazil and erroneously identified it as *Leishmania* (Splendore 2009).

For the next 30 years, *T. gondii*-like organisms were found in several other hosts, including humans.

In 1937 Sabin and Olitsky showed that *Toxoplasma* was an obligate intracellular parasite that could be passaged in laboratory animals by intracranial, subcutaneous and intraperitoneal inoculation of brain homogenates (Sabin and Olitsky 1937). In the same years, following a fatal case of *T. gondii* infection in a three-day old infant, it has been showed that *Toxoplasma* was a parasite capable of and associated with congenital transmission (Wolf *et al.* 1941). In the late 1940s and early 1950s *Toxoplasma* was shown to be involved in inflammatory diseases of the eye (Frenkel and Jacobs 1958).

In 1948 the developing of "The Sabin-Feldman dye test", the first serological technique to detected specific *Toxoplasma* antibodies, made it possible to find out that a high proportion of the human and domestic animal population was infected (Sabin and Feldman 1948). Due to this surprising data, *Toxoplasma* changed from being considered a rare exotic infection into one of more common human parasitic infections, but it was still unknown the mechanism whereby adults were infected.

As the only parasites stages known at that time were tachyzoites and tissue cysts, numerous fruitless studies were performed to demonstrate the relation of *Toxoplasma* to *Leishmania* and to show the presence of an insect vector. It's when Jacobs *et al*. (1960) proved that that bradyzoites in tissue cysts could survive exposure to acid and trypsin that it was first suggested that carnivorism could be a possible mechanism of infection. The role played by carnivorism was then confirmed in 1965 by Desmonts *et al.* in an epidemiological study in a tubercolosis hospital in Paris: it was observed that the incidence of *T. gondii* infection rose from 10% to 50% (and even to 100%) when raw or undercooked meat started to be given to patients for therapeutic purposes. However, the observation that *Toxoplasma* could be transmitted through carnivorism did not explain the widespread infection in herbivores or strict vegetarians; indeed, a study in Bombay, India, found the prevalence of *T. gondii* in strict vegetarians to be similar to that in non-vegetarians (Rawal 1959).

In 1965, Hutchinson first linked *T. gondii* infectivity with cat feaces. He fed tissue cysts to a cat infected with the nematode *Toxocara cati*, collected and floated faeces in a 33% zinc sulphate solution, stored this sample in tap water for 12 months to embryonate *T. cati* eggs and then fed these faeces to mice. The great discovery was that "something" in the faeces, that survived

for over a year in water, induced toxoplasmosis in mice (Hutchison 1965). As neither the tachyzoites nor bradyzoites could survive this treatment, it was clear that it should be a new form of *T. gondii*; initially it was proposed that the parasite might be protected within the egg of the cat nematode, *T. cati*, similar to the transmission of the flagellate *Histomonas* through *Heterakis* eggs (Hutchison 1967). The nematode egg theory of transmission was discarded when *Toxoplasma* infectivity was found in faeces of worm-free cats fed *T. gondii* (Frenkel *et al.* 1969; Sheffield and Melton 1970).

In 1970, almost simultaneously, scientific groups in the USA, UK, Germany and Netherlands resulted in the identification of the resistant form of *Toxoplasma* as a coccidian oocyst with typical coccidian asexual and sexual development occurring in the small intestine of the cat, recognized as the definitive host of the parasite (Hutchison *et al.* 1969; Dubey *et al.* 1970a; Hutchison *et al.* 1970; Overdulve 1970; Sheffield and Melton 1970; Weiland and Kuhn 1970; Witte and Piekarski 1970; Hutchison *et al.* 1971).

Thus, knowledge on the life cycle of *T. gondii* was completed more than 60 years after its first description and still remains the only known member of the genus *Toxoplasma* (Dubey 2008).

Table 1 Taxonomic classification of *T. gondii*

Kingdom	Protista
Subkingdom	Protozoa
Phylum	Apicomplexa
Class	Sporozoasida
Order	Eucoccidiorida
Famliy	Sarcocystidae
Genus	*Toxoplasma*
Species	*gondii*

LIFE CYCLE AND ROUTES OF INFECTION

Toxoplasma gondii is an obligate intracellular parasite that occurs in most areas of the world, capable of infecting virtually all warm-blooded species, including humans.

The life cycle of *T. gondii* is facultatively heteroxenous (intermediate host is not essential for the completion of the life cycle) and includes both sexual and asexual multiplication; the sexual phase takes place only felids, including the domestic cat, and makes them the definitive hosts of the parasite. A large range of vertebrates, including domestic and wild mammals, birds, and humans, are intermediate hosts.

Like other protozoa, its life cycle requires switching between distinct developmental stages.
In the case of *T. gondii*, the stages are :

- the rapidly replicating **tachyzoite**,
- the slower growing **bradyzoites** within tissue cysts;
- the **sporozoites** within oocysts.

The tachyzoite

The term "tachyzoite" (tachos=speed in Greek), coined by Frenkel, describes the stage of the parasite that rapidly multiply in any cell of the intermediate host and in nonintestinal epithelial cells of the definitive host; it has also been called the proliferative form and the endozoite (Frenkel 1973). It is the stage that Nicolle and Manceaux found in 1909 in the gundi (Dubey 2008). The presence of fast-replicating tachyzoites is an indication of an initial infection and an acute disease.

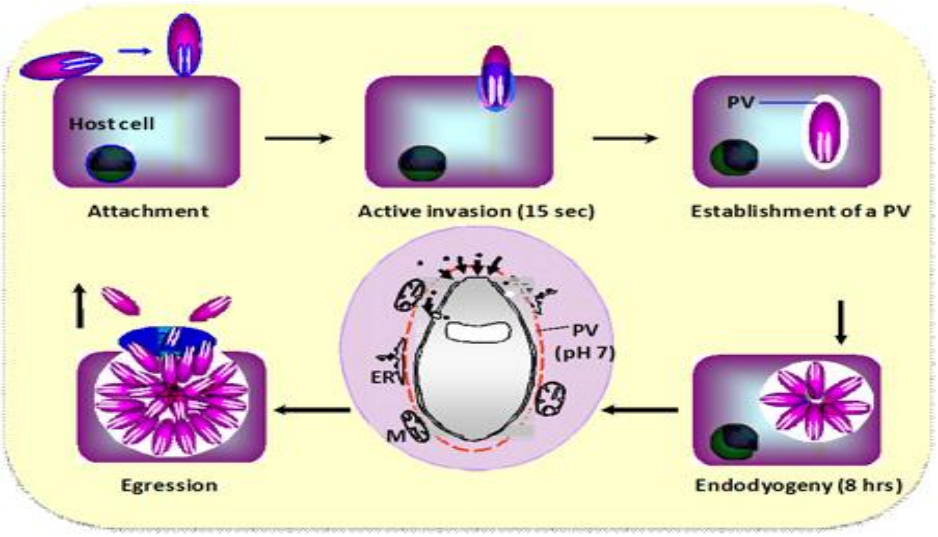
The tachyzoite is lunate, approximately 2 by 6 µm, with a pointed anterior (conoidal) end and a rounded posterior end. Ultrastructurally, the tachyzoite consists of various organelles, inclusion bodies and an outer covering called pellicle; the nucleus is usually situated toward the central area of the cell and contains clusters of chromatin and a centrally-located nucleolus. Although tachyzoites can move by gliding, flexing, undulating, and rotating, they do not have visible means of locomotion such as cilia, flagella, or pseudopodia.
Both sporozoites or bradyzoites differentiate into tachyzoites once that an intermediate or definitive host ingests a sporulated oocyst or a tissue cyst and this conversion takes place in the small intestinal lamina propria within 18 h post infection (Dubey 1998a).

Tachyzoites enter host cells by actively penetrating through the host cell plasmalemma or by phagocytosis. After entering the host cell, the tachyzoite becomes ovoid and is surrounded by a parasitophorous vacuole (PV), which appears to be derived from both the parasite and the host cell (Silva *et al.* 1982; Werk 1985; Bonhomme *et al.* 1992; Morisaki *et al.* 1995).

Tachyzoites multiply asexually within the host cell by repeated endodyogeny (Ferguson 2009) (Fig.1); this is a specialized form of reproduction in which the two progenies form within the parent parasite, consuming it (Sheffield and Melton 1968). Tachyzoites continue to

divide until the host cell can no longer support their growth and ruptures. In this way, the parasite can disseminate and replicate within the new host: it is reported that *T. gondii* organisms circulate in the blood only 1 h after infection (Chinchilla *et al*. 1993).

Fig. 1 *T.gondii* endodyogeny (http://www.charite.de/sysbio/research/toxoplasma/)



In the blood flow, the majority of parasites exist intracellularly, inside infected leukocytes which contribute to the dissemination of the parasite from the general circulation into the peripheral tissue by a "Trojan horse" mechanism (Unno *et al.* 2008). This parasitemia in primarly infected pregnant hosts (both animal and humans) may results in the invasion of the placenta and eventually of the foetus, if some tachyzoites cross the placenta (Tenter *et al.* 2000).

Tachyzoites are rapidly killed outside the host; they are considered sensitive to proteolytic enzymes and they are usually destroyed by gastric digestion (Powell *et al*., 2001). However, a study showed that tachyzoites may occasionally survive for a short period of time (up to 2 h) in acid pepsin solutions, and that oral application of high doses of tachyzoites may cause an infection in mice and cats (Dubey 1998b). It has also been suggested that tachyzoites may enter the host by penetration of mucosal tissue and thereby gain access to the host's circulation or lymphatic system before reaching the stomach (Riemann *et al.* 1975; Sacks *et al.* 1982).

Bradyzoites and tissue cysts

The term "bradyzoite" (brady= slow in Greek) was also coined by Frenkel (1973) to describe the organism multiplying slowly within a tissue cyst in the intermediate host; they are also called cystozoites.

Tachyzoites differentiate into bradyzoites around 10-14 days post infection. It is still not completely clear what is the molecular environment that regulates development from tachyzoites to bradyzoites; it is likely that differentiation into bradyzoites during *in vivo* infection is multifactorial with contributions from host immunity (Lyons *et al.* 2002), . Data

from *in vitro* studies suggest that the bradyzoite differentiation program is genetically programmed after the brief period of rapid division as tachyzoites (Skariah *et al.* 2010).

Bradyzoites differ structurally only slightly from tachyzoites: they are more slender, with a nucleus situated toward the posterior end and they contain several amylopectin granules which stain red with PAS (Dubey *et al.* 1998a). Similar to tachyzoites, bradyzoites are approximately 7 by 1.5 μm in size (Mehlhorn and Frenkel 1980) and divide by endodyogeny (Ferguson and Hutchison 1987).

In the past, the methods used to distinguish tachyzoites from bradyzoites were the resistance of bradyzoites to acid-pepsin digestion and bioassay in cats. Cats fed tachyzoites may become infected and may shed oocysts with a long pre-patent period (>15 days), whereas cats fed bradyzoites shed oocysts with a short (<10 days) pre-patent period. Dubey (199.b) suggests that these cannot be the sole criterion to distinguish between the two stages and that, to accurately determine the cyst-like structures, the identification of molecular markers is required; in fact, stage conversion is associated with the expression of stage-specific surface antigens (Lyons *et al.* 2002). Several stage-specific surface antigens (SAG) have been identified and many of these fall into two distinct families: SAG1 and SAG2. The SAG1 family includes SAG3, bradyzoitespecific recombinant (BSR) 4 SAG-related sequences (SRS) 1–4 proteins, SAG5, SAG5.1 and SAG5.2. Whereas SAG1 and SRS1–SRS3 are present only on tachyzoites, BSR4 is present only on bradyzoites and SAG3 is present on both stages. This family of proteins is likely to play a role in the attachment process before parasite invasion, as proven for SAG1 and SAG3. The SAG2 family comprises four related proteins designated SAG2A (previously SAG2) and SAG2B–SAG2D. Whereas SAG2A and SAG2B are expressed by tachyzoites exclusively, SAG2C and SAG2D are expressed by bradyzoites exclusively (Lyons *et al*. 2002).

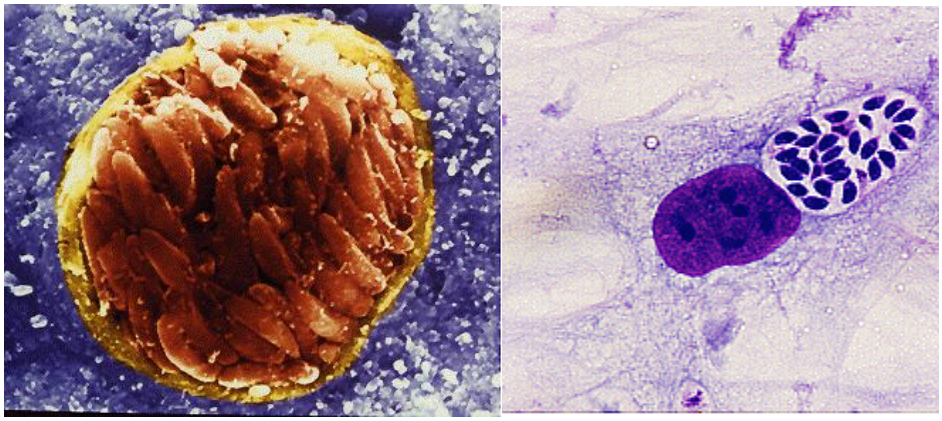
The conversion to bradyzoites takes place within the parasitophorous vacuole (PV), which converts itself into a cyst. In this way, tissue cysts remain intracellular with an elastic and thin wall (<0.5μm), composed either of host cell and parasite materials (Ferguson and Hutchison 1987). Their size is dependent on cyst age and the type of host cell parasitized; young tissue cysts may be as small as 5 mm in diameter and contain only two bradyzoites, while older ones may contain hundreds of organisms.

Although tissue cysts may develop in virtually any cell type *in vitro*, in infected animals they are more prevalent in the neural and muscular tissues, including the brain, eyes, and skeletal and cardiac muscles; they can be also detected in visceral organs as lungs, liver, and kidneys (Dubey 1998a). The ones in the brain are often spheroidal and rarely reach a diameter of 70 μm, whereas intramuscular cysts are elongated and may be 100 μm long.

Tissue cysts are the terminal life-cycle stage in the intermediate host and their finding represent the establishment of a chronic infection; the species of intermediate host may also affect the ability of tachyzoites to convert to bradyzoites and to persist as tissue cysts. In some intermediate host species, such as sheep and goats, they may persist for the life of the host. The mechanism of this persistence is unknown. However, many investigators believe that

tissue cysts break down periodically, with bradyzoites transforming to tachyzoites that reinvade host cells and again transform to bradyzoites within new tissue cysts. This reactivation can results in clinical symptoms and is a serious and life-threatening condition in immune-compromised individuals (Skariah *et al.* 2010).

Fig. 2 On the left: tissue cyst with many bradyzoites within the brain of an infected mouse (scanning electron micrograph (http://cmgm.stanford.edu/micro/boothroyd/test.html). On the right: tachyzoites in a human foreskin fibroblast (HFF) grown in culture (Giemsa stain) (http://cmgm.stanford.edu/micro/boothroyd/test.html).



Enteroepithelial asexual and sexual stages

The sexual development of *T. gondii*, also called the coccidian stage, takes part only in the gut of the definitive hosts, the felines, when an uninfected cat ingests one of the three infectious stages of the parasite, tachyzoites, bradyzoites or sporozoites. Felidae excrete *T. gondii* oocysts in faeces 3 to 10 days after ingesting bradyzoites, ≥ 18 days after ingesting of sporulated oocysts and ≥ 13 days after ingesting tachyzoites. Under experimental conditions, it has been demonstrated that the bradyzoite induced cycle in cats is the most efficient since nearly all experimentally infected cats fed tissue cysts shed oocysts, whereas < 30 % of cats fed tachyzoites or oocysts shed oocysts (Dubey 1998a).

Only the bradyzoite induced cycle in cats has been studied in detail (Dubey and Frenkel 1972). After the ingestion of tissue cysts, contained in a prey animal or in meat, the cyst wall is dissolved by gastrical or intestinal proteolytic enzymes and the bradyzoites released in the gut's lumen; once they penetrate the epithelial cells of the small intestine, they start to replicate asexually by a process called *Toxoplasma* endopolygeny. This development represents an asexual proliferative phase with repeated nuclear divisions followed by the formation of between 8-20 daughters' cells within the mother cell (Ferguson 2009). After five asexual stages of this multiplication, the mature merozoites are released, invade new enterocytes and undergo a repeated cycle of asexual development or differentiate into a sexual stage, either macrogametocyte (female gamete) or microgametocyte (male gamete). The factors involved in deciding the fate of a merozoite are unknown. The initial asexual cycles are required to increase parasite density for two reasons: firstly because the male and female gametes have to be able to find each other; secondly, since each macrogamete only produces a single oocyst, there requires being literally millions of merozoites available to

develop into macrogametes to produce the large number (millions) of oocysts seen in the faeces (Ferguson 2009).

The sexual development starts 2 days after tissue cysts are ingested by the cat (Dubey *et al.* 1998a) and gametes are found throughout the small intestine, more commonly in the ileum, above the nucleus of the host epithelial cell, near the tips of the villi. The female gamete or macrogametocyte, is sub spherical and has two very important functions. Firstly, it has to synthesize and store all the nutritional requirements to allow sporulation in the external environment and sustain the viability of the sporozoites over long periods (in excess of 1 year); secondly, it has to synthesize the specific components necessary to form the oocyst wall (Ferguson 2009). Microgametes are elongated, consist mainly of nuclear material and present two long, free flagella projected posteriorly. Microgametes use their flagella to swim, to penetrate and fertilize mature macrogametes in order to form zygotes; after fertilization, an oocyst wall is formed around the parasite.

Finally infected epithelial cells rupture discharging unsporulated oocysts into the intestinal lumen of the cat (Dubey *et al.* 1998a) which will eventually shed them into the environment or cat litter box.

Sporozoites

Unsporulated oocysts (zygote) are subspherical to spherical with a diameter of 10 by 12 µm. The cytoplasm of the unsporulated oocyst has a large nucleus with amorphous nucleoplasm and a distinct nucleolus (Fig. 3).

Sporulation occurs outside the cat within 1 to 5 days of excretion depending on oxygenation and temperature. Firstly, the nucleus divides twice and two spherical sporoblasts are formed, each with two nuclei. As the sporulation continues, the sporoblasts elongate and sporocysts are formed, each nucleus divides into two and four sporozoites are formed in each sporocyst; a prominent residual body is left after sporozoites are formed and it is enclosed in a single-unit membrane (Ferguson *et al.* 1979). Sporulated oocysts are subspherical to ellipsoidal and are 11 by 13 µm in diameter. Each oocyst contains two ellipsoidal sporocysts that measure 6 by 8 µm; sporozoites are 2 by 6 to 8 µm in size with a subterminal nucleus and, ultrastructurally, they are similar to tachyzoites.

Cats can shed upward of 360 million oocysts in their feces in a single day and the shedding period lasts for a median of 8 days, although it may be as long as 3 weeks (Dabritz and Conrad 2010). Sporulated oocysts can survive for long periods in moist soil, under most ordinary environmental conditions, and in water (Dubey and Beattie 1988).

Fig. 3 Multiple oocysts and a single *Isospora oocyst* (arrow): comparison of the relative size of *Isospora felis* to the *Toxoplasma* oocysts

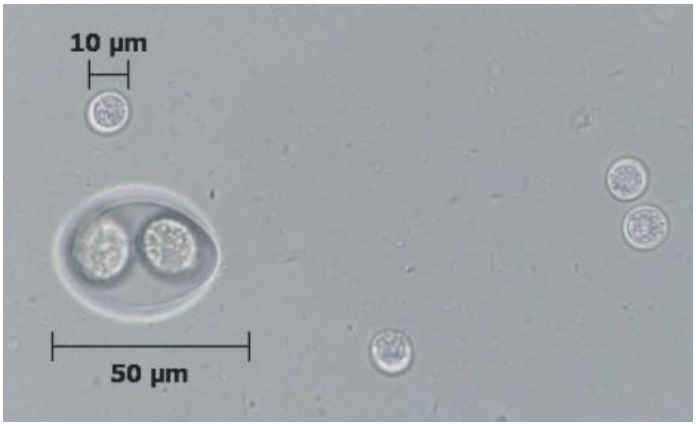
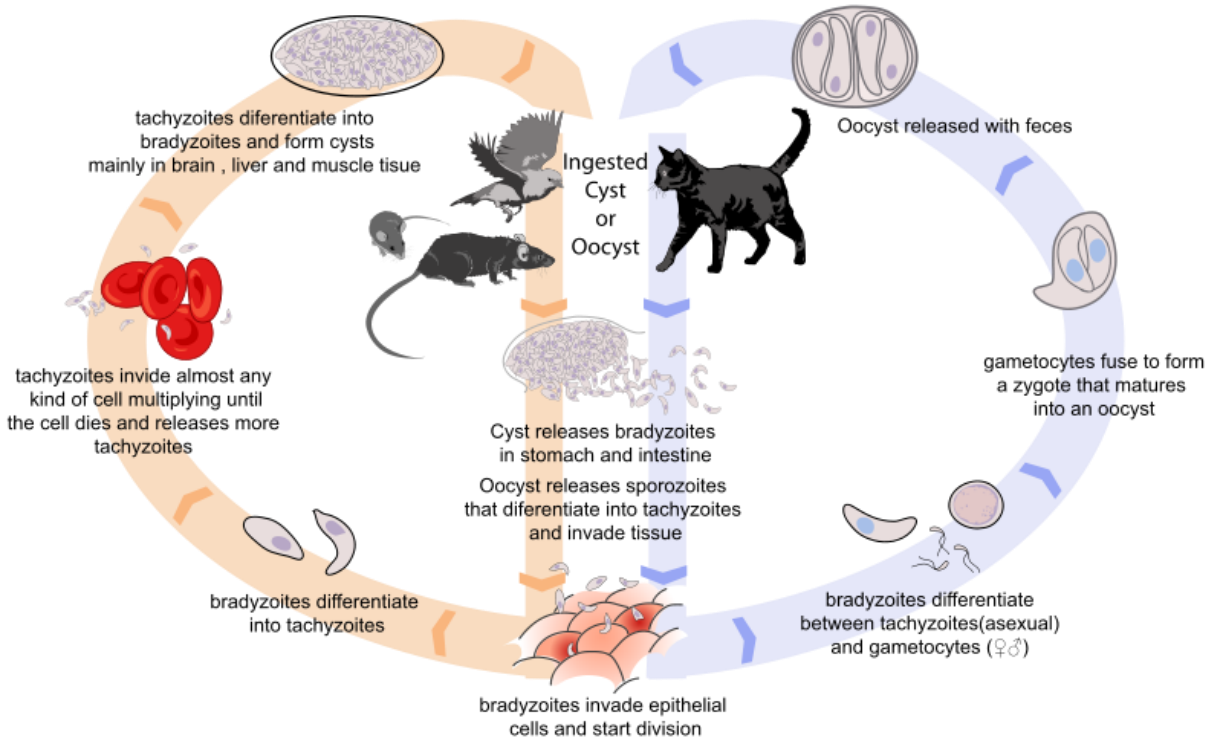


Fig. 4 Life cycle of *T. gondii* (http://www.nzg.ac.za/)



ROUTES OF TRANSMISSION OF *T. gondii*

Tachyzoites, bradyzoites contained in tissue cysts, and sporozoites inside of sporulated oocysts are the three infectious stages in the life cycle of *T. gondii.* All the stages are infectious for both intermediate and definitive hosts which may acquire *T. gondii* infection mainly via one of the following routes:

1. horizontally by oral ingestion of infectious oocysts from the environment (soil or water), i.e. **faecal-oral route**;
2. horizontally via carnivorism by oral ingestion of tissue cysts contained in raw or undercooked meat or primary offal (viscera) of intermediate hosts, i.e. **carnivorism**;
3. vertically by transplacental transmission of tachyzoites to the foetus, i.e**. congenital.**

Faecal-oral route. As reported above, primary infected cats shed into their litter box or in the environment (soil, water) millions of unsporulated oocysts which become infected within 1 to 5 days after excretion. Experimental studies demonstrated that in soil, sporulated oocysts remain infectious over a period of 18 months depending on humidity, temperature and exposure to direct sunlight (Yilmaz and Hopkins 1972; Frenkel *et al.* 1975). In water (Dubey 1998c) and seawater (Lindsay and Dubey 2009) sporulated oocysts remain infective for 6 up to 54 months at temperatures between 4 and 25 °C; oocysts resulted also resistant to experimental freezing, surviving up to 28 days at -21°C (Frenkel and Dubey 1973). As they are highly impermeable, they are very resistant to disinfectants (Kuticic and Wikerhauser 1996; Jones and Dubey 2010). Oocysts are distributed in the environment through wind, rain and surface water, or harvested feeds and they can be distributed mechanically by vectors and transported to water by runoff. Intermediate hosts, especially humans and herbivores, as well as definitive host (Dubey 1998a), can be infected by the ingestion of food or water contaminated with sporulated oocysts.

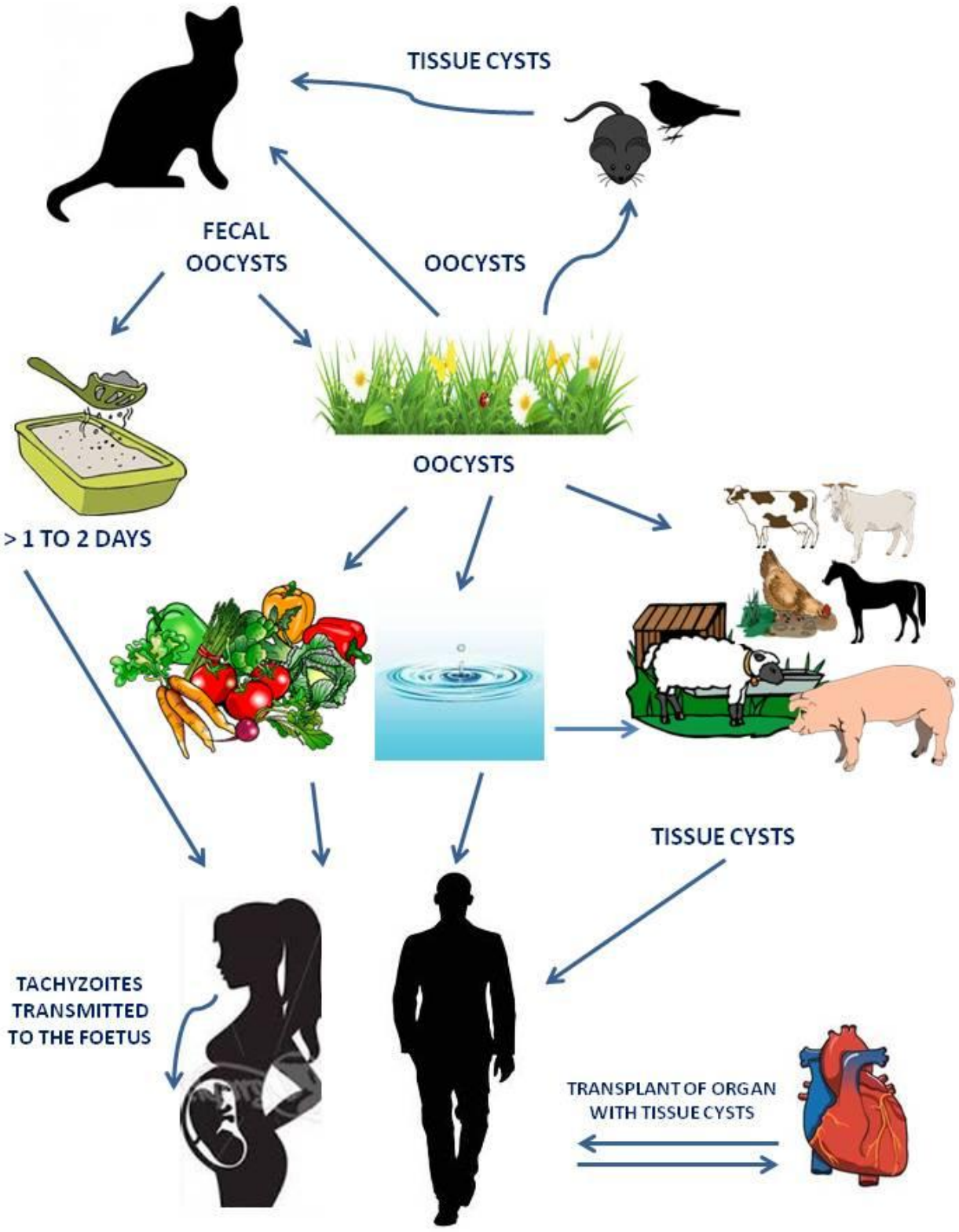
Carnivorism. Tissue cysts are contained in tissues, especially muscles and nervous system, of chronically infected intermediate hosts. Studies have indicated that T. *gondii* tissue cysts in meat are susceptible to various physical procedures such as heat treatment, freezing and salting (depending on the salt concentration and duration (Kijlstra and Jongert 2008)): e.g. freezing to -12 degrees °C for at least 2 days and cooking to an internal temperature of 67 degrees kill tissue cysts in meat (Dubey 1996a). Ingestion of tissue cysts is the main mechanism involved in transmission of the infection in carnivores, including humans. It is clear that in humans, as omnivores, both mechanisms described above play a role in *T. gondii* transmission and it is likely that the lifestyle of the individual plays the most important role in how the parasite is transmitted; the possible sources of acquired infections in humans will be discussed ahead.

Congenital. Transmission of *T. gondii* via tachyzoites can occur in hosts which contract primary *T. gondii* infection during pregnancy: in this case tachyzoites may be transmitted transplacentally to the foetus. Congenital transmission has been documented in various intermediate hosts, as mice, sheep, goats and, most important, in humans.

In addition, tachyzoites have been found in the milk of several hosts, including cats, sheep, goats, cows, camels and mice (Powell *et al.* 2001; Dehkordi *et al.* 2013). Thus now, the report of acute toxoplamosis following the consumption of raw milk has been documented only for caprine milk. In addition to blood and milk, tachyzoites have been detected in other body fluids, including saliva, sputum, urine, tears, and semen but there is currently no evidence of horizontal transmission of *T. gondii* to humans via any of these routes.

In conclusion, *T. gondii* may be transmitted from definitive to intermediate hosts, from intermediate to definitive hosts, as well as between definitive and between intermediate hosts. It is currently not known which of the various routes of transmission is more important from an epidemiological point of view. *T. gondii* life cycle may continue indefinitely by transmission of tissue cysts between intermediate hosts (even in the absence of definitive hosts) and also by transmission of oocysts between definitive hosts (even in the absence of intermediate hosts) (Tenter *et al.* 2000) (Fig. 5).

Fig. 5 Routes of transmission of *T. gondii*



CHARACTERISTICS OF *T. gondii* POPULATION

It has been long known that different strains of *T. gondii* vary in virulence for mice. With the development of the molecular typing techniques, it has been possible to associate differences in virulence to genetic diversity and to investigate the correlation between *T. gondii* genotype and disease manifestation in animals and also in humans (Saeij *et al*. 2005). Several studies have been conducted by multilocus enzyme electrophoresis (MLEE) and restriction fragment length polymorphism (RFLP) on a large set of isolates collected from animals and humans from North America and Europe. Such studies revealed that *T. gondii* population is an unusual striking clonal population, highly structured and dominated by three clonal lineages, type I, II and III. These lineages differ genetically by 1% or less and have been derived from few genetic crosses among closely related parents (Howe and Sibley 1995). Highly similar or identical genotypes were sampled from different hosts in different geographic regions of North America and Europe revealing that there are no strong geographic differences between the two continents' isolated and that similar genotypes can be found in both animals and human infections (Sibley and Ajioka 2008).
Clonality is usually described in protozoan parasites which lack a defined sexual phases in their life cycles, precluding the genetic exchange by meiosis. Although *T. gondii* is one of the few parasitic protozoa with a well-defined sexual cycle, this mode of propagation evidently occurs only rarely. There are few possible explanations for this striking pattern:

1. it is known that intermediate hosts develop strong immunity to primary infection, limiting the chances for simultaneous infection and developing tissue cysts related to one specific strain (Reikvam and Lorentzen-Styr 1976);
2. cats are rarely simultaneously infected with multiple strains, limiting the chances of genetic exchange;
3. the parasite can be transmitted by direct oral infection between intermediate hosts without the necessity of the sexual phase in cats (Sibley and Ajioka 2008).

A proper definition of *T. gondii* virulence is still incomplete because the susceptibility to the infection and to acute disease in different hosts is extremely variable: e.g. mice can die in one day and rats may be full refractory to the infection. As rodents are natural hosts for *T. gondii*, laboratory mice have provided a reasonable model to study the virulence factors associated with different *T. gondii* genotypes. The three lineages, i.e. type I, II and III, differ markedly in virulence in the mouse model; however, little is known about the correlation between *T. gondii* virulence in mice compared with other species.
Type I strains are reported to be uniformly lethal with an infectious dose of a single viable organism in mice; the first human strain of *T .gondii*, called RH strain, was classified into lineage I. Type I virulent strains are found rarely in animals and in humans; in literature they have been associated with acquired ocular toxoplasmosis, congenital infection and cerebral toxoplasmosis in immunocompromised patients (Khan *et al*. 2005; Hunter and Sibley 2012).

Due to their rare isolation, scientists recently called the existence of type I strains into question. Some research groups reported that during genotyping analysis, the use of additional markers transformed a "type I strain" into a non-type I or atypical one. This suggests that the identification of type I strains by monolocus typing, as it was done in the past, needs to be confirmed by additional multilocus typing (Ajzenberg 2010).

Type II strains are commonly isolated in both animal and human cases in North America and Europe (Darde *et al*. 1992; Howe *et al*. 1997) and have an intermediate virulence that varies with mouse strain. As the genotype of *Toxoplasma* strains is strongly linked to the geographical origin of infection, the French *Toxoplasma* Biological Resource Center (Toxo BRC, France) reported that in France type II strains are predominant in cases of congenital toxoplasmosis and in immunocompromised persons, whatever the clinical presentation (Ajzenberg 2010).

Type III are considered completely avirulent in mice and are characterized by low tissue cysts burdens and limited ability to cause infection and human disease (Sibley and Ajioka 2008; Hunter and Sibley 2012).

The analysis of acute virulence of type I strain, performed by classical genetics and by genetic crosses between virulent type I and avirulent type III, revealed that the high virulence of type I is largely due (90%) to a single allele at the ROP18 locus, a gene encoding a rhoptry protein (ROP). Rhoptries proteins (ROP) are discharged by rhoptries (secretory organelles involved in the formation of the parasite-containing vacuole) during the parasite cell invasion. ROP have different destination in the host cell, e.g trafficking to the host cell nucleus, but their specific roles in the parasite biology have not been exactly defined (Sibley and Ajioka 2008). The analysis of features of ROP18 in different *T. gondii* types evidenced that the ROP18 allele is remarkably polymorphic in type I strain whereas its expression level is reduced by more than 100 fold in the type III lineages. Transfection of ROP18 typeI allele in type III strain resulted in a 4-fold increase in lethality in mice (Saeij *et al*. 2005; Sibley and Ajioka 2008). This findings provides evidence that virulence of the type I strains is strongly related to this specific allele unique to this lineage.

A successful *T.gondii* infection depends on the ability of the parasite to cross biological barriers, such as gut epithelia, blood-brain barrier and placenta and access to immunoprivileged sites as the nervous system. Different migration capacities are related to strain-specific differences in parasite dissemination and parasite motility. In vitro studies evidenced that subpopulations of type I strain parasites display a higher migration capacity both in long-distance and across the extracellular matrix than type II and III strains (Saeji *et al.* 2005). Furthermore, in vivo type I parasites migrate more effectively to spleen than the other strains. Genetic analysis supports that the association of migratory capacity and virulence is linked to the same chromosome region related to acute virulence (Barragan and Sibley 2002; Saeij *et al*. 2005).

Growth rate is a common virulence feature in protozoan parasites. *T. gondii* burden is the major contributor to *Toxoplasma* pathogenesis in mice and it is related to an overstimulation of the immune system leading to high levels of T helper cell type 1 (Th1) cytokines, increased apoptosis and organ damage. Several studies have noted that type I strains grow faster than the other strains and that one tachyzoite of type I strain is sufficient to generate high parasite

loads and high levels of Th1 cytokines. This high growth rate might be linked to a higher reinvasion rate of type I parasites compared with the type II or III strains; in fact, type I parasites remain infectious for a longer time in extracellular matrix so are able to disseminate more efficiently than type II and III (Saeij *et al*. 2005). Furthermore, the doubling time (Td) of a strain seems to be related to the appearance of bradyzoites antigen (BAG1) and to the following conversion to the slower growing bradyzoite form. It has been seen that tachyzoites emerged from cells experimentally infected with virulent strains remained firmly on the tachyzoite phase not expressing BAG1 antigen at any time (Jerome *et al*. 1998; Saeij *et al*. 2005). Thus, there seems to be an inverse correlation between the virulence of *Toxoplasma* strains and the expression of bradyzoite genes ('bradyzoiteness') under normal culture conditions.

As reported above, the major parts of the typing investigations were and are performed on strains isolated from North America and Europe, resulting in an overrepresentation by strains isolated in these areas. Recent typing studies are focusing on isolates from animals and humans collected from more remote areas of the world with the purpose to extend the knowledge on *T. gondii* population structure. Surprisingly, strains from South America have been identified as natural recombinant, genetically different from those in North America and Europe; furthermore, in Brazil, *T. gondii* populations resulted to be distinct among different locations and some atypical genotypes have been shown to be highly pathogenic to humans, causing severe cases of ocular toxoplasmosis (Ferreira Ade *et al*. 2006; Khan *et al*. 2006; Dubey *et al*. 2007; Pena *et al*. 2008). More worringly, severe and fatal cases of toxoplasmosis in immunocompetent individuals were linked to one atypical strain in French Guiana (Carme *et al*. 2002; Demar *et al*. 2007). Genetic data on *T. gondii* isolates from Africa are scarce and not sufficient to elucidate the population structure in this continent. To date, studies of isolates collected in several African countries showed a predominance of classical lineages, type I and II in Uganda and predominantly type III in samples from Western and Middle Africa (Lindstrom *et al*. 2008; Velmurugan *et al*. 2008). However, Mercier and colleagues (Mercier *et al*. 2010), identified, new haplogroups, called Africa 1 and 3, from isolates of Gabon area and proposed them as being major lineages in the continent, encouraging further studies with genotype analysis.

TOXOPLASMOSIS IN ANIMALS

The capability of *T. gondii* to infect all warm blooded animals makes it the most successful parasite worldwide. Among the different animal species, there is a wide variety of clinical responses, ranging from unapparent infection to fatal disease with many intermediate situations, such as behavioural changes and congenital diseases. Factors that may influence the outcome of *T. gondii* infection in animals include:

- the parasite strain,
- if the infected species has evolved alongside the cat and
- how the immune system of the host responds to the infection (Innes 1997).

Table 2 Severity of reaction to *Toxoplasma gondii* in intermediate hosts (Innes *et al.* 1997).

Very Severe	Intermediate	Resistant
Marsupials	Human	Cattle
New World Monkeys	Sheep	Horses
Old World Monkeys	Goats	Pigs

Toxoplasma infection in felids

Felids are the only known definitive hosts for *T. gondii*, with domestic cats and a large range of wild felids shown to be capable of shedding oocysts (Dubey 2008).
Cats shed between 3 and 810 million of oocysts per infection in a period of 8 days on average, although this can be extended up to three weeks (Dabritz and Conrad 2010). After the first infection, cats are generally protected against re-shedding, although re-shedding has been observed experimentally upon challenge infection six years after the primary infection, upon administration of high doses of corticosteroids, and after superinfection with the related parasite *Isospora felis* (Dubey 1995). Re-shedding was not observed in Feline Immunodeficiency Virus infected cats challenged with *T. gondii* 66 weeks post primary infection (Lappin *et al.* 1996), therefore it is unclear how the parasite may persist in cats following primary infection.

As definitive hosts, cats are essential in the epidemiology of *T. gondii*, which is demonstrated by the low prevalence of infection in other animal species on cat free islands (Wallace *et al.* 1972; Dubey *et al* 1997). Cats mainly become infected by ingestion of tissue cysts. Kittens can be infected by transplacental passage of the parasite and can possibly also be infected by their mother's milk (Powell *et al*. 2001). Infection through ingestion of oocysts is another possibility, but cats are not very susceptible to oocysts compared to intermediate hosts (Dubey 1996b). Prevalence of infection in cats varies with hunting behavior or other factors such as outdoor access and feeding on raw meat, this latter factor being associated with hunting behavior. In addition, the prevalence of infection increases with age, as *T. gondii* infection persists in the form of tissue cysts. Seroprevalences of up to 74% have been reported. These are generally higher in stray and feral cats compared to pet cats (Tenter *et al*.

2000). Importantly, it has been recently shown that owning a pet cat is not a strong risk factor for human infection. In a review of 14 case-control studies for toxoplasmosis, only one indeed identified a slightly increased risk of infection in cat-owners (Petersen *et al.* 2010). Petting cats is probably not a risk, as no infectious oocysts could be detected on fur clippings collected 7 days after cats had shed millions of oocysts upon experimental infection (Dubey 1995). The risk of accidental ingestion of oocysts while cleaning the cat litter box can be reduced by proper hand hygiene and by eliminating cat feces daily, before oocysts have had the opportunity to sporulate. In addition, cat litter has been shown to reduce oocyst viability (Dubey *et al*. 2011). Accidental ingestion of oocysts is more probable during gardening, playing in a contaminated sandbox, or when eating raw and unwashed vegetables or fruits (Petersen *et al*. 2010).

Felids are thought not to be severely affected by the *T. gondii* infection. About 10 to 20% of cats develop self-limiting, small-bowel diarrhea in the first 2 weeks after experimental inoculate (Lappin 2010). Disseminated toxoplasmosis with uncontrolled replication of tachyzoites in hepatic, pulmonary, CNS, and pancreatic tissues can develop after acute infection or in cats concurrently infected with feline leukemia, feline immunodeficiency, or feline infectious peritonitis viruses, as well as after cyclosporine administration or after renal transplantation (Lappin 2010). In a recent study from Finland, 6 out of 193 cats submitted to post-mortem examination were diagnosed with general toxoplasmosis, demonstrating that *T. gondii* may be more of a health issue for cats than previously thought (Jokelainen *et al.* 2012).
There are other feline species that are more susceptible than domestic cats: female Pallas cats can transmit *T. gondii* to their offspring when persistently infected, which often leads to fatal toxoplasmosis in kittens, and is a common cause for failure of captive breeding programs (Kenny *et al*. 2002).

As cats are generally not affected by the clinical disease, prevention should focus on cats as a source of environmental contamination with *T. gondii*. Although cats are not necessarily a direct risk for human infection, all human infections can be traced back to cat-shed oocysts, either as a direct source of human infection or via infection of food animals causing meat-borne infection in humans. Therefore, reducing the number of oocysts shed in the environment could significantly reduce *T. gondii* infections in both food animals and humans. At the moment only a limited set of control options are available. The first option is to reduce the cat population. This includes timely neutering of owned cats, and a control strategy for stray cats. The second option is to prevent oocysts from ending up in the environment. Cat owners should thus encourage their cats to use the litter box, and dispose of cat litter with household wastes rather than, for example, flushing it down the toilet. The third option is to prevent cats from becoming infected by limiting their hunting behavior, e.g. by keeping them indoors (Woods *et al.* 2003). In addition, cats should not be fed with raw meat. All these options involve changes in the behavior of cat owners or of their cats, which implies that it may not be feasible to achieve a large reduction of the number of oocysts shed into the environment using these methods. Focusing only on high risk locations, by keeping cats away from food animals, vegetable gardens and sandpits may be more feasible.
A vaccine for cats to prevent or reduce oocyst shedding would greatly enhance the

possibilities of *T. gondii* infection control, and should be considered a research priority. An experimental cat live vaccine using oral administration of the T-263 strain, derived from the RH strain, prevented oocyst shedding in 84% of kittens (Frenkel *et al.* 1991) and has been shown to decrease the exposure of pigs to *T. gondii* in a farm-based experiment (Mateus-Pinilla *et al*. 1999). However, this vaccine strain needs to be maintained in mice, limiting the scale of production. Moreover, the vaccine requires frozen storage, and importantly, as a live vaccine, it may be hazardous to the people administering it (Innes *et al*. 2009). A different type of vaccine should thus be favored but, so far, studies are limited. The use of recombinant feline herpesvirus-1 expressing the *T. gondii* rhoptry protein ROP2 did not reduce oocysts shedding (Mishima *et al*. 2002). Intranasal administration of crude native rhoptry proteins reduced oocysts shedding by 87.4% compared to an adjuvant-control group (Zulpo *et al.* 2012), and prevented 2 out of 3 vaccinated cats from shedding oocysts (Garcia *et al*. 2007).

Toxoplasma infection in livestock

In most countries, epidemiological data on *T. gondii* infection in livestock are not regularly monitored and information on its diffusion is mainly obtained from local surveys.
From the review of the seroepidemiological studies published in literature until 2000 (Tenter *et al.* 2000), it has been possible to note that prevalence of antibodies against *T. gondii* vary widely among different animal species with the highest levels of seropositivity reported in sheep and goats and the lowest values in horses and poultry.

Among livestock, sheep and goats are susceptible to congenital toxoplasmosis, which represents one of the main causes of abortion and stillbirth; in other species (pigs, cattle, horses, chickens), the infection usually remains asymptomatic (Dubey and Jones 2008).

Despite the clinical manifestation, the importance of *T. gondii* primary infection in livestock is related to the ability of the parasite to produce tissue cysts in infected animals, which may represent important sources of infection for humans. The organotropism of *Toxoplasma* and the number of tissue cysts produced in a certain organ vary with the intermediate host species: tissue cysts are most frequently observed in various tissues of infected pigs, sheep and goats, less frequently in poultry and horses and only rarely in cattle (Tenter *et al.* 2000; EFSA 2007). Even if the seropositivity of meat-producing animals does not necessarily reflect the risk that those animals pose for their consumers (Tenter 2009), it has been recently demonstrated that in sheep, the antibody concentration was strongly related to the probability of the presence of tissue cysts in meat samples (Opsteegh *et al*. 2010a).
Using intensive farm management together with adequate measures of hygiene, confinement, and prevention, it has been economically possible to produce pigs and poultry free of *T. gondii* infection, although this has been achieved in only a few countries, i.e. in the Netherlands and Denmark (van Knapen *et al.* 1995; Shirley 1997).
These measures include:

a) to keep meat-producing animals indoors throughout their life-time;
b) to keep the sheds free of rodents, birds, and insects;
c) to feed meat-producing animals on sterilized food, and

d) to control access to sheds and feed stores, i.e. no pet animals should be allowed inside them (Tenter *et al*. 2000)

By contrast, production of free-ranging livestock will inevitably be associated with *T. gondii* infection. Animals kept on pastures with an increased pressure of infection due to contamination of the environment with oocysts, such as sheep and goats, show high seroprevalences in many areas of the world, i.e. up to 92 and 75%, respectively (Tenter *et al*. 2000).

Toxoplasma infection in sheep

Toxoplasma gondii antibodies have been found in sheep worldwide; global seroprevalence values have been recently reviewed by Dubey (2009b) revealing that values vary widely among different locations, from 4% to 92%. Since then, seroprevalences of 44.1% (90/204) have been reported in sheep from Grenada and Carriacou, West-Indies (Chikweto *et al.* 2011), 18.6% (71/382) in Sao Paulo, Brazil (Langoni *et al.* 2011), using MAT and 49.3% (248/503) in Spain using a commercial ELISA (Garcia-Bocanegra *et al.* 2013). In a serological survey of *T. gondii* infection in adult breeding sheep in Great Britain, of the 3539 sera collected from 227 flocks, 74% were found to be positive for *T. gondii* specific antibody using latex agglutination (Hutchinson *et al.* 2011).

In relation to different serological tests used, different cut-off and different animal investigated, their comparison should be done with extreme care (Dubey 2009b). However, it is clear that the infection is widely diffused among the global sheep population. Seroprevalence has been shown to increase with the age of the animals, indicating that the major part of sheep acquire infection post-natally; differences in seroprevalence between young and adult sheep are probably related to the oral route of transmission, with the older animals being exposed for longer time (Dubey and Kirkbride 1989; Lunden *et al.* 1994; Dubey 2009b; Katzer *et al.* 2011). Antibody levels are expected to stay elevated for the lifetime of the animal, because the immune response of infected animals does not completely clear the parasite and animals stay persistently infected (Katzer *et al.* 2011).
As sheep are herbivores, the most likely source of infection is represented by oocysts shed by infected cat and contaminating the environment, i.e. pastures, feed stock or water. The importance of ingestion of sporulated oocysts has been supported by risk assessment studies showing a statistically significant association between infection and:

- the presence of cats in the farms (Vesco *et al.* 2007; Abu-Dalbouh *et al.* 2012; Cenci-Goga *et al.* 2013; Guimaraes *et al.* 2013; Mendonca *et al.* 2013).;
- the management (Dubey 2009b; Gebremedhin *et al.* 2013);
- the use of superficial or stagnant water for drinking animals (Vesco *et al.* 2007; Cenci-Goga *et al.* 2013; Gebremedhin *et al.* 2013);
- the age of investigated animals (Vesco *et al.* 2007; Katzer *et al* 2011; Hutchinson *et al.* 2011;)

With regard to sheep farming, animals kept on pastures and raised under estensive or semi-estensive conditions are generally believed to be more likely exposed to *Toxoplasma* oocysts (Tenter *et al.* 2000); however some authors found an increased risk of infection for sheep kept under intensive conditions as farm facilities may provide shelter to various hosts of *T.gondii* (such as cats and rodents) which might be involved in the spread of the infection (Tzanidakis *et al.* 2012).
Also flock size was found to be a significant risk factor for the infection but in relation to the farming conditions under which animals were kept (Cenci-Goga *et al*. 2013).

Once ingested, oocysts excyst in the small intestine, releasing sporozoites which quickly invade and multiply within the cells of the gut differentiating into tachyzoites (Innes *et al.* 2009). A common clinical sign of toxoplasmosis during the early stages of infection is an elevated temperature which may last for a further week, during which time tachyzoites may be detected in the circulation and find their way to the placenta (Dubey and Sharma 1980; Wastling *et al.* 1993). Important observations from several studies have shown that ewes infected during one pregnancy are unlikely to suffer from a recrudescence of the infection in a subsequent pregnancy, suggesting that they are able to develop effective protective immunity against the disease (Buxton *et al.* 2006; Buxton *et al.* 2007; Innes *et al.* 2009). However, the protection is not to consider absolute (Innes *et al.* 2009; Edwards and Dubey 2013): recent studies reported the finding of *T. gondii* DNA in fetuses from persistent infected ewes suggesting a recrudescence of congenital transmission of *T. gondii* but this data require confirmation by other techniques, i.e. bioassay in mice for determining the viability of the parasite (Morley *et al.* 2005; Morley *et al.* 2008).
Congenital transmission from ewes primarily infected during pregnancy to the foetus represents an important cause of abortion and economic losses to sheep industry, reported in New Zeland, Australia, UK and Europe (Dubey and Beattie 1988). The stage of pregnancy when transplacental transmission of *T. gondii* takes place is important in determining the clinical outcome. If the infection occurs early in gestation, when the foetal immune system is relatively immature, foetal death is likely to occur. Infection at mid-gestation can result in birth of a stillborn or weak lamb which may have an accompanying small mummified foetus, whereas infection in later gestation may result in birth of a live, clinically normal, but infected lamb. Characteristic white spot lesions may be observed on placentas from sheep that have aborted their lambs and these areas of necrosis may compromise the function of the placenta to support the fetus during gestation (Buxton 1990). The average annual incidence rates of clinical toxoplasmosis was estimated to range between 1% and 2% (Blewett and Trees 1987) but, according to Dubey (2009b), "actual losses in lambs due to toxoplasmosis are difficult to estimate because the disease is sporadic, only a small number of aborted lambs are submitted for diagnosis and, most importantly, toxoplasmosis does not produce clinical disease in the ewe, not alarming the farmer". In the UK the estimate of the annual cost of toxoplasmosis to the sheep industry has been calculated to be £12.4 million (Gutierrez *et al.* 2012).
In the last decade the scientific community shown increased interest in determining the role played by *T. gondii* in ovine abortion and numerous studies have been conducted worldwide with the aim to investigate the presence of the parasite in placentae or aborted foetuses. The results of these studies are summarized in Table 3.

Table 3 Summary of the *T. gondii*-abortion cases reported in literature.

COUNTRY	DIAGNOSTIC METHOD USED	TISSUE	OVERALL PREVALENCE (n° pos samples /total tested)	REFERENCE
SPAIN	B1 nested PCR IFAT-ELISA Hystology	Fetal brain serum or fetal thoracic fluids sections of fetal brain, heart, liver and placental cotyledons	23.1% (40/173)	Pereira-Bueno *et al.* (2004)
	ITS1 nested PCR	Fetal brain	5% (4/74)	Moreno *et al.* (2012)
HUNGARY	Hymmuno histochemistry	Fetal membranes	8% (5/65)	(Szeredi and Bacsadi 2002)
IRAN	IFAT	Fetal abdominal/thoracic fluids	5.2 % (17/352)	(Razmi *et al.* 2010)
IRELAND	Duplex real-time PCR based on 529 bp repeated element	Placenta Fetal brain, fetal tissues, fetal stomach contents, fetal pleura fluid, fetal hair coat swabs, and vaginal swabs.	10% (51/489)	(Gutierrez *et al.* 2012)
JORDANY	PCR kit (VeTeK™TOXO Detection Kit for the detection of T. gondii by one-step PCR)	Fetal blood and pool of tissues	29.8 % (76/255)	(Abu-Dalbouh *et al.* 2012)
ITALY	ITS1 nested PCR	Fetal brain, skeletal muscle, liver, spleen, abomasum placenta	11.1% (271/2421)	(Masala *et al.* 2003)
	ITS1 nested PCR	Fetal brain, skeletal muscle, liver, spleen, abomasum Placenta	17.1% (63/366)	(Masala *et al.* 2007)
SWITZERLAND	Himmunohystology	Various fetal tissues sections	19% (16/86)	(Chanton-Greutmann *et al.* 2002)

These results confirm once more how *T. gondi* represents a common cause of abortion in

sheep. In global sheep farming a more strictly application of proper prophylactic measures is urgently needed for the prevention of the infection and the economic losses due to missed lactation and lambs.

For reducing losses to the sheep industry, a life vaccine (Toxovax ™) is available in New Zealand and Europe to help prevent ovine congenital toxoplasmosis; this vaccine, initially developed in New Zealand and further studied in Scotland, consists of a modified S48 strain of *T. gondii* that has lost the ability to differentiate into bradyzoites (O'Connell *et al.* 1988) but that can induce protective cell-mediated immune responses in sheep (Buxton and Innes 1995). Toxovax™ is advised to be administered at least 3 weeks prior to mating by subcutaneous inoculation and it induces a protective immunity for at least 18 months with a meat and milk withdrawal period of six weeks following vaccination (Buxton and Innes 1995); care should be taken by those administering it as it is a zoonotic pathogen (Innes and Vermeulen 2006).

Together with the vaccination, other prophylactic measures are fundamental for reducing the environmental contamination and the infection rate of the animals, i.e. the control of the cat population (both stray and pets) in the farm, especially for the 2 months before the lamb season (Dubey *et al.* 1990b), the cover of the feed stock at all times to prevent contamination (Buxton 1990) and, when possible, prevent the access of cats to water sources used for drinking animals. Moreover, strict sanitation measures are necessary during an abortion outbreak, e.g. the separation of aborting ewes or the ones with vaginal discharges and the dispose of aborted fetuses and placentae (Masala *et al.* 2007).

The control of *T. gondii* infection in sheep is also important from a public health point of view since tissue cysts may persist in organs of infected sheep for several months and perhaps for life, representing a source of infection for humans (Uggla and Buxton 1990; EFSA 2013). It has been demonstrated, indeed, that *T. gondii* was still detectable after more than five months p.i in tissues of asymptomatic sheep experimentally infected (Dubey 1984). Regarding the distribution of the cysts, two experimental studies, which examined by PCR and histological techniques the distribution of *T. gondii* in various tissues of experimentally infected sheep, found that the parasite was more frequently detected in the brain, in the heart and in muscle samples (Esteban-Redondo and Innes 1998; Esteban-Redondo *et al.* 1999). According to European Food Safety Authority, the consumption of raw or undercooked mutton represent the main source of infected meat in Southern European countries (EFSA 2007; Kijlstra and Jongert 2008).

Little genetic typing has been performed on *T. gondii* isolates from sheep and published data indicated that Type II is the predominant lineage of the strains isolated. Interestingly, no Type I isolate of *T. gondii* has been found in sheep to date (Dubey 2009b).

Several studies have shown the presence of *T. gondii* DNA in ovine milk samples. Fusco *et al.* (2007) detected *T. gondii* DNA in 3.4% (4/117) of the milk samples (10 samples pooled from

each farm) collected from 117 farms of the Campania region in Italy; additionally, they tested milk samples for anti-*Toxoplasma* antibodies founding 91 positive out of the 117 samples tested. Similarly, in Brazil, *T. gondii* DNA was detected in the milk of 5 (3%) naturally infected sheep, out of the 70 seropositive tested animals (Camossi *et al.* 2011). In a recent study performed in Iran, milk samples from different animal species, including sheep, have been tested for *T. gondii* by cell line culture, ELISA and PCR; using the culture method, the 7.02% (13/315) of ovine milk samples were found to be contaminated, with the positivity confirmed by bioassay in cats too (Dehkordi *et al.* 2013). Thus far, acute toxoplasmosis has not been associated with the consumption of unpasteurized ovine milk; however these results, in particular the positivity in bioassay, suggest that the consumption of raw ovine milk, or dairy products made with raw milk, might be considered a source of infection for humans (Tenter *et al.* 2000).

Toxoplasma infection in goats

Similarly to sheep, *T. gondii* infection in goats has been reported worlwide with seropositivity values varying widely among different countries (from 4% to 77%) depending on the serological techniques used, the cut off values, the animals investigated and management conditions (Tenter *et al.* 2000). Though these differences, it is clear that the infection is widespread among global goats population.
Similarly to sheep, goats are supposed to acquire the infection mainly by the ingestion of oocysts from contaminated pastures or water (Mancianti *et al.* 2013); various studies demonstrated that risk factors associated with *T. gondii* infection in goats are similar to the ones described above for sheep (Tzanidakis *et al.* 2012; Garcia-Bocanegra *et al.* 2013; Kantzoura *et al.* 2013; Lopes *et al.* 2013). Furthermore, goats are thought to more susceptible to toxoplasmosis than sheep as a result of their higher activity and movement compared with sheep, which increases the probability of contact with contaminated sources (Abu-Dalbouh *et al.* 2012).

Congenital transmission from goats primarily infected during pregnancy to their foetuses is similar to that seen in sheep representing a likewise important cause of abortion and neonatal mortality. The stage of pregnancy when transplacental transmission of *T. gondii* takes place is important in determining the clinical outcome. Infection early in gestation can result in foetal death and resorption/abortion while infection in the latter part of the pregnancy may have no clinical effect, with the offspring born normal but infected and immune (Buxton 1998). In experimentally infected goats, doses as low as 10 sporulated oocysts may produce abortion (Dubey 1982). An experimental study showed that infected goats experimentally re-infected with oocysts had a repeat transplacental transmission of the parasite (Dubey 1982). On the contrary, an experiment using 11 pregnant goats, infected and re-infected with high doses of oocysts showed that none of the young born to the previously-exposed goats had congenital toxoplasmosis (Obendorf *et al.* 1990). More studies are required to improve the understanding of the pathogenesis of congenital toxoplasmosis in goats and if the infected animals develop protective immune response. Although abortion and neonatal mortality are

the main clinical signs, adult goats can develop clinical toxoplasmosis involving liver, kidneys and brain (Dubey and Beattie, 1988)

As previously reported for sheep farming (Dubey 2009b), also actual data on the impact *T. gondii* abortion in caprine farming are difficult to obtain and they can only be deduced from the scientific press. An 8-year period diagnostic assessment of caprine abortion cases conducted by immunohistochemical procedures in California, USA, identified *T. gondii* as the causative agent in the 3% (7/211) of cases (Moeller 2001). Recently, an investigation on caprine aborted foetuses, sent to a diagnostic service in Spain, revealed the presence of *T.gondii* DNA in 3.8% (1/6) of the examined samples whereas previously the parasite was detected in 8.3% of goat abortion (Moreno *et al.* 2012). Investigations on abortion during two lambing seasons in Switzerland showed that *T. gondii* was the responsible agent of caprine abortion in the 15% of cases (22/144) (Chanton-Greutmann *et al.* 2002); in Egypt, the rate of abortion due to *T. gondii* reported in a flock of goats was 43.7% (Ahmed *et al.* 2008). Although these rates of detection are noteworthy, the burden of *T.gondii* abortion in goats is not truly assessed as the disease is still under-detected and underreported in relation to the absence of a proper surveillance system.

Regarding the distribution of tissue cysts, early studies on distribution of *Toxoplasma* in tissues of goats experimentally and naturally infected demonstrated that tissue cysts frequently develop in liver and kidneys but that persist longer in skeletal muscle than in brain (Dubey 1980b; Dubey *et al.* 1980).
Recently, parasites have been be detected by a quantitative PCR in various tissues of experimentally infected goats, with the highest concentration of tissue cysts found in lung and brain tissue (Jurankova *et al.* 2013). Furthermore, the parasite has been isolated by bioassay in mice from goats' hearts destined to human consumption in USA; the isolates were subsequently grown in cell culture for genotyping studies, revealing a dominance of Type II and Type III and also the presence of atypical genotypes (Dubey *et al.* 2011). Genotyping data on *T. gondii* strains from goats worldwide is very limited. Dubey (1980a) isolated a mouse virulent strain (GT1) from muscles of a goat from Ohio, USA. Ragozo *et al.* (2009) isolated viable *T. gondii* from tissues of 12 out of 26 seropositive goats from Brazil: these 12 isolates were grouped into five atypical genotypes and clonal Types II and III were absent. Recently, Mercier *et al.* (2011) isolated *T. gondii* from 10 seropositive goats from Dienga, Gabon, Africa; all 10 isolates were avirulent for mice and Type III by *T. gondii* microsatellite markers.
Regardless of genotypes identified, all of these studies suggest that goats can be important hosts for *T. gondii* transmission to humans.

The control of toxoplasmosis is similar to that in sheep and biosecurity measures should be conducted in farms to prevent contact between cats, rodents and feed bins.
Toxovax™ vaccine is not licensed for use in goats; however, when it has been tested, protection was observed against abortion but the 25% of the vaccinated group had stillbirths after challenge with sporulated oocysts (Chartier and Mallereau 2001).

The excretion of *T.gondii* tachyzoites in caprine milk was studied for the first time in 1980 by inoculation of mice with milk collected from experimentally and naturally infected goats

(Dubey 1980b; Dubey *et al.* 1980): the parasite was successfully isolated from milk sampled from experimentally infected animals (Dubey *et al.* 1980) whereas no isolation was achieved from naturally infected goats (Dubey 1980b). In Iran milk's samples collected from healthy naturally infected goats have been recently tested for *Toxoplasma* infection by cell line culture and the positive ones confirmed by bioassay in cats: 18% (10/180) of the samples tested positive by the culture method and had positive bioassay too (Dehkordi *et al.* 2013). Other studies documented the presence of *T.gondii* DNA in the milk of naturally infected goats (Bezerra *et al.* 2013; Mancianti *et al.* 2013). These findings, in particular the positivity in bioassay, together with the reports of acute human toxoplasmosis following the consumption of milk of naturally infected goats (Sacks *et al.* 1982; Chiari Cde and Neves 1984; Skinner *et al.* 1990), highlight the importance of caprine milk as a source of infection for human. Moreover, a recent case-control study of adults infected with *T. gondii* in USA identified drinking unpasteurized goats' milk as a risk factor for recently-acquired toxoplasmosis (Jones *et al.* 2009). Nevertheless feeding of goat whey was also identified as a source of *T. gondii* infection in pigs in the Netherlands (Meersburg *et al*., 2006).

Toxoplasma infection in sheep and goats in Italy

According to the National Institute of Statistics (ISTAT), in Italy there are 7.5 million small ruminants, in particular about 6.8 million of sheep and 861,000 of goats (http://www.istat.it). Small ruminants are predominantly reared in Central-Southern Italy: Sardinia is the Italian region with the highest percentage of heads of small ruminants, owning approximately half of the total Italian sheep stock and more than 20% of the total heads of goats. Following Sardinia, Sicily, Lazio and Tuscany own respectively 10.4%, 9.4% and 8.1% of the total sheep stock whereas Calabria, Sicily and Basilicata own 15.1%, 13.2% and 10.7% of the total goats population, respectively (http://www.izs.it/IZS 2013).

In Southern Italy, the traditional handling sheep methods are represented by transhumance and by permanent handling system. Transhumance, or "big transhumance" in relation to the high number of animals (1000-3000) present in each flock, is a seasonal migration between winter and summer pastures which was very practised in the past but less common nowadays, with the exception of Sardinia. Under this system, sheep are grazed on the plains during the falling-winter season whereas they are kept on mountain pastures during the summer. In relation to the high number of heads in each flock and to the nomadic characteristic of the system, animals are unlikely to be closely supervised by the shepherd and can be uncontrolled for long-short periods (Idda *et al.* 2010). Under the permanent handling system, flocks of 500-1000 heads are permanently kept outside on fenced pastures, almost exclusively living on graze, receiving very little food supplement and drinking water coming from surface sources (local streams or lakes) or ground waters, like springs and wells (Vesco *et al.* 2007).
Throughout Italy, goats are predominantly reared under extensive conditions, using natural pastures; furthermore in relation to their capacity for adaptation to very different environments, they can be reared also under extreme conditions, as barren lands, in particular in Southern Italy (De Luca, 2004).

In Northern Italian regions, sheep and goats farming is not as widespread as in Southern because these species are not very important in the livestock industry. The only exception is represented by the Alps, where small ruminants' husbandry is a productive livestock sector, in particular for local cheese-making (De Luca, 2004).

In Table 4 are summarized data on *T. gondii* seroprevalence in sheep and goats published in Italy over the last 3 decades and reviewed by Rinaldi and Scala (2008). The prevalence values are highly heterogeneous and difficult to compare since various assays have been used and animals sampled were of different ages and reared under different farming conditions. Considering that almost all the studies have been performed in the Central-Southern regions where sheep farming is more concentrated, it might be interesting to investigate the presence of *T. gondii* antibodies also in animals rose under different conditions in other parts of Italy.
The role played by *T. gondii* in ovine and/or caprine abortion has only been documented in Sardinia by Masala *et al.* (2003; 2007): in two consecutive surveys, the authors analysed by PCR aborted foetuses collected throughout the region. In the first study (1999–2002), *T. gondii* DNA was found in the 11.1% (271/2471) of tissues from ovine aborted foetuses, with placenta tested positive in 31.5% of cases; among caprine tissues, DNA was found in the 6.4% (23/356) of samples with the placenta found positive with the highest frequency again (50%; 6/12). In the second survey (2003–2005), authors examined again abortion specimen collected from 107 farms (98 ovine and 9 caprine) in northern Sardinia: *T. gondii* DNA was found in 18.1% (53/292) ovine foetuses and in 13% (3/23) of caprine samples. Few years later, Zedda *et al.* (2010) monitored by serology an outbreak of toxoplasmosis occurred in a Sardinian farm where abortion event involved 7.2% of 430 pregnant sheep; authors found a very high serological positivity (45.99%) in the animals and also anti-toxoplasma IgG in the sera of one of the farmers, in addition to *T. gondii* DNA in aliquots of grain and pellets taken from feed stocks.
All these data clearly indicate that in Sardinia clinical toxoplasmosis plays an important role in the economic losses due to lamb mortality and to missed lactation, that are estimated about 10 million Euro/year (Masala *et al.* 2003). However, according to the authors, the application of prophylactic measures for reducing environmental contamination, like reducing the number of stray cats and/or keep covered sheep feed and/or isolate aborting ewes and dispose aborted foetuses and/or placenta, is almost impossible as the breeding conditions are still linked to primitive and nomadic herding practices (transhumance) with animals remaining out of individual control for as long as 24h and sometimes even longer without any chance of control (Masala *et al.* 2003; Masala *et al.* 2007; Zedda *et al.* 2010).

Vesco *et al.* (2007) and Cenci-Goga *et al.* (2013) assessed risk factors for *T. gondii* infection in sheep flocks raised in Sicily and Tuscany, respectively. Either the presence of cats in the farms (Vesco *et al.* 2007) and their access to water used by sheep were found positively associated with farm-level seroprevalence (Cenci-Goga *et al.* 2013); furthermore, in both studies the use of stagnant or surface water for drinking animals resulted to significantly increase the risk for a sheep to be seropositive, confirming that contamination of water (and feed) with oocysts is fundamental in the epidemiology of the disease. In Sicily, the seroprevalence increased with age from 39.7% in animals less than 1 months to 57.5% in adult sheep; all the lambs were

found to be IgM negative, indicating that in these animals IgG were the maternal transmitted one through colostrum and confirming the low frequency of congenital infection (Innes *et al.* 2009). However it is noteworthy that no fall in seroprevalence was observed after the first month of life, thus indicating that the infection happens soon after weaning due to a very high pressure of infection in pastures (Vesco *et al.* 2010).

With regard to the use of the vaccine (Toxovax ™), currently registered as a prophylactic measure against abortion in sheep, as far as we know, such procedure is not practiced in Italy (Cenci-Goga *et al.* 2013).

Risk factors for *Toxoplasma* infection in goats have not been assessed in none of the published study; however Mancianti *et al.* (2013) hypothesized a high environmental pressure in the pasture and a probably presence of a stray cats population as possible explanation of the noteworthy seroprevalence value (60.6%) found in goats raised in Tuscany.

Although the consumption of dairy products made from raw ovine and/or caprine milk is a common eating habit in Italy, the role of ovine and, more important, caprine milk as a potential source of infection for human has not been exhaustively studied yet. Only two studies (Fusco *et al.* 2007; Mancianti *et al.* 2013) detected the presence of *T. gondii* DNA in Italian ovine and caprine milk samples, respectively: *T. gondii* DNA was found in the 3.4% (4/117) of examined ovine samples and in the 13% (10/77) of caprine milk. Even if a positive PCR does not demonstrate the presence of viable tachyzoites, these findings can hypothesize the possibility of transmission of *Toxoplasma* to humans through consumption of raw milk.

As far as we know, genetic typing from *T.gondii* isolates in Italy has been performed in goats by Mancianti *et al.* (2013) and in sheep abortion by Chessa *et al.* (2014). In the first study, conducted in Tuscnay, genetic characterization revealed the presence of classical clonal type isolates (III and I) from most samples (8/10) but also the presence of atypical genotypes from 2 samples. This is difficult to explain as the animals were born and lived uniquely in the Italian farm; the only possible explanation of the presence of atypical strains might be the occurrence of a recombination event in cats sharing the same area with the examined goats. In the second study, conducted in the Sardinia region, *T. gondii* DNA was found in 5 placentas, 14 brains, and 2 livers by PCR analysis and all isolates displayed Type II alleles, indicating that the Type II *T. gondii* may be associated with ovine abortion.

Table 4 Seroepidemiological studies on *T. gondii* prevalence in Italy

SPECIES	REGION	N° EXAMINED ANIMALS	SEROPREVALENCE (%)	DIAGNOSTIC METHOD	REFERENCES
SHEEP	SICILY	1,876	49.9	ELISA IgG	(Vesco *et al.* 2007)
	SARDINIA	7,149	28.4 9.9	IFAT IgG IFAT IgM	(Masala *et al.* 2003)
		29,886	19.2 5.4	IFAT IgG IFAT IgM	(Tola *et al.* 2006)
		1,043	51.3	ELISA	(Natale *et al.* 2006)
		524	45.99	IFAT (IgM+IgG)	(Zedda *et al.* 2010)
	CAMPANIA	1170	28.5	IFAT IgG	(Fusco *et al.* 2007)
	TUSCANY	630	33.3	IFAT IgG	(Cenci-Goga *et al.* 2013)
	LAZIO	198	95	MAT	(De Capraris and Gravino 1981)
	APULIA AND BASILICATA	244	68.9	IFAT	Puccini *et al* 1983
GOATS	SARDINIA	2,445	12.3 5.6	IFAT IgG IFAT IgM	(Masala *et al.* 2003)
		4,562	11.7 4	IFAT IgG IFAT IgM	(Tola *et al.* 2006)
	TUSCANY	127	60.6	MAT	(Mancianti *et al.* 2013)

Toxoplasma infection in pigs

Similarly to what reported for small ruminants, the prevalence of the infection in pigs varies either globally and among the classes surveyed (market pigs versus sows, indoor pigs from biosecure housing systems versus free-range) as the prevalence of infection in pigs is also influenced by management systems. Dubey (2009a), reviewing worldwide information on prevalence since 1988, reported that in the USA the application of good management measures in pigs farming leaded to a drastic decline of the prevalence of infection in the last decade, e.g. in breeder pigs seroprevalence dropped from 42% in 1983-1984 to 20.8% in 1992. Since the review published by Dubey (2009a), the seroprevalence of *T. gondii* in slaughter pigs from Ireland has been recently observed at 4.7% (15/317) (Halova *et al*. 2013). In Mexico a prevalence of 12.7% (136/1,074) has been observed (Alvarado-Esquivel *et al.* 2011) and between 2.7% (162/6238) and 12.5% (38/305) in North America and Brazil, respectively (Hill *et al.* 2010; Samico Fernandes *et al*. 2012). A study across ten regions of China noted an overall seroprevalence of 53.4% (434/813) (Yu *et al*. 2011).

Signs of severe clinical symptoms following natural *T. gondii* infection are uncommon and disease reports in the literature are generally described as sporadic outbreaks. One of the first identified cases occurred in the US in 1951 where fifty percent of the infected pigs died showing cough, diarrhea, weakness, lack of coordination and tremors (Farrell *et al.* 1952). More recently a lethal outbreak of *T. gondii* was reported in fattening pigs from China with morbidity and a mortality rate of 57% (549/960) and 2% (19/960), respectively (Li *et al.* 2010): further investigations linked this outbreak to ingestion of contaminated feed as cats were found to reside within the feed warehouse. Clinical disease in fattening pigs has also been reported in two regions of Northern Italy, Lombardia and Emilia-Romagna with death occurred 2-4 days after initial symptoms (Gelmetti *et al.* 1999).

Porcine abortion caused by *T. gondii* infection has also been reported, although again with a rare occurrence in this livestock species. Although a number of other outbreaks of porcine abortion and severe clinical disease in naturally infected pigs have been reported, disease is generally subclinical. A more commonly observed symptom is a slight increase in rectal temperature during the initial stages of infection, which may persist for 1-2 days. More acute clinical symptoms, although rare, are likely to be linked to the age, the immune status, and even the breed of the infected animal (Dubey 2009a). Pigs generally go onto develop efficient protective immunity and quickly recover from infection without any obvious clinical signs. Clinical symptoms in pigs which have been experimentally infected with *T. gondii* are similar to those described above.

The source of infection in pigs can be via either oocyst contamination from soil or feed, or from consumption of tissue cysts from infected rodents or other small mammals harboring tissue cysts: contamination of feed or soil with oocysts is thought to be the main source of infection anyway (Lehmann *et al.* 2003). Transmission of *T. gondii* to pigs from the consumption of infected rodents has also shown to be a direct source of infection, particularly in animals reared outdoors (Kijlstra *et al.* 2004), which have generally been shown to have a higher prevalence of the parasite. A study by Dubey and collaborators have shown the prevalence of *T. gondii* in an outdoor reared pig farm from Northern USA to be 90.9%

(30/33), which is significantly higher than the figures reported for pigs raised in conventional indoor housing (Dubey *et al.* 2012). A similar trend was also showed in a study examining the prevalence of *T. gondii* in Argentinean sows (Venturini *et al.* 2004) and in Dutch ones; in particular, van der Giessen *et al.* (2007) found the prevalence of *T. gondii* in conventionally raised pigs in the Netherlands to be 0.38% (1/265), while the animals which were reared outdoors had a prevalence of 5.62% (10/178). It should also be noted that the age of the animal is important as an increase in age is linked to an increase in *T. gondii* seropositivity (Berger-Schoch *et al.* 2011; Halova *et al.* 2013) and that industrial pigs are more likely to have a short life.

As clinical toxoplasmosis is not considered a problem in pigs, the main reason to implement a control strategy to prevent exposure to *T. gondii* in this species is to reduce the number of parasites entering the human food chain and their subsequent transmission.
Together with sheep and goats, pigs are known to harbor a greater number of tissue cysts compared to other animals such as cattle and horses (Tenter *et al.* 2000), therefore undercooked or raw pork from infected animals is potentially a great source of infection for foodborne toxoplasmosis in humans.

To our knowledge, there is no identification system for individual pigs destined for human consumption neither in the USA nor in Europe, since pigs are not tested for *T. gondii* infection at slaughter in any country (Dubey 2009a). Currently, the most successful control measures are those which involve good on-farm management, such as ensuring that cats are not allowed to enter feed and grain stores and that they have limited access to pig farms (Gamble *et al.* 1999). The important role of cats in transmission of the parasite to pigs has been demonstrated in an experimental vaccination trial in cats in Illinois, USA (Mateus-Pinilla *et al.* 1999). The authors demonstrated that a decreased number of infected pigs was observed when cats living around the farms under investigation had been vaccinated against *Toxoplasma,* using live bradyzoites of the attenuated mutant T-263 strain, derived by mutagenesis from the RH strain, capable of preventing oocyst shedding by cats.
Effective rodent control is also vital as pigs are omnivores and can consume rodents or rodent cadavers if they are present within their housing. A study examining a 4 month rodent control program in three organic pig farms, followed by longitudinal analysis over seven months, showed that the levels of *T. gondii* infection in pigs fell from 10.9% (10/92) to 3.3% (4/122) and that the prevalence of infected pigs rose again after the control campaign had stopped (Kijlstra *et al.* 2008). Therefore any on-farm control strategies must concentrate on cat and rodent control, both within the farm and the area used for food storage.
Another control strategy consists of vaccinating the pigs against the parasite to prevent tissue cyst formation, hence reducing the transmission of *T. gondii* from infected pigs into the food chain. Although a licensed vaccine for this purpose is currently unavailable, research has shown that vaccination using live strains of the parasite has had more success than a killed vaccine approach (Innes *et al.* 2011).

Toxoplasma infection in pigs in Italy

The Italian pig sector counts about 9.000.000 of heads with more than 80% of animals that is concentrated in Northern Italy.

Lombardy is considered the leader region owning 45.5% of the total pigs' population, followed by Emilia-Romagna and Piedmont owning 17.6% and 10.6%, respectively. In these regions, animals are predominantly reared under intensive methods: 74% of the pigs' herds raise more than 2000 animals and the density of animals can reach 3000 pigs/km^2. On the contrary, pigs herds of Central and Southern of Italy are predominantly "back yard" holdings, with an average of 27 and 13 animals raised in each farm, respectively (Bellini *et al.* 2010).

Little information is available on *T. gondii* infection in Italian pigs as in the last decade only four serological studies have been conducted on pigs from farms located in different parts of Italy.

In Sardinia, Sicily and Umbria, respectively regions of Southern and Central Italy, different groups of researchers, using different serological methods and investigating animals raised under different conditions, found a similar seroprevalence value of 16% (Scala *et al.* 2008; Villari *et al.* 2009; Veronesi *et al.* 2011).

Despite the economic importance of the swine breeding in Northern Italy, only one study analysed the serological status of pigs intensive reared in this area (Giacomini *et al.* 2013): the seroprevalence value found was 4.4%, that is low if compared to the 16% found in Centre and South Italy, but is noteworthy if compared to the value found in the Netherlands, where the prevalence of *Toxoplasma* in intensive farming raised swine was 0% (Kijlstra *et al.* 2004). However no data were reported by Giacomini *et al.* (2013) on management conditions of the investigated farms and it is therefore not possible to further analyse the differences between Italian and Dutch intensive breeding conditions.

Villari *et al.* (2009) and Veronesi *et al.* (2011) performed risk assessment studies on *T. gondii* in pigs farms located in Sicily and Umbria, respectively. In both studies the presence of cats had no influence on the serological status of the animal; authors supposed that other factors, such as the presence of infected rodents and the absence of use of coveralls or boots or footbaths before entering the stables, could have masked the influence of exposure to cats as risk factors. Moreover, the access to carcasses of dead animals, the lack of application of all-in-all-out housing (Veronesi *et al.*2011), the absence of rodent control and the use of water drawn from wells for drinking animals (Villari *et al*. 2009) resulted to significantly increase the risk for a pig to be seropositive. These findings clearly indicate that, in these regions, in addition to the presence of high levels of environmental contamination, the swine production systems lacks of proper hygienic conditions and management systems. The high level of environmental contamination in Sicily was also confirmed by the difference in seroprevalence found between imported and locally reared animals: animals from France and Spain showed an overall seroprevalence of 0.7% whereas locally raised pigs revealed a seroprevalence value of 16.4% (Villari *et al*. 2009).

The high risk of exposure to *T. gondii* in pigs is a consequent public health risk as raw or undercooked pork meat may represents an important source of human toxoplasmosis. As pigs are not tested for *T. gondii* infection at slaughter in Italy, the only available method for

decrease the risk for humans infection is to improve management systems and introduce modern intensive farming systems (Kijlstra and Jongert 2008).

With regard to clinical toxoplasmosis, in October 1994 Gelmetti *et al.* (1999) reported 4 outbreaks of disease with high morbidity (50-60%) and mortality (10-42%) in 2 different pig herds located in Northern Italy. The origin of infection still remains unknown as the 4 farms did not have any common feed sources or exchange of animals. Considering that either the presence of cats (3/4), poor hygienic conditions of the husbandry (2/4) and the absence of a program of rodents control (3/4) have been reported by the authors, the high rate of morbidity and mortality could have been due to the ingestion of a very high number of tissue cysts (infected rodents) or oocysts (high environmental contamination) or, eventually, to the circulation of a highly pathogen strain of *T. gondii.*

As far as we know, no studies have documented *T. gondii* genotypes circulating in pigs reared in Italy.

T*oxoplasma* infection in cattle, horses and chickens

Being mammalian, also cattle and horses can act as intermediate hosts of *T. gondii*; as herbivores, they become infected only by the ingestion of food or water contaminated with sporulated oocysts or by congenital transmission.

In cattle, seroprevalence values are generally high (up to 92%) but in infected animals tissue cysts are found rarely as they are unlikely to persist for long time; furthermore, only few positive bioassay results have been reported for naturally infected cattle (Dubey 1986) or beef samples (Aspinall *et al.* 2002; Dubey *et al.* 2005). On the contrary, PCR based detection of *T. gondii* in bovine samples is relatively common (e.g. More *et al.* 2008; Santos *et al.* 2010; Opsteegh *et al.* 2011b) but, as already reported, the detection of *T. gondii* DNA does not necessarily mean the presence of viable parasites and a risk for human infection.
In cattle, natural *T. gondii* infection does not appear to cause clinical disease or abortion (Dubey 1986); however, in a recent study, 16 out of 60 gravid dairy cows tested positive for *T. gondii* by bioassay of their foetuses, showing the occurrence of transplacental transmission of the parasite (de Macedo *et al.* 2012).
Beef is usually considered of low risk for human infection (Dubey and Thulliez 1993; Tenter *et al.* 2000; Kijlstra and Jongert 2008) even if a recent quantitative microbial risk assessment conducted in the Netherlands demonstrated that, when consumed raw, beef can contribute to 67.6% of predicted meat-borne infections (Opsteegh *et al.* 2011a).
Milk from infected cows has always been considered of negligible importance in the transmission of the infection (Dubey 1986); however a recent Iranian study detected *T. gondii* also in bovine milk's samples by cell line cultures and, more importantly, by bioassay in cats, calling into question the role played by bovine raw milk in the transmission of the infection (Dehkordi *et al.* 2013).

Under natural conditions, the prevalence of *T. gondii* antibodies in horses is generally low, with variations related to the serological methods used and animals investigated; recent

serological studies conducted in Greece, Mexico and Spain found seroprevalence values ranging from 1.8% to 13.3% with higher prevalence in animal kept in rural environment compared to animals kept indoors in urban settings (Kouam *et al.* 2010; Alvarado-Esquivel *et al.* 2012; Garcia-Bocanegra *et al.* 2012).
Currently, there is no evidence that *T. gondii* infection causes clinical disease in either naturally or experimentally exposed horses. This animal species is thus considered resistant to toxoplasmosis (Dubey and Jones 2008). Transplacental infection of the fetus with *T. gondii* and resulting abortions are considered possible but rare, with only one documented case (Aleandri *et al.* 1978).
Few successful attempts of isolation of viable *T. gondii* parasites from naturally or experimentally infected horses have been performed in the past (Zardi *et al.* 1964; Al-Khalidi and Dubey 1979); more recently, two groups of researchers isolated the parasite from pooled samples of muscles and brain using bioassay in mice, in Egypt and Brazil respectively, (Shaapan and Ghazy 2007; Evers *et al.* 2013). These findings suggest that also horsemeat can be considered a source of *T. gondii* infection for humans, mainly in countries where horsemeat is part of the culinary tradition and where people eat it rare or undercooked.

Chickens are susceptible to *T. gondii* infection but are generally considered to be resistant to clinical toxoplasmosis (Dubey *et al.* 2005; Hill and Dubey 2013). Free ranging chickens, feeding from the ground, are frequently exposed to the parasite oocysts present in soil (Hill and Dubey 2013); this risk is further enhanced by the location where free range chicken are kept, which is often in close proximity to human habitation, increasing the likelihood that cats and oocysts are present. Seroprevalence data for *T. gondii* infection in back yard and free range chicken is widely available and has been summarized in a recent review, which describes international seroprevalence data ranging from 30% to 100% (Dubey 2010). On the contrary, prevalence data for chickens reared indoors are rare (Dubey *et al.* 2005). In the United States *T. gondii* has been isolated frequently from the tissues of backyard chickens but no viable *T. gondii* was recovered from chicken meat samples obtained from shops using bioassays (Dubey *et al.* 2005). This findings highlights the zoonotic potential of free range chickens if consumed undercooked and also when handling their raw meat. Thus, free ranging chickens are a relevant and excellent epidemiological target for studies of the prevalence of *T. gondii* oocysts in the environment (Dubey *et al.* 2007). Furthermore in relation to their resistance to toxoplasmosis, to their feeding habits and to the fact that they are an invaluable source of viable parasites, samples from chickens have been collected all over the world and have been used for broadening the knowledge on *T. gondii* genotypic diversity (Lehmann *et al.* 2006). Analysis of these samples revealed a far greater diversity within *T. gondii* population than that previously expected: Type II confirmed to be the prominent type encountered in North America and Europe but to be either absent or very rare in South and Central America, where a greater diversity in the form of atypical strains was observed (Lehmann *et al.* 2006; Dubey and Su 2009; Rajendran *et al.* 2012) .

Toxoplasma infection in wildlife species

Although infection is common in wildlife, descriptions of symptomatic diseases are rare and infection is often discovered following post-mortem examinations. Exceptions concern animal species that have evolved in environments where there are no cats. As a result, many of these species are very susceptible to *T. gondii* infection and exhibit clinical symptoms of toxoplasmosis, which are often fatal (Innes *et al.* 1997). Wildlife species infected with *T. gondii* are important as a source of infection for humans. They are also studied for epidemiological reasons. The consumption of raw or undercooked venison and game meat (wild boars, hares, bears and grouse) containing tissue cysts has been demonstrated as a source of infection for humans (Tenter *et al.* 2000; Ross *et al.* 2001); moreover, handling and evisceration of game may also present a risk for human infection, especially for hunters and their families (Dubey 1994).

In cervids (roe deer, red deer, fallow deer) the seroprevalence has been found to vary considerably depending on the serological technique used, the host species and the geographical location: it ranges from 14 to 60% in wild roe deer, from 1.6 to 24% in fallow deer and is around 15 % in red deer (Aubert *et al.* 2010; De Craeye *et al*, 2011). *Toxoplasma gondii* infection is common in wild boars and seroprevalence in different European areas ranged from 23% to 55% (Beral *et al.*2012). These high prevalence percentages are difficult to compare because they were obtained using different serological methods. However, these studies do confirm the high frequency of *T. gondii* infections in wild boars and stress that eating raw or undercooked wild boar meat is a real risk for acquiring infection. This is of particular importance in countries where traditional raw and salted pork products are consumed, especially if they contain wild boar meat (Richomme *et al*. 2010).

Foxes are used frequently as a sentinel species to give an indication of how frequently *T. gondii* occurs in a geographical area, due to the predicted frequent exposure of the host to the parasite based on their carnivorous nature (Burrells *et al*. 2013). Seroprevalence of *T. gondii* in foxes ranged considerably between European countries: two independent studies found a seroprevalence of 20% in British foxes (Buxton *et al.* 1997; Hamilton *et al.* 2005), while 64% of foxes in France (Aubert *et al.* 2010), 68% in Hungary (Jakubek *et al.* 2007), 79% in Germany (Herrmann *et al.* 2012) and 98% in Belgium (De Craeye *et al.* 2010) tested positive.

The variability in the seroprevalence in the different wildlife species is likely to be influenced by their diet and habitats. Wild foxes and boars are exposed to tissue cysts because of their feeding habits, which may include small mammals, such as rodents and carrion or birds, all of which can be *T. gondii* carriers in their tissues. In addition to this, foxes and boars may also be exposed to oocysts contaminating soil or water (Herrmann *et al.* 2012). Cervids, that feed mainly on grass, leaves, young shoots and berries, are therefore less likely to be exposed to and ingest tissue cysts however they can be infected by drinking contaminated water or eating grass contaminated with oocysts (De Craeye *et al.* 2010). Therefore, wildlife species with different feeding habits are good indicators of different sources of *T. gondii* contamination. The geographical differences in *T. gondii* prevalence within wildlife host species may vary according to their habitat, the presence or absence of domestic or wild felids

and the climate. The contact between wildlife and domestic animals increases the opportunity of parasite exchange between the sylvatic and domestic transmission cycle (De Craeye *et al.* 2010; Beral *et al*. 2012).

Animal species that have evolved in isolation of *T. gondii* or had only very limited contact with the parasite are particularly susceptible to *T. gondii* infection and can exhibit clinical symptoms of toxoplasmosis, which are often fatal. This is well documented for marine mammals, in particular for Southern sea otters, macropods from Australia, New World monkeys and lemurs from Madagascar (Conrad *et al.* 2005; de Camps *et al.* 2008). Encephalitis caused by *T. gondii* is one of the major causes of death of sea otters, often found associated with shark attacks as the infection makes them less able to evade attacks. In the same way, *T. gondii* infection in Australian marsupials may make them more prone to predation causing declines in the population in the wild (Parameswaran *et al.* 2009); this is a particular threat when the infection causes declines and die-offs in endangered species, like the eastern barred bandicoot (Obendorf *et al.* 1996) or the brush-tailed rock-wallaby (Barnes *et al.* 2010).
Nevertheless, as *T. gondii* infection in marsupials is not always fatal but can be long-term latent and reactivated during time of stress, like captivity, toxoplasmosis has been associated with widespread pathology and death in several collections of captive marsupials in zoos worldwide (Canfield *et al.* 1990; de Camps *et al.* 2008; Parameswaran *et al.* 2009).
The description of severe and fatal toxoplasmosis in lemurs and New World monkeys is mainly based on zoo animals where animals were thought to be infected following exposure to oocysts brought in their enclosures or following the ingestion of undercooked infected meat, respectively (Dubey *et al.* 1985; Innes 1997; de Camps *et al.* 2008).

Toxoplasma infection in rodents

Natural *T. gondii* infections in wild rodents are common and these prey species are likely to play a substantial role in the transmission of the parasite to the definitive host, ie. felids, resulting in the amplification of the parasite and shedding of millions of environmentally stable oocysts that are infectious to all warm blooded animals.

There are numerous studies that report behavioral changes in rodents infected by *T. gondii*; these changes are predicted to aid predation by cats and to increase transmission rates of the parasite. Behavioral alterations that are described include deleterious memory and learning effects (Witting 1979), increases in time spent in the open (Hay *et al.* 1984), being less neophobic (fear of novelty) (Berdoy *et al.* 1995) and inhibition of fear of cats and cat kairomones (Berdoy *et al.* 2000; Vyas *et al.* 2007). Immuno-histochemistry studies in mice showed that *Toxoplasma* proteins may directly interfere with neuronal function which may significantly contribute to altered behavior of the host (Haroon *et al.* 2012). A recent study also showed that *T. gondii* induces a significant increase in dopamine metabolism in neural cells which may help to provide a mechanism for parasite induced behavioural changes (Prandovszky *et al*. 2011). Although these behavioral changes have been observed, they are not consistently seen in all infected animals, which raise the question whether these behavior

changes are chance events rather than adaptive manipulations of the host by the parasite (Worth *et al.* 2013). Studies are continuing to investigate how these behavior changes are achieved: whether this is due to uneven brain colonization (Berenreiterova *et al.* 2011) and how parasite infection can inhibit neuronal function (Haroon *et al.* 2012)

TOXOPLASMOSIS IN HUMANS

The acknowledgment of *T. gondii* as a human pathogen is dated 1939, when Wolf and colleagues identified *Toxoplasma* as the causative agent of encephalomyelitis in a three day old infant (Wolf *et al.* 1939). In the wake of this detection, during the next decade other scientists discovered new pathological aspects of the parasite: *Toxoplasma* was demonstrated to be capable of and associated with congenital transmission (Wolf *et al.* 1941) and it was shown to be involved in inflammatory diseases of the eye, e.g. adult retinochoroiditis (Wilder 1952; Frenkel and Jacobs 1958).
In the same periods, Sabin and Feldman introduced the methylene blue dye test, i.e. Sabin-Feldman test (Sabin and Feldman 1948), a serological technique that opened the door for extensive epidemiological studies on the frequency of infection in both animals and, more important, in humans. Since then, it has been estimated that up to one third of the world human population has been exposed to the parasite (Tenter *et al.* 2000).

The European Food Safety Authority (EFSA) in "The Community Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents, Antimicrobial Resistance and Foodborne Outbreaks in the European Union in 2005" reported that toxoplasmosis has the highest human incidence among zoonotic parasitic diseases. Despite that, it is considered an under-detected and underreported disease as very few European States have a routine surveillance system for toxoplasmosis and the only data available are laboratory-confirmed clinical cases that are not sufficient to assess the true disease burden (EFSA 2007).
From the review of the scientific literature on human toxoplasmosis, it is possible to obtain seroprevalence estimates of infection in various parts of the world; however, as different serological techniques have been used to obtain this information, they are difficult to compare and may not reflect nationwide prevalences differing from the true prevalence of infection. Nevertheless, these data can be useful if interpreted as estimates, reflecting the different levels of prevalence among similar populations in different region of the world. In the UK and US estimates vary between 9.1% - 11% of the population are predicted to be infected, in South America figures range from 6.1% - 77.5%, in Europe from 8.2% - 63.2% , and in Asia and Oceania 0.8% - 63.9% (Pappas *et al.*, 2009). A steady decrease in the incidence of human toxoplasmosis assessed by antibody levels has been reported among adults over the past two decades in the United States and Northern Europe (Jones *et al*. 2007). This observed decline in *Toxoplasma* seroprevalence has been attributed to the introduction of modern farming systems resulting in a lower prevalence of *Toxoplasma* cysts in meat in combination with an increased use of frozen meat by consumers (Tenter *et al*., 2000; Diza *et al*., 2005; Kijlstra and Jongert 2008; Torrey and Yolken 2013).

CLINICAL ASPECTS OF *Toxoplasma* INFECTION IN HUMANS

For an opportunistic parasite such as *Toxoplasma,* expression of the disease is mainly dependant of host immunity and genetic background. It is usual to consider that in immunocompetent individuals the infection is mild symptomatic or totally asymptomatic and

that clinical sever manifestation occurs only in immunocompromised patients or congenitally infected children. However, a part of the variation in disease manifestation, even in immunocompetent humans, may be related to the parasite itself: inoculum dose, infecting stage, route of infection, and parasite genotype.

Immunocompetent individuals

T. gondii primary infection in children and adults is asymptomatic in most patients: the most typical clinical manifestation is isolated cervical or occipital lymphadenopathy that may persist for weeks or months; severe manifestation as encephalitis, myocarditis, polymiositis, pneuomonitis and hepatitis are very rare and infrequent (Montoya and Liesenfeld 2004). However, fatal cases due *T. gondii* infection have been reported in immunocompetent individuals from South America (French-Guiana) and have been associated with one atypical highly virulent strain (Carme *et al.* 2002; Demar *et al.* 2007). Recently other 2 cases of severe toxoplasmosis in immunocompetent individuals caused by atypical strains have been described in France; for both patients the ingestion of infected meat imported from Brazil and Canada resulted to be the more likely cause of infection (Pomares *et al.* 2011). Furthermore, in the last years it has been recognized the possibility that ocular toxoplasmosis in immunocompetent individuals may be a consequence of postnatally acquired infection (Khan *et al.* 2006).

Immunocompromised individuals

The majority of *Toxoplasma* infection in immunocompromised hosts, such as individuals receiving cortocosteroids or cytoxic drugs, patients with hematological malignancies, transplants or AIDS, can be life-threatening and happens almost always as reactivations of previous infections or reactivation of tissue cysts in transplanted organs and in bone marrow transplants; primary infection is thought to be a rare event (Mele *et al.* 2002). The most affected site is the Central Nervous System (CNS) with clinical presentation that ranges from asymptomatic reactivation to severe disseminated disease with encephalitis, meningoencephalitis or, more commonly, tumor lesions with a mass effect. Motor syndrome, consciousness disturbances, seizures and focal signs are common manifestations that are clinically indistinguishable from other CNS complications; infection can also involve lungs and eyes. In AIDS patients *Toxoplasma*-encephalitis was an important cause of death (10-30% of cases) but thanks to the introduction of highly active antiretroviral therapy (HAART) and immune reconstitution the incidence of disease in AIDS patients is declining in developed countries (EFSA 2007).

For patients receiving a transplant of a solid organ, especially heart (a predilection site for *T. gondii*) the risk of toxoplasmosis can be reduced by serological screening of the donor and the recipient and if necessary by a prophylactic treatment (EFSA 2007) with cotrimazole.

Prenatal *Toxoplasma* infection and congenital toxoplasmosis (CT)

Immunocompetent women that contract *T. gondii* during pregnancy can transmit the infection to the foetus. The most likely mechanism of the vertical transmission is that tachyzoites may invade placenta during temporary parasitemia of the mother, replicate within cells and some of these may cross the placenta and enter the foetal circulation or foetal tissues (Remington

and Desmonts 1990). While the risk of intrauterine infection of the foetus increases during pregnancy, the effects on the foetus are more severe if transmission occurs at an early stage of pregnancy. According to the "Systematic Review on Congenital Toxoplasmosis" study group (Thiebaut *et al.* 2007), the transmission occurs in about 15% of women infected at 13 weeks of pregnancy, 44% at 26 weeks and 71% at 36 weeks; overall, about a third of infected mothers give birth to an infant with toxoplasmosis. At early stages of pregnancy, about 10% of prenatal infections result in abortion or neonatal death due to encephalomyelitis (Remington and Desmonts 1990); up to 10% of congenitally infected newborns show the classical triad of symptoms, i.e. retinochoroiditis, intracranial calcifications and hydrocephalus. About 12-16% of these newborns die from the disease and the surviving ones can suffer from mental retardation to other neurological deficiencies (Tenter *et al.* 2000). On the contrary, infants infected during late stages of pregnancy are asymptomatic at birth but they may develop clinical symptoms later in life, e.g. eye (retinochoroiditis, strabismus and blindness), CNS (convulsion, mental retardation) or ear (deafness) deficiencies.
In immunocompetent women that acquire primary *T. gondii* infection 4-6 months before conception or earlier, protective immunity usually prevent vertical transmission to the foetus; on the contrary, immunocompromised pregnant women previously infected can transmit congenitally the parasite.

Ocular toxoplasmosis (OT)

Chorioretinitis is the most common clinical manifestation of ocular toxoplasmosis: the classic signs of infection are retinal scars and white-appearing lesions often associated with vitritis (Pfaff *et al.* 2013). The presence of *T. gondii* in eye tissues was not considered to be a threat to health in immunocompetent individuals as ocular toxoplasmosis (OT) was commonly supposed to affect exclusively congenitally infected patients. In the last decade, OT following acquired infections has been frequently documented; interestingly multi-center and retrospective studies of clinical cases of ocular toxoplasmosis revealed that ocular toxoplasmosis was the result of postnatal infection in at least two thirds of patients, when it was possible to determine the origin of the infection, (Gilbert and Stanford 2000; Delair *et al.* 2008). Due to the difficulty to differentiate with absolute certainty between congenital and acquired infection in case of clinical OT, the percentage of acquired cases is probably underestimated.

Ocular toxoplasmosis (OT) is reported to be more common in South and Central America, the Caribbean and parts of tropical Africa compared with Europe and Northern America. Ocular disease in South America is more severe than in other continents with lesions that are larger, more numerous, more recurrent and more likely to damage vision due to the presence of extremely virulent atypical genotypes of the parasite. OT in South America is a leading cause of blindness in the population, both in children and in adults (Ajzenberg 2011; Petersen *et al.* 2012).

Further research on the role of *Toxoplasma* strains as well as basic studies on parasite virulence is needed to explain why OT is so severe in some countries, such as Brazil. With the availability of ocular fluid specimens obtained for diagnostic purposes and the development

of advanced proteomic techniques, a biomarker fingerprint that is unique for an eye with toxoplasmosis may become available; it is hoped that such a biomarker analysis may also be able to distinguish between acquired versus congenital disease (Kijlstra and Petersen 2013).

Latent toxoplasmosis

One of the most important scientific finding of the past 20 years has been the evidence of an association between latent toxoplasmosis and severe psychiatric and neurological diseases, in particular schizophrenia; the increased prevalence of toxoplasmosis in schizophrenics has been reported in more than 50 studies and/reviews (Torrey *et al.* 2007; Torrey *et al.* 2012). A strong proof of the causative role played by *T. gondii* comes from a recent magnetic resonance imaging (MRI) study showing that in a subpopulation of *Toxoplasma* infected patients was present a decreased density of grey matter in particular parts of the brain which was not present in *Toxoplasma*-free schizophrenics (Horacek *et al.* 2012). Additional confirmation of this relation comes from a recent study which proved that having antibodies against *T. gondii* resulted a risk factor for the development of schizophrenia (odds ratio=2.73) (Torrey *et al.* 2012). The major responsible for the association between toxoplasmosis and schizophrenia seems to be dopamine, a neurotransmitter which is usually found at high levels in schizophrenic patients. In brains of *Toxoplasma*-infected patients, dopamine seems to be produced by brain cells responding to interleukins produced as a consequence of *T. gondii* infection; furthermore, other studies showed that *Toxoplasma* can produce and express in tissue cysts the key enzyme for the synthesis of dopamine, releasing it into surrounding tissues (Gaskell *et al.* 2009; Prandovszky *et al.* 2011; Flegr 2013).

Latent toxoplasmosis has been demonstrated to cause a wide range of behavioral changes in humans and animals, in particular rodents. These alterations are named "parasite manipulation" in animals or "parasite constraint" in humans (Webster 2001); particularly in rodents which are intermediate hosts predated by cats, *Toxoplasma* alter host behavior for its benefit, prolonging their reaction times and changing their native fear of the odor of cats into an attraction. In various independent studies involving infected humans, the same behavioral changes, such as the prolongation of reaction times, have been reported also in humans and thought to increase the probability of traffic or work accidents in this patients (Flegr *et al.* 2002; Yereli *et al.* 2006; Flegr *et al.* 2009). Significant high levels of anti-*T.gondii* antibodies have been also reported in individuals with obsessive-compulsive disorders, depression and anxiety (Dalimi and Abdoli 2012); in particular depression is likely to be responsible for the increased risk of suicides in psychiatric patients with high levels of anti-*Toxoplasma* antibodies (Okusaga *et al.* 2011). Nevertheless, a correlation study showed that the incidence of suicide in Europe correlates with prevalence of toxoplasmosis in any particular country (Flegr 2013).

In the end, results of many other studies suggest that latent toxoplasmosis could play an important role also in bipolar disorders (Pearce *et al.* 2012), personality disorders (Hinze-Selch *et al.* 2010), Parkinson's disease (Miman *et al.* 2010a), Alzheimer's disease (Kusbeci *et al.* 2011), obsessive-compulsive disorders (Miman *et al.* 2010b), cryptic epilepsy (Yazar *et al.* 2003), autism and even brain tumors (Flegr 2013).

POSSIBLE SOURCES OF ACQUIRED INFECTION

Humans can act as intermediate hosts of *T.gondii*; being omnivores, they can acquire the infection by the ingestion of either tissue cysts present in meat or sporulated oocysts contaminating food or water. Other routes of infection are reported in literature, but they are considered less common, such as the inhalation or inoculation of tachyzoites and the accidental ingestion of tachyzoites eliminated in secretions and excreta of infected animals, e.g. placentas from infected abortions (Jones *et al.* 2009). Since acquired infection with *T. gondii* is increasingly being recognized as a severe cause of acute ocular toxoplasmosis in immunocompetent adults, the focus of education for the prevention of the infection must be expanded to human infections in general (Peterson *et al.* 2010).

Meatborne infection

In Europe, three large case-control studies have pinpointed uncooked meat as the most important risk factor for pregnant women (Kapperud *et al*., 1996; Baril *et al*., 1999; Cook *et al*., 2000). The largest study conducted by Cook *et al.* (2000) estimated that consumption of not-well-cooked meat was the cause of infection in 30–60% of pregnant women with acute toxoplasmosis.

Due to the varying organotropism of *T. gondii* in different animal species, the number of tissue cysts produced in a certain organ varies greatly among food producing animals (Dubey and Jones 2008); however, tissue cysts are generally more prevalent in muscle and nervous system compared to internal organs, thus the consumption of e.g. livers and kydneys are considered less likely sources of infection (Tenter *et al* 2000). In response to natural infection, most farm animals that are seropositive for *T. gondii* have been shown to *harbor* infectious parasites in their meat, with the exception of beef (Tenter *et al.,* 2000; Kijlstra and Jongert 2008); 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).

All the case control studies cited above (Kapperud *et al*., 1996; Baril *et al*., 1999; Cook *et al*., 2000) have identified the consumption of mutton/lamb meat as a highly significant risk factor for contracting *T. gondii* in pregnant women. Sheep are considered particularly vulnerable to *T. gondii* infection as they are usually kept on pastures contaminated with sporulated oocysts; in relation to the environmental burden of oocysts, seroprevalence in sheep can exceed 90% (Tenter *et al.* 2000). A recent PCR based study on sheep meat demonstrated that serological screening can be used to estimate the number of animals that *harbor* tissue cysts and that, therefore, seropositive sheep can pose a risk to their consumers (Opsteegh *et al*. 2010a). Raw or undercooked lamb meat is considered a delicacy in certain countries such as France and it is considered an important source of infection in that country (AFSSA, 2005); the consumption of raw/undercooked lamb was identified as a statistically significant risk factor (OR 3.13) in the European multicentre study conducted in 2000 by Cook and colleagues. A recent study by Dubey and colleagues (2008) found 77.9% (53/68) lamb hearts positive in bioassay in mice. However, as the probability of acquiring the infection increases with the age of the animals, adult sheep are more likely to be seropositive than lambs (Dubey *et al*. 2009b); however, mutton probably poses a smaller risk of infection to the consumer than lamb meat as it is often consumed well cooked (Kijlstra and Jongert 2008).

One outbreak of acute toxoplasmosis has been described following the consumption of undercooked pork (Choi *et al.* 1997). Pork meat from breeder pigs is no longer considered as the major source of acquired toxoplasmosis since, with the introduction of intensive farming, the infection has been practically eliminated in many countries (Kijlstra *et al*., 2004; van der Giessen *et al*., 2007; Jones and Dubey 2012). However, the animal friendly production systems is becoming more and more popular and this may cause a re-emergence of pork meat as an infectious meat source (Kijlstra *et al.*, 2004; van der Giessen *et al*., 2007; Hill *et al.* 2010). In fact, in a recent study viable *T. gondii* has been isolated from 17 of 33 organically raised pigs from Michigan (Dubey *et al*. 2012).

The ingestion of beef has not been considered important in the epidemiology of *T. gondii* because cattle are not a good host for this parasite (Dubey and Beattie 1988). However, only relatively small amounts of beef have been tested for viable *T. gondii* parasites with negative results. Multi-center epidemiologic studies have demonstrated that the consumption of undercooked beef can be considered a risk factor for *T. gondii* infection in humans (Baril *et al.* 1999; Cook *et al.* 2000) because beef is often mixed with other types of meat in minced meat preparations that are consumed raw or undercooked (Kijlstra and Jongert 2008).

Similarly to pigs, meat from indoor raised chicken has been demonstrated not to pose risk for the consumers, as viable *T. gondii* has not been isolated by bioassay from any of the 2094 chicken meat samples tested from retail stores in the United States (Dubey *et al*. 2005). On the contrary, the prevalence of *T. gondii* in free-range chickens is very high (United States, 17%–100%) and the recent trend of consumers demanding meat from organically grown free range poultry will probably increase the risk of acquiring infection from poultry (Jones and Dubey 2012).

The presence of cysts in edible tissues from horses has been demonstrated (Al-Kjalidi and Dubey 1979). The role of horses as a source of *T. gondii* infection depends on regional preferences for horse meat, the preparation method and the seroprevalence of horses used for consumption (Gill, 2005; Kijlstra and Jongert 2008). Raw or undercooked horse meat is frequently consumed in countries such as Belgium, Italy, France and Japan (Gill, 2005). In France, severe human toxoplasmosis has been epidemiologically linked to ingestion of horse meat imported from Canada and Brazil (Pomares *et al.* 2011) and viable *T. gondii* has been isolated from US horses slaughtered for export (Al-Khalidi and Dubey 1979).

The consumption of goat's meat is very popular in developing countries. In a recent study, the seroprevalence of *T. gondii* antibodies in goat meat destined for human consumption in the United States was found to be 53.4% (Dubey *et al*. 2005).

An emerging risk is the increasing popularity of game meat such as roe deer, wild boar or kangaroo, as a study from the European Food Safety Authority has recently estimated that approximately half of the game produced in Europe may be seropositive for *T. gondii* (EFSA, 2007). Epidemiological studies and several outbreaks have identified the handling and consumption of raw or undercooked game as a source of toxoplasmosis (Cook *et al*., 2000; Ross *et al*., 2001; Carme *et al.*, 2002; Dubey and Jones, 2008). Due to high demand in certain countries there is an intense trade of both farmed and natural game, partly via frozen import or, more risky for acquiring the infection, fresh meat imports (Reinken, 1998). Earlier studies have indicated that kangaroos are highly susceptible to *T. gondii* infection and kangaroo meat

was implicated as the cause of an outbreak of toxoplasmosis in Australia in 1994 (Kijlstra and Jongert 2008).

The relative importance of risk factors and the type of meat associated with it varied among different countries (Cook *et al.* 2000). For example, in France consumption of undercooked beef was a stronger risk factor than consumption of undercooked lamb (Baril *et al.* 1999), in Norway consumption of undercooked lamb was a stronger risk factor than consumption of undercooked pork (Kapperud *et al.* 1996), whereas in Poland consumption of undercooked pork was the principle risk factor identified in the study (Paul 1998). These findings may reflect differences in eating habits of consumers or different prevalences of infection in meat-producing animals in these regions.

Production of *Toxoplasma* free meat is thus directly related to the application of specific actions both on farms and animal's level, i.e. the adoption of proper hygienic condition and prophilactic measures for the prevention of infection in food-producing animals (e.g. rodents controls, application of intensive production system, storage of feed stock, use of tank waters, animal vaccination) together with an effective control of the cat population (keep cats indoor, neuterization, vaccination) (Tenter *et al.* 2000; Kijlstra and Jongert 2008). Since such measures might require in some cases the eradication of breeding habits or the total renovation of the farms, they are difficulty accepted by farmers in absence of a National Eradication program of toxoplasmosis. At the same time, the monitoring of the meat at the slaughterhouse is likewise difficult, as there is no general agreement among the tests to be used; no standardized reference sera or other reference materials are available and there is no laboratory certification program (Kijlstra and Jongert 2008). Therefore, the most reliable method to assure non-infectivity of meat is represented by pre-kitchen procedures involving a combination of treatments (Lopez *et al.* 2000; Leroy *et al*. 2005). Freezing for 2 days at -20 °C is sufficient to inactivate the parasite (Kijlstra and Jongert 2008). In general, freezing can inactivate the *T. gondii* tissue cysts, but proper timing and temperature are necessary for a 100% parasite killing efficiency, as for example tissue cysts remain viable up to 22 days at -1 and -3.9 °C and 11 days at -6.7 °C (Kotula *et al.,* 1991). Also heating could inactivate tissue cysts. At 50 °C it takes 1 h to inactivate tissue cysts whereas immediate destruction takes place when the internal temperature of meat reaches a temperature of 67 °C (Dubey *et al*., 1990a). Cooking infected meat in a microwave does not guarantee killing of the parasite, most probably due to uneven heating (Lunden and Uggla, 1992). Numerous studies have addressed the effect of curing procedures such as salting, smoking or fermentation on tissue cyst survival. Curing of lamb meat with salt and sugar for 64 h at 4 °C or smoking salt-injected meat at temperatures not exceeding 50 °C for 24–28 h were effective in killing *T. gondii* (Lunden and Uggla, 1992). Studies by Dubey have shown that 6% NaCl can kill the isolated tissue cyst, independent of the temperature used (Dubey, 1997a). Isolated tissue cysts can survive for 56 days in a solution of 0.85% salt, 49 days at 2% and 21 days at 3.3% (Dubey, 1997). More recent data have shown that injection of >2% NaCl and/or >1.4% lactate salt solutions into experimentally infected pig meat could kill the parasite whereas a 1% NaCl solution provided variable results and the addition of tripolyphosphate salts had no effect on parasite viability (Hill *et al*., 2004, 2006). PCR studies have demonstrated the presence of *T. gondii* DNA in commercially available swine sausages but bio-assays did not detect the

presence of viable tissue cysts in these samples (de Oliveira Mendonca *et al*., 2004). However, there is too much variability in these procedures to make a safety recommendation on salts, cured or smoked meat preparation (Jones and Dubey 2012). Also irradiation at 0.4–0.7 kGy or high-pressure processing at 300–400 MPa can inactivate *T. gondii* tissue cysts in meat; however, the effects of irradiation and of high pressure treatment on color and texture have limited consumer acceptance (Kijlstra and Jongert 2008; Jones and Dubey 2012).

Water and contaminated food and soil

The percentage of *T. gondii* infections acquired by tissue cysts versus oocysts is not known; however, humans may frequently become infected by ingesting or inhaling the *T. gondii* oocysts directly. Recently Hill and colleagues (2011) have recognized the oocyst-specific antigen and Boyer and colleagues (20011) have used it for testing serum samples of mothers of congenitally infected infants in the US in order to differentiate oocyst- versus tissue cyst–induced infection. The study demonstrated that 78% of these women (59/76) had oocysts-acquired infections.

The oocysts burden in the terrestrial and aquatic environments is influenced by 3 factors: the oocysts loading, the oocysts survival and their transport overland and in waterways (VanWormer *et al.* 2013). The oocyst loading depends on the felid distribution, availability of prey or other food resources and duration and quantity of oocysts shedding (VanWormer *et al.* 2013). Studies have reported that approximately 1% of cats are shedding oocysts at any given time and that they excrete oocysts for a median of 8 days with a total of up to 55 million oocysts per day (Dabritz and Conrad 2010). The total number of oocysts shed by a single cat varies widely from 3 to 810 million; in USA the annual quantity of oocysts deposited on soil was estimated at 779-1328 oocysts/m^2 (Dabritz and Conrad 2010). The oocysts are remarkably stable, especially if they are deposited in shady, moist, and temperate conditions. Oocysts maintained experimentally at 48°C in seawater or freshwater remained viable for 24 and 54 months, respectively (Dubey 1998c; Lindsay and Dubey 2009); oocysts also survived for over a year in vials of 2% sulfuric acid at 48°C (Frenkel and Dubey 1972) and up to 28 days of freezing at -21°C (Frenkel and Dubey 1973). Transport and potential accumulation of *T. gondii* in soil and water is determined by the combination of felid defecation behavior, environmental characteristics and surface properties of oocysts. With felid defecation behavior it is intended the habits or not to bury the feces and the location of defecation; in fact if feces are buried under the soil, fewer are mobilized in the environment whereas if feces are left exposed, as it is reported in feral cat colonies, they are more likely to be transported (Ruiz and Frenkel 1980). Location of defecation can enhance oocysts transport and the likelihood for humans to acquire the infection from the environment; felids, in particular feral and wild, have been reported to defecate near waterways, running streams and/or shallow ponds, enhancing the transport of oocysts into aquatic environments (VanWormer *et al*. 2013). In 1979, an outbreak occurred in US Army soldiers during a 3-week training exercise in Panama (Benenson *et al*., 1982) and the source of infection was recognized to be unfiltered, iodine treated water from streams contaminated by jungle cats. Furthermore, cats select places with loose soil in order to better cover their feces, as gardens or children's play areas (sandboxes or sandpiles) (Torrey and Yolken 2013). A Japanese study quantified the frequency of cat defecations in three uncovered urban sandboxes by monitoring the places for almost 5

months with night lights and camcorders and identified a total of 961 cat defecations (Uga *et al.* 1996). Using this number, Torrey and colleagues (2013) estimated that the hypothetical accumulation of *T. gondii* oocysts per square foot in each sandbox was between 55.184-1.677.852 oocysts. The substrate, the vegetation and the climate at the defecation sites also affect oocyts transport: while permeable soils facilitate vertical percolation with a reduced number of oocysts mobilized, asphalt and concrete surfaces can lead to increased mobilization of pathogens (VanWormer *et al.* 2013).

The transport of oocysts into aquatic systems is also directly influenced by precipitation patterns, in terms of duration, intensity and frequency. The role played in oocysts transport by excessive rainfall and runoff into water reservoir was demonstrated in a large toxoplasmosis outbreak in the Victoria area of British Columbia in 1995 (Bowie *et al*., 1997; Burnett *et al*., 1998); 100 persons reported acute toxoplasmosis after drinking unfiltered water from a reservoir of the Greater Victoria area that had been contaminated by *T. gondii* oocysts following high precipitations. Similarly, in Santa Isabel do Ivai, Brazil, waterborne *T. gondii* was thought to be responsible for an outbreak involving 155 persons served by an underground tank reservoir delivering unfiltered water (de Moura *et al*., 2006). It was supposed that runoff contaminated with *T. gondii* oocysts entered the reservoir because a cat with a litter of kittens lived on top of the underground tank reservoir that was not adequately sealed.

The oocysts burden in both terrestrial and aquatic environment is also influenced by the way of disposal of the cat litter boxes by cat owners; Dabritz and colleagues (2006) reported that among Californian cat owners it was a common habit to dump the contents of cat litter boxes on the property or to flush it down the toilet. This last practice was recognized later as one of the causes of *T. gondii* oocysts contamination of the Morro Bay region that was causing death among sea otters. In order to prevent soil or water contamination, cat feces should not be flushed into the municipal sanitation system, but rather bagged and disposed in trash. Furthermore, feces should not be left for more than one day in the litter box in order to prevent their sporulation (Kapperud *et al.* 1996).

To reduce the risk of infection from soil some hygienic measures should be adopted by pregnant women and humans in general, i.e. wearing gloves during gardening, (Torrey *et al.* 2013) or avoid eating unwashed fruits or vegetables (Kapperud *et al*. 1996). A recent study investigated the effectiveness of two soaking solutions in inactivating oocysts from experimentally contaminated lettuce samples (El-Tras *et al*. 2011). Either soaking the vegetables in water at 65°C or in vinegar solutions at 45°C for 1 minute was efficient to inactivate *T. gondi* sporulated oocysts. The acid pH of the vinegar solution (pH 4.2) neutralizes oocysts charge and lead to flocculation and subsequent accumulation of *T. gondiii* oocysts. Studies have shown that also cockroaches and flies may carry oocysts from cat feces to unprotected food (Wallace 1971, 1972) and that *T. gondii* oocysts may even infect humans who pet dogs that have rolled in cat feces (Frenkel and Parker 1996). In that cases cover the food and wash accurately hands are hygienic measures of fundamental importance.

With regard to water, as the level of contamination is not usually known, it should be avoided drinking unfiltered water. Toxoplasmosis outbreaks due to the consumption of unfiltered water occurred in many areas of the world, in particular where surface water was used for

human consumption without any purification, including Brazil (Heukelbach *et al.,* 2007; Cavalcante *et al*., 2006; Boia *et al*., 2008), India (Palanisamy *et al*., 2006; Hall *et al*., 1999), Poland (Jones and Dubey 2010), Turkey (Ertug *et al.,* 2005), Granada (Asthana *et al*., 2006), Taiwan (Hung *et al*., 2007), and China (Lin *et al*., 2008). Guidelines for prevention of *T. gondii* infection (Lopez *et al.* 2000; Leroy *et al.* 2005) suggest that, if untreated surface water represents the main source of drinking water, filtering (absolute 1 lm filter) or boiling will help eliminate *T. gondii* oocysts. Chlorine treatment will not inactivate *T. gondii,* but a long exposure (3 hours) to tincture of iodine (2%) will be effective. It is also possible that ingestion of inadequately treated water or inadvertent ingestion of recreational water from streams, lakes, or ponds may be involved in some human infections (Jones and Dubey 2010). Shellfish and other filter feeders consumed raw can be considered another potential source of infection as they have been experimentally shown capable of retain and filtrate oocysts from water (Arkush *et al.* 2003; Lindsay *et al*. 2004). Travelers should also avoid drinking untreated or inadequately treated water and be aware that in some areas of the world municipal water in not adequately treated or filtered. When possible, access of cats to the areas around drinking water tanks or reservoirs should be limited.

HUMANS TOXOPLASMOSIS IN ITALY

Italy lacks a nationwide epidemiological surveillance system for toxoplasmosis (Bénard *et al.* 2008; Mosti *et al.* 2013); serological tests for toxoplasmosis are performed as a routine prenatal screening during pregnancy, with a first evaluation at the 13th week, followed by monthly testing of pregnant women who are seronegative for toxoplasmosis (total 5–7 tests), as indicated by Italian legislation (Pinto *et al.* 2012). Surveillance on congenital toxoplasmosis (CT) is only confined to a regional programme in the Campania region, which has been running since 1997 with the aim to monitor the burden of CT through the monitoring of IgG levels in living newborn babies (Stagni *et al.* 2009). Therefore, epidemiological data about the seroprevalence of *T. gondii* in the general Italian population are not available whereas data about seroprevalence in pregnant women are sparse and limited to the area where the results of the routine screening have been statistically analyzed (Buffolano *et al.* 1996; De Paschale *et al.* 2008; Thaller *et al.* 2011; Mosti *et al.* 2013).

Recently, a retrospective studies conducted in the Tuscany region (Carrara and Lucca district) investigated the seroprevalence for *T. gondii* in individuals referring to two hospitals for serological tests; all the serum samples considered have been tested for both IgG and IgM anti-*Toxoplasma* antibodies (Pinto *et al.* 2012). The total sample size was 10,352, with the majority (92.6%) of individuals that were female, mostly (95.2%) of childbearing age. The high rate of women that underwent to serological analysis indicates both a good awareness of the risk posed to pregnant women by toxoplasmosis and an easy access to prenatal screening for women resident in that area. Overall, a total of 2,216 subjects, corresponding to 21.4% of the sample, were found to be positive for IgG antibodies and among women in reproductive age (9,132), the prevalence detected was 19.4% (1,766). The *Toxoplasma* IgG seropositivity showed a significant positive correlation ($p<0.0001$) with age: the very low prevalence was observed in younger age groups and markedly increased in older people. The rising trend with age reflects the continuing risk of exposure and infection in adult life.

Another retrospective study, conducted in the same area estimated the seroprevalence of *T. gondii* infection in both general population and women of reproductive age (13 177 subjects) over a 4-year period (2007-2010) in order to evaluate the trend of exposure to the parasite (Mosti *et al*. 2013). The data showed a decline in IgG prevalence from 31% in 2007 to 24.2% in 2010. More interestingly, a significant ($p < 0.0001$) reduction over time of IgG seroprevalence was also observed in females of the 16-45 years age group, i.e. from 30.2% in 2007 to 23.6% in 2010. This decline in women of reproductive age has become evident in Italy since 2001: seroprevalence shifted from 48.5% in 1995 (Valcavi *et al*. 1995) to 31.4% in 2001 (Vezzo *et al.* 2001) and to 21.4% in 2005 (De Paschale *et al.* 2006). This finding is also in agreement with the decreasing trend reported in several EU countries (Berger *et al.* 2009; Stricker *et al.* 2009; Hofhuis *et al.* 2010) as well as in the USA (Jones *et al*. 2007). The decline has been attributed to lower prevalence of *Toxoplasma* cysts in meat, as a result of modern farming systems, in combination with an increased use of frozen meat by consumers (Tenter *et al*., 2000; Diza *et al*., 2005; Kijlstra and Jongert 2008; Torrey and Yolken 2013).

Risk factors for toxoplasmosis during pregnancy have been assessed in two studies conducted in two hospitals of Naples and Rome (Buffolano *et al.* 1996; Thaller *et al*. 2011). In the first study, the 12% (42/3518) of investigated women resulted recently infected (IgM+IgG

positive) and the 39% (380/3518) previously infected (IgG positive and IgM negative). Eating cured pork, raw meat and gardening resulted significantly associated with IgM positivity. Tissue cysts are reported to be inactivated after curing meat with more than 10% added salt and sucrose and at a temperature of conservation between 18°C and 22°C (Lunden and Uggla 1992); however homemade cured pork products prepared following traditional recipes usually contain low concentrated salt solutions (<1%), are stored at less than 12°C and are often eaten within 10 days of slaughter (Buffolano *et al.* 1996). These conditions do not assure the inactivation of tissue cysts in the meat. Furthermore, these products are often prepared with meat from farm animals kept outdoors together with internal organs, as livers or kidney, which contain tissue cysts. The consumption of homemade cured pork preparation, together with gardening and eating raw meat, resulted to be the most likely source of infection for primarily infected (4.8%; 113/2356) pregnant women also in the study conducted in the Rome district (Thaller *et al.* 2011). These findings clearly suggest that if susceptible pregnant women avoid eating cured pork or raw meat, the risk of primary infection could be reduced. It is, however, concerning that these habits are still well-established among Italian pregnant women as they reflect an inappropriate divulgation of the epidemiology of the disease and of the hygienic measures necessary for the prevention of infection. Guidelines for prevention of infection with *T. gondii* have been developed by the Centers for Disease Control and Prevention in the USA (Lopez *et al.* 2000) and by the EUROTOXO project (Leroy *et al.* 2005). In relation to the serious consequences of both congenital and acquired toxoplamosis, a widespread diffusion of this information should be provided by the National Health System throughout the Country, with particular attention to the rural areas and to women in reproductive age. Furthermore, extension of the CT monitoring system, which is solely running in the Campania Region, across the country, could help to define a future strategy for the prevention of CT (Stagni *et al.* 2009).

LABORATORY DIAGNOSTIC

In relation to the peculiarity of its life cycle, *T. gondii* can be detected by direct and indirect methods in almost all warm blooded animals (including humans), in tissues destined to human consumption and in environmental samples. The substrates of detection vary from serum samples, cat feces, fluids present in the thoracic cavity of aborted fetuses, tissues from aborted fetuses, milk, extracts of infected tissues, liquid drained from muscles after cycles of freezing and thawing and samples of soil or water.

DIAGNOSIS OF TOXOPLASMOSIS IN ANIMALS

T gondii infection in animals can be diagnosed directly by Polymerase Chain Reaction (PCR), mice or cell culture isolation, histology and indirectly by serological methods. Direct methods are considered too expensive and inadequate for routine application, as they are time consuming and they do not often lead to a rapid and sensitive diagnosis. Indirect serological tests are considered more feasible and they are largely used for surveillance and monitoring of *Toxoplasma* in animals, though standardized methods are still not available.

DIRECT METHODS

Isolation of the agent

Direct isolation of live parasites is still considered the "gold standard" method for diagnosis of toxoplasmosis. Attempts of isolation of *T. gondii* are usually performed from aborted ovine and caprine fetuses and fetal membranes by inoculation of laboratory mice. Tissues samples are homogenized and then intraperitoneally inoculated in mice; in case of very virulent strains, tachyzoites can be detected in peritoneal fluids 3-4 day p.i, whereas the detection of cysts in mice brain by histological examination requires 40 days (Piergili-Fioretti 2004). Failure to demonstrate tissue cysts does not rule out a positive diagnosis, so serum from infected mice may be analyzed for the presence of antibodies against *Toxoplasma*; if this analysis is also negative, the infection is unlikely (OIE, 2008). Although mice bioassays are still considered the definitive method of diagnosis of *T. gondii* (Gutierrez *et al.* 2010), this method is labour intensive, time consuming, expensive, relies upon the submission of fresh material to the diagnostic laboratory and is generally impractical in diagnostic situations.

Tissue sections

Tachyzoites and tissue cysts can be found in tissue sections and organs fixed and stained with hematoxylin and eosin and immunohystochemistry. In animals that die with acute toxoplasmosis, focal mononuclear inflammation, with or without focal necrosis, may be seen in a number of tissues, including the liver, heart and lungs. In cases of abortion and stillbirth in sheep and goats, affected placental cotyledons typically contain large foci of coagulative necrosis that may have become mineralised with time (OIE, 2008). Any associated inflammation is characteristically slight and nonsuppurative. Well preserved samples of

placental cotyledons may show moderate edema of the mesenchyme of the fetal villi with a diffuse hypercellularity due to the presence of large mononuclear cells. Sometimes small numbers of intracellular and extracellular toxoplasms are visible, usually on the periphery of a necrotic area or in a villus that is in the early stages of infection. The *Toxoplasma* tachyzoites appear ovoid, 2–6 µm long, with nuclei that are moderately basophilic and located centrally or towards the posterior end; primary and secondary lesions may also develop in the fetal brain (OIE, 2008). Histological examinations of biological samples show a lack of reliability since the parasite is not evenly distributed in tissues and animals can be infected with few parasites (Piergili Fioretti, 2004; Cenci-Goga *et al.* 2011).

PCR-based Methods

Parasite DNA can be extracted and purified from several tissues, including tissues from aborted fetuses, placenta, the central nervous system, blood and fluids. The specificity of this test is almost 100%, but the difficulty to extract DNA and concentrate large sample quantities results in limited sensitivity (Alfonso *et al*., 2009). The main target regions are the B1 repetitive sequence (Burg *et al.* 1989), the P30 (SAG1) gene (Savva *et al.* 1990), 18S ribosomal RNA (rRNA) (Ellis, 1998) and the 529 bp fragment (Homan *et al*. 2000). The sensitivity of the PCR is dependent on the copy number of the target sequence: P30 is present in 1 copy, B1 in 35 copies, rRNA in 110 repeat units and the 529 bp fragment is repeated 200-300 fold. In order to improve the detection limit, both B1 gene and 18S ribosomal RNA are often amplified by nested PCR (Homan *et al.* 2000). Real time-PCR is a very sensitive and rapid technique that allows direct detection and quantification of the parasite in biological samples. Of the PCR targets described for *T. gondii*, the 35-copy number B1 gene and the 18S ribosomal RNA have been the most widely used for *T. gondii* diagnosis in ovine and caprine aborted fetuses and tissues and placenta has been identified as the main target organ of *T. gondii* (Hurtado *et al.* 2001; Masala *et al.* 2003, 2007; Pereira-Bueno *et al.* 2004; Ahmed *et al.* 2008; de Moraes *et al.* 2011; Moreno *et al.* 2012). Recently, real-time PCRs have emerged as reliable methods of *T. gondii* diagnosis in animals (Switaj *et al.*, 2005); this is a very sensitive and rapid technique that allows direct detection and quantification of the parasite in biological samples. Gutierrez and colleagues (2010) used a quantitative real time PCR based on 529 bp elements for detecting *T. gondii* DNA in aborted fetuses and placenta from experimentally infected sheep. This assay leaded to the identification of infected fetuses even when typical histological lesions were not detected or in cases where the fetal membranes were not available for submission to the laboratory. The placenta was confirmed to be the target sample, resulting positive from the earliest time points and having the highest levels of infection; fetal brain and lung resulted the samples of choice for detection of ovine toxoplasmosis when placenta was not available. That real-time PCR provided a valuable addition to the methods currently available for routine diagnosis of ovine abortions.

Coprological Tests

Coprological tests for the detection of oocysts in feces from naturally infected cats are of little significance because of the short patency (15 days). However, fecal samples from experimentally infected cats, e.g. following bioassay, can be processed by common flotation methods and by microscope examination of the oocysts (Mancianti *et al.* 2010).

INDIRECT METHODS

In absence of a "gold standard" serological technique for detecting anti-*T.gondii* antibodies in animals, a broad range of serological methods can be used for testing animals' sera. The use of one technique rather than another is influenced by different factors, such as the animal species to be investigated, the number of samples to be tested, the experience of lab technicians and the availability of specific instruments. The major part of *T. gondii* serological screening published in literature involved sheep and pigs, as they are food producing animals where tissue cysts are most frequently observed (Tenter *et al.* 2000). In particular for sheep, serological titre has been demonstrated strongly related to the probability of the presence of tissue cysts in meat samples (Opsteegh *et al.* 2010a); however, standardized validated serological tests are not currently available for correlating seropositivity to the presence of infective meat. When performing a serological survey, the use of serological methods with high performance characteristics is of paramount importance in order to achieve a correct identification of infected and non infected animals.

The Sabin-Feldman dye test (DT) is considered the most specific test for toxoplasmosis, but it is rarely used for the diagnosis of toxoplasmosis in animals in relation to its technical difficulty and to the use of live virulent tachyzoites as antigen (Dubey 1997b). Therefore, a modified agglutination test (MAT) was described by Dubey and Desmont (1987) using as antigen tachyzoites inactivated with formalin, instead of live ones. The limitation of MAT observed by Minho and colleagues in Brazil (2004) in serological screenings were the long incubation time (overnight) and the cost of the antigen, as MAT employs a large amount of formalin-treated whole tachyzoites. However, it should be considered that with MAT could be tested a great number of sera (96-wells plate) and the reading of the results can be performed directly by visual inspection without the need of specific instruments. When MAT has been compared to DT for detection of *T. gondii* infection in naturally infected sows, it resulted more sensitive (Se 82.9% vs 54.4%) than DT but equally specific (90.2% vs 90.8%)(Dubey *et al.* 1995). Again, MAT has been evaluated, together with other serological methods, in comparison with DT for the diagnosis of *T. gondii* infection in sheep, revealing the highest Se (96%)(Shaapan *et al.* 2008). Since a commercial MAT kit is available (Toxo-Screen DA, bioMérieux, France), this is one of the most commonly test used today for pets and wild animals because it is easy, safe and does not require species-specific reagents (Cenci-Goga *et al.* 2011). The Se and Sp values claimed by the manufacturer and obtained by comparison with DT are 96.22% and 98.8%, respectively (Toxo-Screen DA manufacturer's instruction, bioMérieux, France).

Indirect Immunofluorescence Test (IFAT) is a simple and widely used method. Whole killed *Toxoplasma* tachyzoites are first incubated with diluted test serum, then the specie-specific fluorescent- labeled antibodies is added and finally the results are viewed with a fluorescence microscope. According to what reported in the OIE Terrestrial Manual (2008), a serum is defined positive when at least 80% of tachyzoites are surrounded by an unbroken band of green fluorescence; however, a non specific polar fluorescence in tachyzoites may affect the reading results. In IFAT sheep and swine sera are usually screened at 1/64 and 1/16 dilution. An advantage of IFAT is that not requires specific trained lab technicians, can be performed for several dilutions of sera in order to determine the end-point titre and can also detect IgM.

However, differences in the performances of the test (Se and Sp) may depend on the *Toxoplasma* strain used and on the subjectivity in interpreting the fluorescence reaction (EFSA, 2007; Cenci-Goga *et al*. 2011); furthermore, some species-specific conjugates may be difficult to find. Different commercial IFAT kits are available; for example, Mega Cor (Diagnostic, Horbranz, Austria) claims both Se and Sp > 99% for its product, but Cenci-Goga and colleagues (2013) could not find confirmation of this when they tested sera from naturally infected sheep. Macri and colleagues (2009) compared the performances of a commercial MAT kit for the detection of *Toxoplasma* specific IgG antibodies in dog and cat sera using a commercial IFAT kit as reference method. MAT showed an "almost perfect" agreement with IFAT in detecting positive and negative results in cat sera, whereas only a "substantial" agreement was observed in dog sera due to false negative results. Minho and colleagues (2004) compared IFAT and MAT for detection of antibodies against *T. gondii* in experimentally infected pigs and these tests revealed excellent agreement in detecting antibodies during the infection period.

The Enzyme-linked Immunosorbent assay (ELISA) is another test widely used for examination of animal sera. Ninety-six wells plates may be coated with antigens of various origins, e.g. soluble antigen preparation made from *Toxoplasma* RH strain tachyzoites (OIE, 2008), purified tachyzoite surface antigen (SAG) (Hosseininejad *et al.* 2009; Tzanidakis *et al.* 2012;), P-30 cuticular antigen (Berger-Schoch *et al.* 2011) and crude rhoptry *Toxoplasma* proteins (Garcia *et al*. 2006). Several ELISA kits are commercially available for detection of *T. gondii* antibodies (both IgG and IgM) in different animal species. ELISAs are suited for testing large numbers of samples, e.g. routine screening on farms or at slaughterhouse, but similarly to IFAT, require species-specific enzyme-labeled conjugate and a spectrophotometer for reading the results. Shaapan and colleagues (2008) evaluated the sensitivity and specificity of IFAT, MAT and in-house ELISA in detecting anti-*Toxoplasma* antibodies in sheep sera by comparing the results with these techniques with those of DT, considered as a reference test; MAT had the highest sensitivity (96%), followed by ELISA (90.1%) and IFAT, which demonstrated the lowest sensitivity (80.4%). On the other hand, IFAT had the highest specificity (91.4%), followed by MAT (88.9%) and ELISA (85.9%). Similarly, Garcia *et al*. (2006) evaluated performances of MAT and in-house rhoptry ELISA in experimentally infected pigs by comparison with IFAT: both tests resulted highly specific (100%) but ELISA was more sensitive (94.3%) than MAT (87%). Mainar-Jaime and Barbéran (2007) used MAT and ELISA for testing sheep sera, evidencing a very high degree of agreement between the two serological tests.

DIAGNOSIS OF HUMAN TOXOPLASMOSIS

T gondii infection can be diagnosed indirectly with serological methods and directly by PCR, isolation (mouse inoculation or cell culture) and histology. Whereas indirect serological methods are widely used in immunocompetent patients, definitive diagnosis in immunocompromised people is mostly undertaken by direct detection of the parasite. Direct demonstration of the organism (mouse inoculation, cell culture, or PCR for *T gondii* DNA) from cerebrospinal fluid, blood, and urine, and ophthalmologic testing, radiological studies, and examination of cerebrospinal fluid could assist diagnosis of congenital disease (Montoya and Liesenfeld 2004).

DIRECT METHODS

PCR can be used for detection of *T. gondii* in body fluids and tissues. In particular PCR amplification of the 35-fold repetitive B1 gene has been successfully used to diagnose congenital (Grover *et al.* 1990) ocular (Montoya *et al.* 1999), cerebral and disseminated (Brezin *et al.* 1990; Dupouy-Camet *et al.* 1993; Mele *et al.* 2002) toxoplasmosis. The use of molecular biology on amniotic fluid, fetal blood and peripheral maternal blood has revolutionized prenatal diagnosis of congenital toxoplasmosis by enabling early diagnosis (Piergili Fioretti 2004). Peripheral blood, cerebrospinal fluid, and urine should be considered for PCR examination in any newborn suspected to have congenital disease. In immunocompromised patients suspected to have localized or disseminated toxoplasmosis, performing PCR from blood (buffy coat), body fluids (including bronchoalveolar lavage or cerebrospinal, pleural, ascitic, peritoneal, or ocular fluids), bone-marrow aspirate, or tissues can be an important diagnostic aid (Montoya 2002). However, a positive result of brain tissue PCR may not differentiate between a patient with toxoplasmic encephalitis and an individual with different brain pathology but who is chronically infected (latent infection) with *T gondii* (Montoya and Liesenfeld 2004).

Attempts of *T. gondii* isolation from blood or body fluids should be undertaken in case of suspected acute infection and for strain typing; viable parasites can be isolated by mice inoculation or cell cultures. Furthermore, tachyzoites can be demonstrated in tissues section or smears of body fluid, e.g. bronchoalveolar lavage or cerebrospinal fluid, during primary acute infection or reactivation of previously acquired latent infection (Montoya and Liesenfeld 2004).

INDIRECT METHODS

The detection of IgM and IgG helps in differentiation of acute (IgM only or both IgM and IgG) or chronic infection (IgG only). In adults, IgM antibodies arise within the first week of infection, rapidly increase, and thereafter decline and disappear at highly variable rates. IgG arise within 1–2 weeks after infection and persist for the lifetime of an individual (Montoya and Liesenfeld 2004). Detection of IgG antibodies to *T gondii* should be done in pregnant women and immunocompromised patients in order to identify women at risk and patients at risk for reactivation of a latent infection, respectively. The Sabin-Feldman dye test, immunofluorescent antibody test, ELISA, IgG avidity test, and agglutination and differential agglutination test can be used for detection of IgG antibodies (Montoya and Liesenfeld 2004)..

The Sabin-Feldman dye test is the so called "gold standard" serological test for detection of anti- *T. gondii* antibodies in humans (EFSA, 2007); it is based on the inability of tachyzoites to absorb the dye when, tested with anti-*Toxoplasma* anti-serum, the immunocomplex has been formed. Both sensitivity and specificity of the test are high, but the method requires live tachyzoites, is expensive and needs specialized laboratory and equipment (Cenci-Goga *et al*. 2011). Immunoenzymatic methods (ELISA) are available for the detection of IgG and IgM and also for testing the avidity (functional affinity) of IgG antibodies in pregnant women in order to discriminate between recently acquired infection and those obtained in the more distant past (Hedman *et al.* 1989). Presence of high avidity antibodies essentially rules out a recently acquired infection (e.g. in the last past 3–4 months) whereas low avidity antibodies are indicative of an old acquired infection. For diagnosis of congenital infection in newborns, the IgM immunosorbent agglutination assay (ISAGA) is frequently used in relation to its high sensitivity and specificity (Desmonts *et al.* 1981). Detection of IgG antibodies in the newborn's serum could be attributed to congenital infection as well as to the passage of mother's antibodies through colostrum. Therefore, testing for IgM and IgA antibodies will help in identifying congenitally infected babies (Decoster *et al.* 1991). In babies resulted IgG positive but IgM and IgA negative, the use of IgG/IgM western blots of mother-infant pairs can prove useful (Pinon *et al.* 2001). In case of maternally transferred IgG, antibodies usually decline and disappear within 6–12 months.

Access ® Toxo IgG and IgM (Beckman Coulter Inc., USA), Radim Toxo IgG and IgM (Radim, Pomezia, ROMA), Toxo IgG avidity (TechnoGenetic- Bouty company-Sesto San Giovanni, Milano BEIA) are serological kits that have been utilized in recent Italian epidemiological studies (Mosti *et al.* 2013; Thaller *et al.* 2011).

METHODS OF *Toxoplasma* DETECTION IN FOOD AND WATER

MEAT

Risk-factor analysis indicats that 30 to 63% of human infections can be attributed to the consumption of undercooked meat (Cook *et al.* 2000). Besides pre-harvest food safety measure-s to apply for monitoring *Toxoplasma* infection in food-producing animals, post-harvest strategies at slaughter and during food processing have become more and more important in recent years. However, there is no general agreement among test to be used, no reference materials and no laboratory certification programs for monitoring the parasite burden in meat destined to human consumption (Kijlstra and Jongert 2008). Thus, the "gold standard method" for determining the infectivity of meat samples is still considered bioassays using either mice or cats (Kijlstra and Jongert 2008). As sentenced above, bioassays are laborious, time consuming and cannot be used for testing large numbers of samples. Therefore, PCR based methods to detect *T. gondii* in meat samples have been developed but they resulted to be less sensitivity in comparison to bioassays (da Silva and Langoni 2001; Garcia *et al.* 2006). This is mainly related to the inhomogeneous distribution of tissue cysts in combination with the small size of the sample analyzed; in fact for PCR analysis, DNA is usually isolated from 50mg of samples whereas in bioassays 50 to 500 g of meat samples are fed to a cat or inoculated into mice after artificial digestion. In 2010a, Opsteegh and colleagues

developed a PCR-based method that can be used as an alternative to the bioassay in quantitative screening of large number of meat samples; this method combines homogenization of a large sample with specific magnetic capture of *T. gondii* DNA. This magnetic capture real- time PCR resulted comparable in terms of sensitivity to bioassay for experimentally infected pigs' tissues. Furthermore, when PCR results of seropositive sheep meat samples have been compared to ELISA results, antibody concentration resulted strongly correlated to the probability of a positive PCR from meat and, consequently, to the presence of infective parasite. The correlation between the presence of tissue cysts and antibodies against *T. gondii* has been shown also by Dubey *et al.* (2008) in lambs.

An innovative matrix that it is used at the slaughterhouse for the identification of *T. gondii* positive carcasses is muscle fluids or meat juice samples. Meat juice samples are obtained after a cycle of freezing and thawing from meat samples (usually 25 g of diaphragms or hearts) collected at the slaughterhouse. Monitoring programs based on muscle fluid serology have been successfully implemented for *Salmonella* (Mousing *et al.* 1997), *Trichinella* (Nockler *et al.* 2005) and Aujesky virus (Le Potier *et al*. 1998) diagnosis in slaughtered pigs. Numerous serological studies compared results obtained testing serum samples and meat juice collected from the same slaughtered sheep or pig in order to find out the reliability of muscle fluids for detecting anti-*Toxoplasma* antibodies and for identifying infected animals (Wingstrand *et al.* 1997; Lunden *et al.*2002; Halos *et al.*2010; Berger-Schoch *et al*. 2011; Villena *et al.*2012; Ranucci *et al.* 2012). In Sweden, the correlation between the 2 types of samples tested by ELISA was found to be excellent in pigs (Wingstrand *et al.* 1997; Lunden *et al.*2002). In Italy, the average concordance between serum and meat juice from finishing pigs tested by IFAT was substantial (Ranucci *et al.* 2012). Also Villena *et al.* (2012) in France found a substantial agreement between ELISA results on diaphragm fluids and MAT on heart fluids from slaughter sheep, confirming that this matrix can be considered relevant for surveys of toxoplasmosis seroprevalence in meat. In order to improve the performance characteristics of serology methods when testing meat juice samples, Mecca and colleagues (2011) proposed to adjust fluids testing dilution in relation to their blood content.; this solution resulted indeed in a decreased number of false negative meat juice samples, demonstrating that reactivity of meat juice samples is dependent on the blood concentration of the tissue. Interestingly, Villena *et al.* (2012), in samples from slaughtered sheep, found a significant correlation between increasing MAT titres from muscle fluids and the probability of isolating live parasites from the heart. This finding is in accord to what reported by Dubey and colleagues (2008) in lambs and by Opsteegh and colleagues (2010a) in sheep, suggesting that consuming undercooked meat from seropositive sheep may pose a real risk to consumers.

These results on muscle fluids are promising; however, further studies investigating the correlation of meat juice results and infectivity of the meat are needed in order to evaluate the future application of muscle fluids serology for the identification of "*Toxoplasma* free" meat products (Mecca *et al*. 2011).

MILK

The excretion of *Toxoplasma* in milk can be evidenced by a bioassay using cat and mice, by cell line cultivation, by capture ELISA and by PCR (Dehkordi *et al.* 2013). Similarly to what reported for meat, the "gold standard" methods for determining the infectivity of milk and

dairy products are still bioassays or cell line cultures. Detection of *T. gondii* DNA is only indicative of the presence of parasite without giving any certain information on infectivity and on the risk posed to consumers.

WATER

The directive 98/83/CE on the quality of water for human consumption does not contain specific regulatory measures for water in regard to contamination with *Toxoplasma* oocysts. Therefore, no standardized methods are available. The main difficulty is represented by the recovery of oocysts from large volumes of water. Methodologies published in literature are based on the experience gained from other coccidians such as *Cryptosporidium* and involve concentration of oocysts using filtration of large volumes, centrifugation or flocculation of the samples (Karanis *et al*. 2013). Direct identification of *T. gondii* by microscopy is not reliable due to a high resemblance with oocysts from other coccidian species. Therefore, PCR based methods are commonly used for detecting the presence of *T. gondii* DNA in concentrated water samples (Villena *et al*. 2004; Kourenti and Karanis 2006). To date, the only method able to show the presence of infective material in water samples is represented again by mouse bioassay; however, scientists are working on the development of a quantitative reverse transcription PCR method to differentiate between viable and nonviable oocysts detected in environmental samples (Karanis *et al.* 2013).

DEVELOPMENT AND APPLICATION OF AN IN-HOUSE INDIRECT ELISA FOR ANTI-*T. gondii* ANTIBODY DETECTION IN SHEEP SERA COLLECTED IN THE FORLI-CESENA DISTRICT

PREFACE

Emilia-Romagna (E-R) region owns 1.17% and 1.13% of the Italian sheep and goat stock, respectively (www.istat.it), for a total of more than 86.000 heads kept in 3331 flocks (http://www.izs.it/IZS). Forli-Cesena and Bologna are the regional districts with the highest concentration of ovicaprine heads, owning, respectively, more than 23.000 and 13.000 animals (http://www.izs.it/IZS). According to the number of animals reared, ovicaprine farms can be classified (Cortini, 1999) as: "in backyard" (1-20 animals), semi-professional (50-100 animals) and professional (more than 100 animals); farms holding 1-5 animals are registered for self-consumption and don't have the permission to sell either meat or dairy products. From the analysis of the OviCaprine National Registry (http://www.izs.it/IZS), in E-R region backyard and semi-professional farms are the most widespread (94.5%; 3150/3331) with 44% (1400/3150) that are registered for self-consumption; professional holdings represent only 4% of the total (5.4%; 181/3733). Forty-nine per cent (49.3%; 1643/3331) of the flocks are registered for meat production whereas dairy flocks are only the 4.6% (154/3331) of the total. The 88.7% of meat producing ovicaprine farms are "in backyard" whereas the 55% (85/154) of milk producing flocks owns more than 100 animals. Throughout the region, intensive management (65%; 2161/3331) is more widespread than extensive and only 4 flocks are transhumant ones (0.4%; 4/3331).

In 2011 the E-R territory has been recognized as having an official ovicaprine brucellosis-free status (Deliberazione della Giunta Regionale 27/06/2011, n. 917); since then, according to European guidelines, the routine serological controls on the flocks are performed on a biennial frequency. The laboratory assigned to the regional serological screening is the Laboratory of Serology of the Istituto Zooprofilattico Sperimentale of the Lombardy and Emilia-Romagna (IZSLER), Bologna section. Thus, all the ovicaprine sera collected by Veterinary Officers from each flock of the regional territory are sent to this center; the only exception is represented by Piacenza district, which send samples to the central section of the IZSLER, in Brescia. Thanks to collaborations between the Laboratory of Parasitology of the Department of Veterinary Medical Sciences and the IZSLER, Bologna section, it has been possible to have access to the remainder of the ovicaprine serum samples after the serological screening for brucellosis.

The aim of this collaboration was to collect sera from animals from all the regional districts and, among them, to select a statistically significant number of flocks to test for the presence of anti-*T.gondii* antibodies in order to have, for the first time, data on the prevalence of the infection in sheep and goats of the E-R region.

Between October 2012 and January 2013, a total of 6026 ovicaprine sera representing 504 flocks have been collected and stored at -20°C; from each flock 50% of sera have been sampled. Of the 504 flocks, 189 were from Forli-Cesena, 92 from Bologna, 52 from Modena, 44 from Parma, 40 from Reggio-Emilia, 39 from Ravenna, 33 from Rimini and 17 from Ferrara district. Since previous information on *T. gondii* prevalence was not available for the calculation of a valid statistical sample set of flocks, we considered a 50% expected prevalence, a confidence level of 95% and an accepted error of 5%; using a web sample size

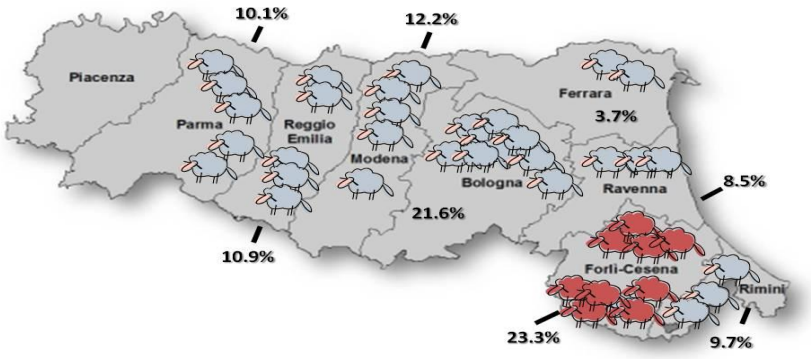
calculator (RaoSoft Inc.) the sample size was calculated as 345 flocks, from which all collected serum samples should be tested. The sample size of flocks (345) to be tested was then stratified by districts, according to the proportion of flocks in each district, as reported in Table 1.

Table 1 Summary of flocks present and number of flocks to be tested for each district

DISTRICT	N° OVICAPRINE FLOCKS PRESENT IN EACH DISTRICT (2012)	RELATIVE PERCENTAGE (%)	N° OVICAPRINE FLOCKS TO BE TESTED
Forli-Cesena	725	23.3%	80.4
Bologna	673	21.6%	74.5
Modena	379	12.2%	42.1
Reggio Emilia	339	10.9%	37.6
Parma	315	10.1%	34.8
Rimini	303	9.7%	33.5
Ravenna	264	8.5%	29.3
Ferrara	115	3.7%	12.8
Total (w/o Piacenza)	3113	100	345

Due to the high number of samples, we decided to use a rapid, easy and economic serological method for the screening. Thus, we firstly worked on the development of an in-house indirect ELISA for the detection of antibodies against *T. gondii* in sheep and goats sera. Even if serological seropositivity of meat-producing animals does not necessarily reflect the risk that those animals pose for their consumers, it has been recently demonstrated that in sheep, the antibody concentration was strongly related to the probability of the presence of tissue cysts in meat samples. This suggested that serological screening can be used as a tool for estimating the number of seropositive animals which are likely to harbor tissue cysts. In light of this, we used this in-house ELISA for testing a batch of sheep sera collected in the Forli-Cesena district, in order to evaluate not only of the distribution of infection in sheep flocks of the Emilia-Romagna region, but also the risk for humans to acquire the infection by consumption of undercooked mutton.

Fig.1 Concentration of ovicaprine herds in each district (%)



MATERIALS AND METHODS

DEVELOPMENT AND VALIDATION OF AN INDIRECT ENZYME-LINKED IMMUNOSORBENT ASSAY FOR THE DETECTION OF *T. gondii* ANTIBODIES IN SHEEP AND GOATS' SERUM SAMPLES

1. Reference sera

Flock 1. In December 2011, 104 serum samples were collected from a sheep dairy flock located in Palesio, Bologna's district. All the animals were older than 6 months. This flock has been chosen as no prophylactic measures were adopted for preventing the transmission of the infection to sheep, i.e. a population of cats was steadily present on the farm and have free access to feed store and to pastures. Sheep were kept indoors during the winter season and on the pasture during spring-summer.

Flock 2. In 2012, two-hundred and forty-four serum samples were collected from a dairy flock located in Forli-Cesena district. The flock was composed of more than 600 animals that were kept under intensive management conditions. No further information on the management conditions was available.

All 348 reference sera were primarily tested by Indirect Immunofluroescence Antibody Assay (IFAT) and then used in different steps of the ELISA validation process.

2. Indirect immunofluorescence antibody assay (IFAT)

IFAT was performed using slides spotted with whole RH strain tachyzoites (Mega Cor Diagnostic, Horbranz, Austria) as antigen and fluorescein isothiocyanate-labelled rabbit anti-sheep IgG (whole molecule, KPL, Gaithersburg, Maryland, USA) diluted 1:20 in PBS plus 1% Evan's blue, as conjugate. Sera were screened at a dilution of 1:64 (cut-off) as recommended in guidelines of the OIE (2008); positive sera from Flock 1 were serially diluted two-fold to define the end-point titre, i.e. the highest dilution of serum that still gave a positive reaction. Positive and negative controls were included in each assay and the slides were examined under a fluorescence microscope at 400X magnification (Diaplan Microscope, Leitz, Milan, Italy). A non specific polar fluorescence (green fluorescent cap at the parasite pole) may be present, therefore only a bright, linear, peripheral fluorescence of the tachyzoites was considered as positive.

Although the manufacturer claimed both Sensitivity (Se) and Specificity (Sp) to be >99%, we adopted a Se = 80.4% and Sp = 91.4%, according to what had been reported by other authors (Shaapan *et al.* 2008; Cenci-Goga *et al*. 2013) for calculation of the true prevalence (TP) from apparent prevalence (AP). The formula used was the following (Rogan and Gladen 1978):

$$TP = \frac{AP + Dsp - 1}{DSn + DSp - 1}$$

3. In-house indirect enzyme-linked immunosorbent assay (ELISA)

3.1 Process of assay validation

According to Jacobson (1998), the development and validation of our in-house indirect ELISA assay followed these steps:

- determination of the antigen;
- determination of the control serum;
- determination of the reagents;
- optimization and standardization of reagents and protocols;
- determination of the performance characteristics of the assays.

3.1.1. Antigen. We used the water-soluble antigen fraction of *T. gondii* tachyzoites of the S48 strain that was kindly provided by the Moredun Research Institute (Edinburgh, Scotland). The stock protein concentration was 2.6 mg/ml.

3.1.2. Sheep and goats serum controls. Five sheep sera, selected among samples from Flock 1, have been selected on the basis of IFAT results, i.e. three positive serum samples (2 with titer 512 and 1 with titer 256) and two negative samples. A *T. gondii* positive goat sera sample was kindly provided by Professor Francesca Mancianti from the Faculty of Veterinary of Pisa's University; the sample tested positive in Indirect Immnofluorescence Antibody test with an antibody titre ≥512.

3.1.3. Reagents. Donkey anti-sheep IgG and rabbit anti-goat IgG labeled with horseradish peroxidase (HRP) were used as enzyme-antibody conjugate and the Sure Blue TMB was used as substrate (Table 1). Donkey anti-sheep conjugate have been tested against goat positive control in order to evidence cross reactivity with goat IgG. The TMB substrate is a ready to use solution that develops a deep blue soluble product when reacts with horseradish peroxidase labeled conjugates. The substrate reaction was stopped by adding of 2M H_2SO_4.

3.1.4. Optimization of reagents and protocol. Optimal dilutions for ELISAs were established using checkerboard titrations with different dilutions of antigen, sera and HRP-conjugate (Fig.1). Antigen was diluted in bicarbonate buffer (Table 2) and tested at 1:500, 1: 1000 and 1:2000 dilutions. Flat-bottom Greiner Medium binding 96-wells plates (cat n° 655001) were coated with 100 µl/well of diluted antigen by overnight incubation at 4°C. Unoccupied hydrophobic sites on the coated wells were blocked by the addition of 250 µl/well of blocking buffer (PBS/1%BSA, Table 2). Serum samples were tested at the dilution of 1:100 and 1:200 in PBS/0.05%Tween20/1%BSA (Table 2); 100 µl of diluted serum was added to each well in duplicates and incubated at 37°C for 1 hour. HRP-conjugate was tested at the dilution of 1:5000, 1:6000, 1:7000 and 1:8000 in PBS/0.05%Tween20/1%BSA and incubated at 37°C for 1 hour. In order to test nonspecific binding of enzyme conjugate and the stability of the substrate, two samples without serum, where the analyte only and where the analyte as well as the enzyme-conjugate were substituted with dilution buffer respectively, were included on each plate. The Sure Blue TMB substrate was added (100 µl per well) and its

incubation time was evaluated monitoring the OD values of the samples at 650nm using a microplate reader (Multiscan EX version 1.1, Labsystems, DASIT, Milan, IT). The substrate reaction was stopped by the addition of 100 µl of 2M H_2SO_4 when OD values of the positive controls were ≥ 0.60. At the end of each incubation step, with the exception of TMB incubation, the excess reactants were washed away by washing each plate 4 times with the wash buffer (PBS/1%BSA, Table 2) (Table 2). The wash buffer was dispensed as 250 µl per well by multichannel pipette and plates were allowed 2 minutes to soak with buffers for the disruption of low affinity nonspecific interactions. The excess buffer was tapped out by banging the plate upside down on dry paper towels. The combination of dilutions that achieved the maximum spread in activity between negative and high positive samples, with a minimal background activity, was considered as optimal.

Fig. 1 Checkerboard titrations' template

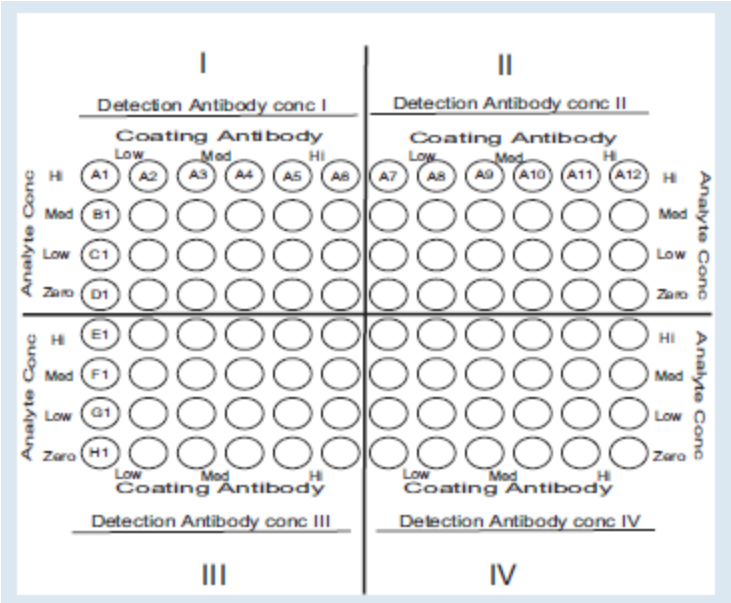


Table 2 List of reagents used

REAGENT	
Enzyme-antibody conjugate	Horse Radish Peroxidase labeled Donkey anti-sheep IgG (Sigma Aldrich, A3415)
Substrate	Sure Blue TMB (KPL, 52-00-02)
Stop solution	2M H_2SO_4
Bovine Serum Albumine (BSA)	Sigma Aldrich (A7906_10G)
Tween 20	Sigma Aldrich (P1379-500)

Table 3 Composition of buffers and dilutors used

BUFFER/DILUTOR	NAME	COMPOSITION	
Coating Buffer	Bicarbonate buffer	Na_2CO_3	1.59g
		$NaHCO_3$	2.93g
		deionized water	1000ml
		pH 9.6	
Blocking buffer	PBS/1%BSA	NaCl	7.650 g
		Na_2HPO_4	0.724 g
		KH_2PO_4	0.210 g
		Deionized water	1000ml
Serum and conjugate diluents	PBS/0.05%Tween20/ 1%BSA	BSA	1g
		NaCl	7.650g
		Na_2HPO_4	0.724g
		KH_2PO_4	0.210g
		Deionized water	1000ml
		Tween 20	0.5 ml
Wash Buffer	PBS/0.05%Tween 20	BSA	1g
		NaCl	7.650g
		Na_2HPO_4	0.724g
		KH_2PO_4	0.210g
		Deionized water	1000ml
		Tween 20	0.5 ml

3.1.5. Standardization of the protocol. Preliminary evidence of repeatability was obtained by evaluating results from four replicates of each control sample within each plate (intraplate variation) and between plates (interplate variation), on four different occasions. For the evaluation of both intraplates and interplates results, coefficients of variation (CV), i.e. standard deviation of replicates divided by mean of replicates, were calculated. Raw absorbance values were used at this stage of validation. CV with values less than 20% indicated adequate repeatability at this stage. If excessive variation (CV >30%) was evidenced for the majority of samples within and/or between runs, a backward step to the optimization of the conditions was performed.

3.1.6. Validation of the ELISA for sheep sera

The determination of ELISA's performance characteristics, i.e. the determination of cut-off and DSe/DSp, was performed by testing a panel of sheep sera and analyzing the results with two different statistical methods. Thus, the indirect ELISA was evaluated first by comparison with IFAT, i.e. the cut-off point was calculated using IFAT results of the panel of reference sera of known reactivity; for this ELISA's DSe and DSp values were called relative values. Secondly,

the cut-off point was calculated on a statistical basis, i.e. fitting a binary mixture model based on the frequency distributions of the ELISA results.

3.1.6.1 Comparative serology

A panel of known infected and uninfected sera was necessary for validating the ELISA by comparison with IFAT. The number of sera has been calculated within statistically defined limits, using the following formula (Jacobson 1998):

$$n = \frac{(DSe\ (or\ DSp))(1-DSe\ (or\ DSp))(c)2}{e2}$$

n is the number of known infected and uninfected animals; DSe is the worst case assumption of ELISA diagnostic sensitivity (and DSp the worst case assumption of ELISA diagnostic specificity); *c* is the confidence interval for the estimate; *e* is the percentage of error, expressed as decimal.

Estimating an 88% of DSe and a 90% of DSp with a 95% confidence interval and ±5% of error allowed the number of known infected and uninfected animals required was 162 and 138, respectively, for a total of 300 samples. A total of 318 sera were selected from Flock 1 and Flock 2 based on the IFAT results, i.e. 178 infected and 140 uninfected sera. ELISAs were performed following the optimized protocol; raw OD-values were corrected first for blank measurement, i.e. the average OD-value for blank controls on a plate was subtracted from the OD-values of the sera on that plate. Then blank-corrected OD values were normalized calculating for each sample (OD_x) a standardized blank corrected OD value using the formula proposed by Lind *et al.* (1997):

$$OD_{corr} = [OD_{(x)} - OD_{(N1)}] \times \overline{OD\ P1}\ /\ OD_{(P1)} + \overline{OD\ N1}$$

where $OD_{(P1)}$ and $OD_{(N1)}$ are the blank-corrected OD values obtained on the actual plate for serum P1 and N1 and $\overline{OD\ P1}$ and $\overline{OD\ N1}$ are the means OD-values obtained for the P1 and N1 at the end of the experiment (16 plates), respectively. A plate was retested if the CV of duplicates of control sera (P1 and N1) was >20%. Individual sera were retested if CV was above 20% (Opsteegh *et al.* 2010). Raw OD-values have been corrected by blank substraction and normalized as described above. Since antibody titers are usually lognormally distributed (Jacobson 1998; Thrusfield, 2005), ODc-values were log-transformed; then the frequency distribution of the log transformed ODc- values was calculated in order to test if the population was normally distributed, i.e. to confirm the presence of two components, one for seropositive and one for seronegative animals. To assess performance of the ELISA test, the cut-off point, and following DSe and DSp, have been calculated generating a ROC curve using IFAT test as criterion-reference standard (XLSTAT data analysis and statistical application for Microsoft Excel®; Addinsoft). In the ROC curve analysis, sensitivity and specificity are computed across all the possible threshold values and the optimal cut-off value is the logODc-value at which the number of animals scored correctly is maximized (Greiner *et al.* 2000). For measuring the discriminatory power of the ELISA, the area under the curve (AUC) for the ROC-curve has been calculated. The closer the curve is located to upper-left hand corner, the larger is the area under the curve and the better the test can discriminate between diseased

and non-diseased (Greiner *et al*. 2000). Since the AUC can have any value between 0 and 1, the relationship between the area under the ROC curve and the diagnostic accuracy was evaluated according to Swets (1988): AUC≤0.5 test non-informative; 0.5<AUC<0.7 less accurate tests; 0.7<AUC<0.9 moderately accurate tests; 0.9<AUC<1 highly accurate tests; AUC=1 perfect test. The degree of agreement between the results from the 2 tests was quantified using κ statistic. The strength of the agreement for the kappa coefficient was evaluated as follows: ≤0 = poor; 0.01–0.20 = slight; 0.21–0.40 = fair; 0.41– 0.60 = moderate; 0.61–0.80 = substantial; and 0.81–1.00 = almost perfect. The McNemar test was applied to the serological test results, and the p values were calculated. The test was considered as significantly different from the reference test when $p < 0.05$ (Dohoo *et al.* 2003).

3.1.6.2 Analysis of mixture distributions: intrinsic cut-off calculation

In order to obtain absolute values of sensitivity and specificity for the ELISA, the calculation of the cut-off point was based solely on the distribution analysis of the log ODc-values from the panel of standard sera (Jacobson, 1998). Under the assumption of a normal distribution of the sampled population, a binormal mixture model was fitted to the observed frequency distribution of the log-transformed ODc-values based on maximum likelihood in Mathematica (Wolfram Research, Champaign, IL, USA). The model estimated two means, two standard deviations and the prevalence; this estimated prevalence was equivalent to the area under the curve of the positive distribution divided by the total area under both curves. For the cut-off calculation, a ROC curve based on the distribution from the binormal mixture model was constructed. The optimal cut-off resulted as the log transformed ODc-value at which the DSe and DSp values were maximized; in addition the AUC was calculated. Standard sera were then classified as positive or negative based on the intrinsic cut-off. IFAT results were then deemed correct or incorrect relative to the ELISA, which were considered the new standard of comparison and IFAT performance characteristics were revaluated. McNemar's χ^2-test and the Kappa statistic were used to test the level of agreement between the ELISA results obtained using the intrinsic cut-off and the IFAT.

APPLICATION OF THE IN-HOUSE INDIRECT ELISA

In this preliminary part of the serological survey, we focused on sheep flocks from Forlì-Cesena district. Thus, 10 flocks were randomly selected among flocks holding more than 20 animals, i.e. semi-professional (20-100 animals) and professional flocks (more than 100 animals) (Cortini, 1999) and all the sera collected from each holding (50% of the total) were screened with the in-house indirect ELISA developed, for a total of 375 sera. Table 4 summarizes information on size, type of production and management system of each tested flock.

Using the cut-off value determined by binormal mixture analysis, the overall and in-flock apparent prevalence (AP) of infection were calculated. The overall AP was calculated as the total of positive sera on the total of tested sera whereas the in-flock AP was calculated as positive sera on the total sera tested for each flock. Then, the AP was used for the estimation of the true prevalence (TP) using the following formula (Rogan and Gladen 1978):

$$TP = \frac{AP + Dsp - 1}{DSn + DSp - 1}$$

For the statistical analysis, flocks were categorized into 3 groups according to the number of animals present in each: 20-50 animals, 50-100 animals, more than 100 animals. Proportions of positive animals were compared using the Chi-Square test (χ^2); a p value of <0.05 was considered statistically significant.

Table 4 Size, production and handling system of investigated flocks

	Total sheep present in the flock	Production system	Handling system	N° tested sera
1	40	MEAT	EXTENSIVE	20
2	89	MEAT	INTENSIVE	45
3	102	MEAT	INTENSIVE	51
4	29	MEAT	EXTENSIVE	15
5	261	MILK	INTENSIVE	131
6	50	BOTH	INTENSIVE	25
7	80	MEAT	INTENSIVE	40
8	23	MEAT	INTENSIVE	12
9	32	MEAT	INTENSIVE	16
10	50	MILK	INTENSIVE	25

RESULTS

IFAT RESULTS FROM REFERENCE SERA

Among the 348 reference sera tested by IFAT, the overall *T. gondii* apparent prevalence was 58.6% (204/348); in particular, 121 sera out 244 (49.6%) from Flock 1 and 83 out 104 (79.8%) from Flock2 were positive for the presence of anti-*T. gondii* antibodies. According to the Rogan and Gladen estimator (TP= AP+ (0.914-1)/0.804 + (0.914 -1); Rogan and Gladen 1978), the true prevalence was estimated to be 58.4%. In Table 5, antibody titres of the 83 positive sera from Flock 1 are summarized.

DEVELOPMENT OF AN IN-HOUSE INDIRECT ELISA PROTOCOL

Checkerboard titrations were performed in order to establish optimal dilutions for the antigen preparation, serum and HRP-conjugate. The combination of dilutions that achieved the maximum spread in activity between negative and high positive samples, with a minimized background activity used antigen diluted 1:1000 in bicarbonate buffer (2.6 µg/ml), serum sample diluted 1:200 in PBS/0.05%Tween20/1%BSA and HRP-donkey anti-sheep IgG diluted 1:5000 in PBS/0.05%Tween20/1%BSA. Since the background signal did not decrease beyond an OD value of 0.055, a further washing with 250 µl/well of PBS was included; in this way, the background signal decreased to an OD value of 0.04. The incubation time of the substrate, monitored by reading of the plates at 650nm, ranged from 5 to 7minutes, in relation to the laboratory conditions, i.e. shorter with higher room temperatures (>25°C) and longer with cooler temperature (≤23°C). The donkey anti-sheep IgG conjugate showed a strong cross-reaction with goat IgG as no great differences in OD values were observed when goat positive control were tested with anti-sheep (OD 1.2) or anti-goat conjugate (OD 1.3). The optimized in-house indirect ELISA protocol is reported below.

In-house indirect ELISA protocol.

Coat a 96-well plate with 100 µl/well of 2.6 µg/ml solubilized *T. gondii* RH-antigen diluted in bicarbonate buffer (Table2) and incubate overnight at 4°C. Wash the plate 4 times with washing buffer (250 µl/well PBS/0.05% Tween20), one time with PBS (250 µl/ well) and tap dry. Block non specific binding sites by incubation for 1 h at 37°C with 250 µl/well of PBS/1% BSA (Table 2) and wash. Dilute sera 1:200 in 1%BSA/PBS/0.05% Tween 20 and add the dilutions in duplicate (100 µl/well) to the plate. Incubate the plate for 1h at 37°C and wash; add 100 µl/well of 1:5000 diluted (1%BSA/PBS/0.05% Tween 20) HRP-labeled conjugate and incubate the plate 1h at 37°C. After washing, reveal the peroxidase activity by adding 100 µl/well of substrate and incubate the plate at room temperature. Monitor OD value of positive control by reading at 650 nm. Stop the reaction by adding 100 µl/well of 2M H_2SO_4 when OD values of the positive control is ≥ 0.6 (5-7 minutes). Leave the plate to equilibrate in the dark for 5 minutes and then read at 450 nm by using a spectrophotometer. Include on each plate one positive (P1) and one negative (N1) serum control diluted 1:200 next to two serum blank samples. In the serum addition step, substitute the analyte with dilution buffer

(1%BSA/PBS/0.05% Tween 20) in both blank wells and in the enzyme-conjugate addition step, in one blank serum samples substitute the conjugate with dilution buffer (1%BSA/PBS/0.05% Tween 20). Correct raw OD-values first for blank measurement, i.e. subtract the average OD-value for blank controls on the plate from the OD-values of the sera on that plate. Then, normalize blank-corrected OD values by calculating for each sample (OD_x) a standardized blank corrected OD value using the formula proposed by Lind *et al.* (1997):

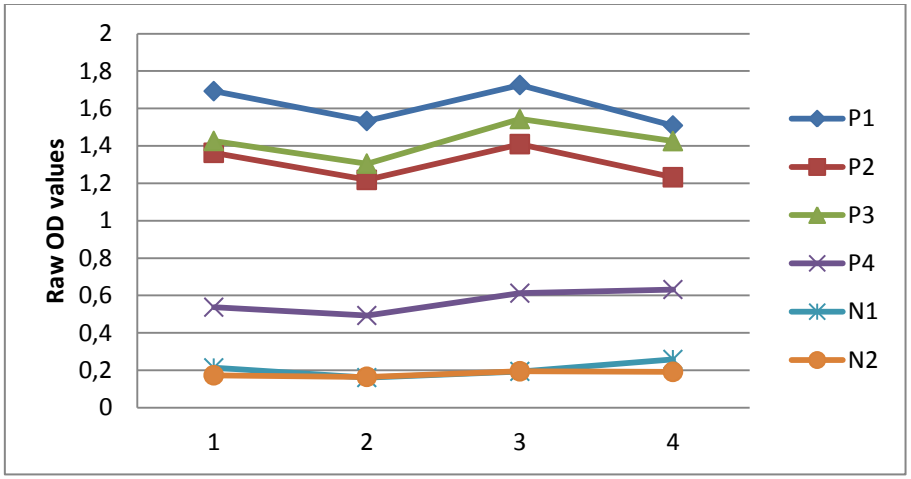
$$OD_{corr} = [OD_{(x)} - OD_{(N1)}] \times \overline{OD\ P1} \ / \ OD_{(P1)} + \overline{OD\ N1}$$

where $OD_{(P1)}$ and $OD_{(N1)}$ are the blank-corrected OD values obtained on the actual plate for serum P1 and N1 and $\overline{OD\ P1}$ and $\overline{OD\ N1}$ are the means OD-values obtained for the P1 and N1 at the end of the experiment, respectively. Retest the plate if the CV of duplicates of control sera (P1 and N1) is >20%. Retest individual sera if CV of duplicates is > 20% (Opsteegh *et al.* 2010B).

Standardization of the optimized protocol

Preliminary evidence of repeatability of the protocol was obtained by evaluating results from four replicates of each control sample within each plate (intraplate variation) and between plates (interplate variation), in four different occasions. For all control samples, intra and interplates coefficients of variation (CV) were <20%, indicating good reproducibility of the protocol. CV of all positive controls (P1-P4) were lower (<10%) compared to CV of negative controls (N1-N2 <20%). Average OD values trend from each control sample is shown in Graph1.

Graph 1. Average OD values trend from each control sample run in four different occasions



ELISA evaluation by comparison with IFAT

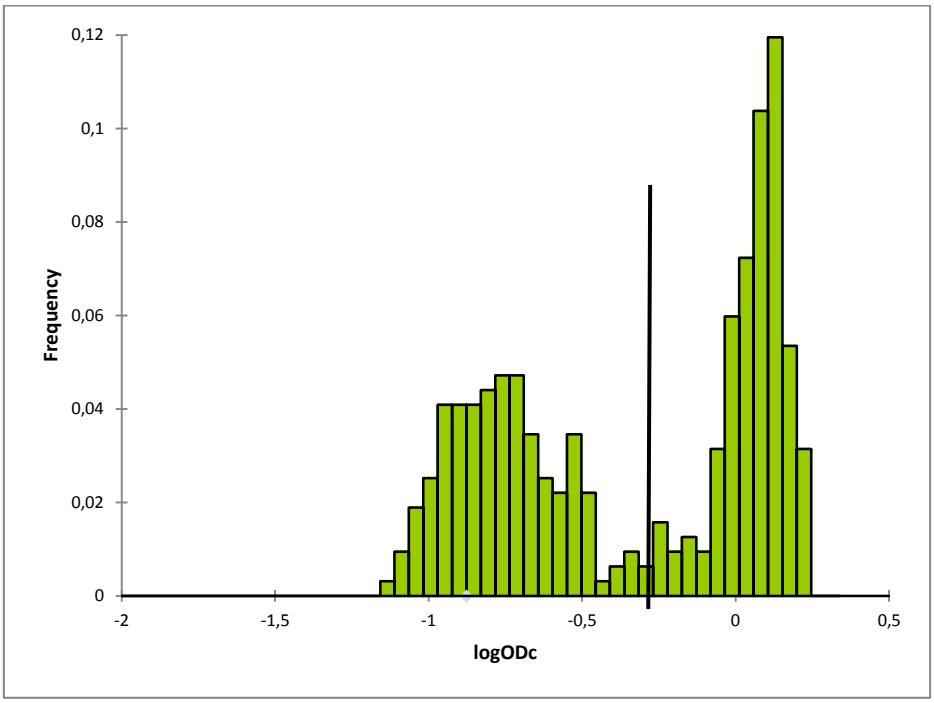
The logODc-values frequency distribution of the 318 standard sera clearly showed two components (Graph 2). Assessment of the ROC curve showed an AUC = 0,968 relative to the ELISA (Graph 3); this is very close to an AUC of 1, which indicates a perfect test (Swets 1988). The ROC analysis showed an optimal cutoff point at logODc-value -0.292 and at this point the estimate of sensitivity and specificity was 91% and 97.9%, respectively. Since these values are obtained by comparison with IFAT, they are considered relative values. Using this cut-off

point, 166 samples (52.2%) were scored as positive and 152 as negative with the ELISA. From the comparison of the two assays, using IFAT as reference, 20 samples gave discordant results, i.e. 4 resulted false positive and 16 false negative in ELISA (Table 6). McNemar test showed that results obtained by both techniques were significantly different ($p<0.000$); estimated κ value indicated an "almost perfect agreement" between the two tests ($\kappa=0.874$).

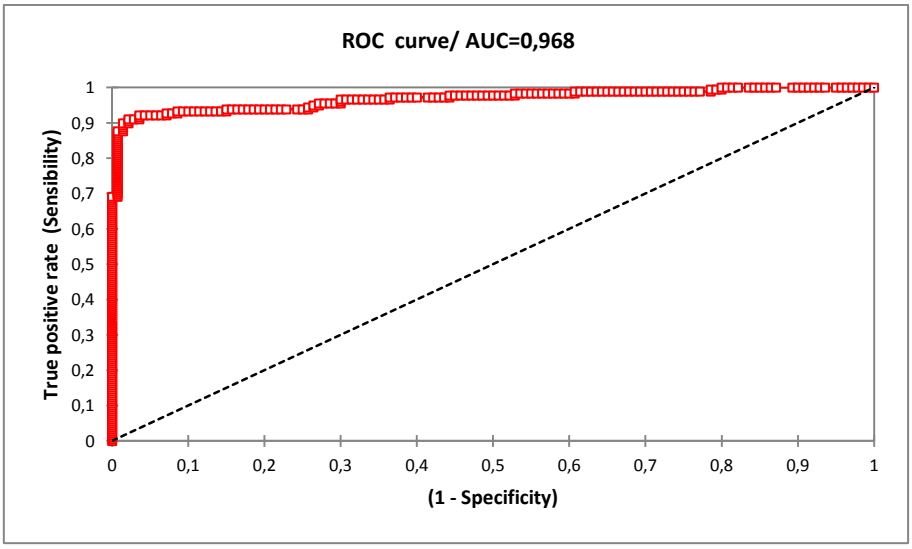
Table 5 Contingency table considering IFAT as reference test

		IFAT (Reference)		
		POSITIVE	NEGATIVE	Total
ELISA	POSITIVE	162	4	166
	NEGATIVE	16	136	152
	Total	178	140	318

Graph 2 Frequency distribution of ELISA logODc-values of standard sera. The vertical line indicates the optimal cut-off value calculated by comparison with IFAT using ROC-curve analysis



Graph 3 ROC curve for *T. gondii* ELISA calculated using IFAT test as criterion-reference standard



Analysis of mixture distributions

From the binormal mixture analysis (Graph. 4), the optimal intrinsic cut-off was estimated at a logODc-value -0.157 and at this point ELISA sensitivity was estimated at 99.4% and specificity at 99.8%. ROC-curve analysis revealed a very good discriminatory power of the ELISA (AUC = 0.999). At this cut-off point, 156 sera (49%) were scored as positive and 162 as negative. Considering the ELISA as reference test, IFAT diagnostic performances were recalculated in DSe 99.3% and a DSp 85.8 % (Table 7). Again, McNemar test revealed a statistical difference in results obtained with the 2 tests (P=0.000) but κ values showed an "almost perfect agreement" (κ=0.849) between them.

Graph 4. Frequency distribution of observed logODc values in ELISA and fitted normal distributions (lines). The vertical line indicates the cut-off point calculated by ROC-curve analysis based on binormal mixture model

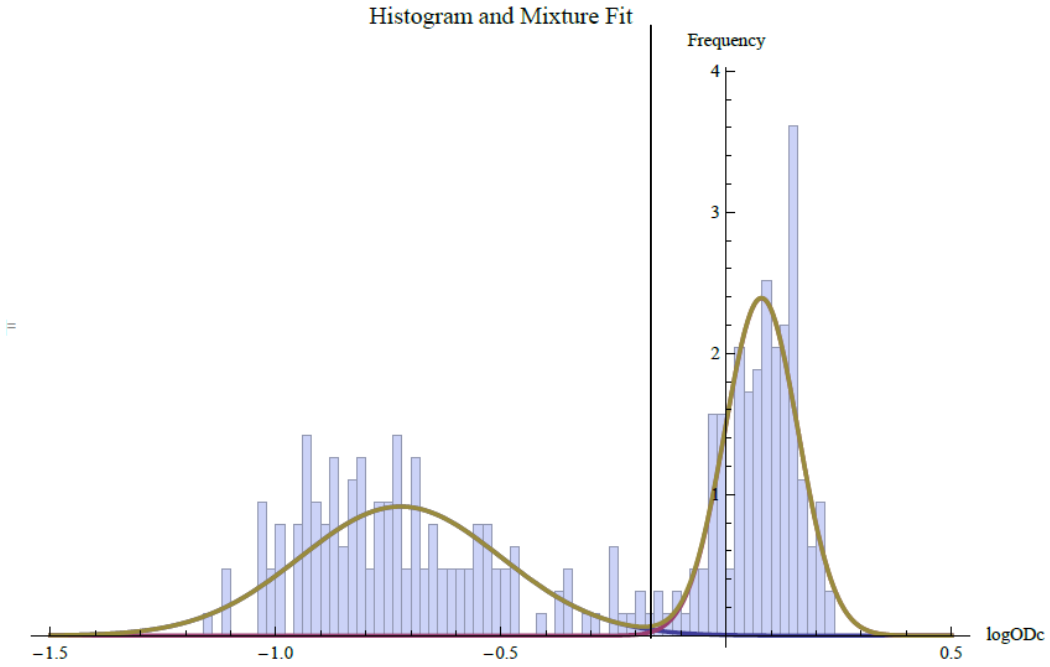


Table 6 Contingency table considering ELISA as reference test

		ELISA (reference)		
		POSITIVE	NEGATIVE	Total
IFAT	POSITIVE	155	23	178
	NEGATIVE	1	139	140
	Total	156	162	318
		DSe=155/156=99%	DSp=139/162=85.8%	

SEROLOGICAL ANALYSIS OF SHEEP FROM FORLI-CESENA DISTRICT

Both overall apparent and true prevalence for *T. gondii* in sheep from Forli-Cesena district was estimated at 41.9% (157/375). All the investigated flocks harbored at least one positive animal (100%). The in-flocks prevalence values (both apparent and true) ranged from 4% (1/25) to 60.3% (79/131); six flocks had more than 30% seropositive animals (Table 8). Seroprevalence increased together with herd size, from 25.4% (15/59) in herds of less than 50 animals to 53.3% (97/182) in herds with more than 100 animals (p=0.000; χ^2=20.1) (Table 9). Seroprevalence was higher in dairy sheep (51.9%) than in meat production ones (38.7%) or in two-fold aptitude sheep (4%) (p=0.000; χ^2=22.03) (Table 10).

Table 7 In-flocks *T. gondii* prevalence.

	Total sheep present in each flock	Production system	Management system	N° tested sera	Apparent prevalence (n°infected animals/ total tested)
1	40	MEAT	EXTENSIVE	20	15% (3/20)
2	89	MEAT	INTENSIVE	45	55.6% (25/45)
3	102	MEAT	INTENSIVE	51	35.3% (18/51)
4	29	MEAT	EXTENSIVE	15	46.7% (7/15)
5	261	MILK	INTENSIVE	131	60.3% (79/131)
6	50	TWO-FOLD APTITUDE	INTENSIVE	25	4% (1/25)
7	80	MEAT	INTENSIVE	39	43.6% (17/39)
8	23	MEAT	INTENSIVE	12	25% (3/12)
9	32	MEAT	INTENSIVE	16	12.5% (2/16)
10	50	MILK	INTENSIVE	25	8 % (2/25)

Table 8 Herd size and infection with *T. gondii.*

HERD SIZE	*%T. gondii* INFECTED ANIMALS (INFECTED/ALL)
<50	25.4% (15/59)
50-100	33.6% (45/134)
>100	53.3% (97/182)

Table 9 Type of production and infection with *gondii*

PRODUCTION	*% T. gondii* INFECTED ANIMALS (INFECTED/ALL)
MILK	51.9% (81/156)
MEAT	38.7%% (75/194)
TWO-FOLD APTITUDE	4% (1/25)

DISCUSSION

Since to our knowledge *T. gondii* prevalence in ovicaprine population of the Emilia-Romagna region has never been investigated, the aim of this study was to fill this gap. Furthermore, since the 50% of ovicaprine farms are registered for meat production, serological results in this category can also give an indication of the possible role of sheep and goat meat in human infections.

Considering the high number of flocks (and relative sera) to test, we decided to first develop an indirect ELISA, which is a semi automated serological method that gives rapid, objective results and does not require skilled personnel. Although several commercial ELISA kits are available, the development of an in house ELISA is more economical but also created more challenges. The main issue encountered in the development process involved the optimization of the washing step;, it was performed by hand, in absence of an automated plate washer. Therefore, the excess reactants and the high and/or low-affinity interactions between reactants were difficult to remove and to disrupt, respectively, resulting in a high background signal in blank serum well (raw OD values ≥0.05). Since there are a variety of reasons for high background signal, e.g. instability of reactants, the inclusion of two serum blank samples on each plate for testing the stability of the enzyme-conjugate and of the substrate, led us to quickly identify the real problem, i.e. the washing step. Following the recommendations of the "Troubleshooting ELISA guide" (KPL, 2013), we achieved an effective washing of the plates by increasing the number of washing steps at each stage, from 4 to 5, and by using a buffer without detergent (PBS only) in the final wash. This way, raw OD values of blank serum wells decreased to 0.04. According to the literature, another critical factor that can affect both the reactivity and the reproducibility of an ELISA (Jacobson 1998; KPL, 2013) is the temperature at which the assay is carried out; in fact even 1°C temperature difference may result in an enzyme's change of activity. With regard to this, we noticed that the incubation time of the substrate ranged from 5 to 7 minutes in relation to a room temperature < or > 25°C, respectively. Thus, to avoid stopping the substrate reaction too late or too soon, we monitored the incubation time by reading the plates at 650 nm and we stopped the substrate reaction solely when OD value of the positive control at 650 nm were ≥0.6, as reported by the TMB Stop Solution datasheet (KPL, Gaithersburg MD, USA). This shrewdness, together with the use of pre-warmed reagents, led to a good reproducibility of the assay. The evidence of a strong cross-reaction between anti sheep-conjugate and goat IgG was not surprising since between these 2 species there is a well-documented interspecies cross-reactivity (Karol *et al.* 1978). Since, in our Country, sheep and goats are often raised together, future serological screening will be performed more easily and quickly by using a single enzyme labeled conjugate for both species.

The estimates of the performance characteristics of our indirect ELISA was performed using two methods, i.e. by comparison with another serological method, the IFAT, and by a mixture distribution analysis. The first is a traditional widely used method, which considers a better-known diagnostic test as a "gold standard", or as a "standard of comparison", for defining performance characteristics of the new test. In our study, IFAT has been first considered as

the standard of comparison and the number of infected and uninfected serum samples necessary for the validation of the ELISA has been selected on the basis of IFAT results. ROC analysis revealed a very good discriminatory power of the ELISA and, according the optimal cut-off point, good performance characteristics, i.e. 91% of DSe and 97.9% of DSp. However, since a "gold standard" serological method for the diagnosis of *T. gondii* in animals is still lacking, each test used as the standard of comparison for evaluation of a new test is far from perfect as it has its own established levels of false positivity and false negativity which can be a source of error in estimates of diagnostic sensitivity (DSe) and specificity (DSp) of the new assay. Furthermore, the estimates of DSe and DSp for the new assay are only relative estimates, which might be an under or over estimate (Jacobson 1998; Mainar-Jaime and Barberan 2007). An indication of DSe and DSp underestimation can derive when the new assay gives a high percentage of false positive or false negative results; in our case, 16 samples gave false negative results and 4 tested false positive in the ELISA. Differences in results between the two tests were also demonstrated by the McNemar's test ($p<0.000$). Considering that other authors reported low values of DSe (80%) and DSp (91%) for IFAT (Shaapan *et al.* 2008; Cenci-Goga *et al.* 2013), we decided to assess the diagnostic characteristic of the ELISA fitting logODc- values in a binormal mixture model, since the binomial distribution of ELISA data was assessed previously (Graph 2), followed by a ROC curve analysis based on the distribution of the values. The ROC curve analysis confirmed an optimum discriminatory power of the ELISA (AUC=0.999) and estimates of ELISA characteristics were surprisingly high, i.e. DSe 99.4% and DSe 99.8%. This finding clearly confirms how estimates of diagnostic performances can be affected by diagnostic performances of the method used as standard of comparison. As suggested by Jacobson (1998), we reversed the roles of the two tests, making the ELISA the standard of comparison for IFAT and the relative IFAT DSe and DSp were 99% and 85.8%, respectively. We can ascribe the low specificity of the IFAT to the difficult interpretation of nonspecific fluorescence that can occur when testing hemolytic or lipid rich sera. In fact the main disadvantage of this serological method is the subjective reading of the results, since interpretation of a positive sample depends on the number of tachyzoites with total peripheral fluorescence per field and on the number of fields observed (Minho *et al*. 2004). In our experience, we found it challenging to reach the right discrimination between negative sera showing nonspecific fluorescence and positive sera with a low antibody titre (≥64 or ≥128). Again, the McNemar's test showed a significant difference in the proportion of positive results between the two methods ($p<0.000$) but the calculated κ value was very high either using IFAT or ELISA as standard for the comparison; since the κ value depends on the prevalence of the disease in the population analyzed (Mainar-Jaime and Barberan 2007), it is not considered an unbiased indicator of agreement between tests (Jacobson, 1998). Therefore, we confirm that the analysis of mixture distributions represents a powerful approach for an unbiased estimation of seroprevalence, in absence of a serologic-al gold standard test, as previously reported (Greiner *et al.* 1994; Jacobson 1998; Opsteegh *et al.* 2010b). We achieved to develop an easy, good performing serological method that could clearly distinguish between seropositive and seronegative sera and that can be used to achieve the principal aim of this study, i.e. to obtain preliminary information on the prevalence of *T. gondii* infection in sheep flocks from the Forli-Cesena district, i.e. the district

with the highest concentration of sheep. This is the first report of the presence of the parasite in this area; therefore, we don't have data for comparison and for evaluating the trend of the disease in time. We found an overall true seroprevalence of 41.9% and all investigated flocks harbored at least one seropositive animal (100%). Although these data are surprising and noteworthy, they have to be considered as preliminary since only 10 flocks were investigated. Very few studies have been conducted in Italy investigating *T. gondii* seroprevalence in sheep and goats (Rinaldi and Scala 2008); despite that, published data are difficult to compare since various serological assay were used and investigated animals were kept under different management conditions. However, the highest seroprevalence value (49.9%; 937/1876) was found by Vesco *et al.* (2007), analyzing, by a commercial ELISA, sheep sera collected in Sicily. Other *T. gondii* serological surveys have been conducted in Sardinia, Campania and Tuscany and the proportion of infected animals were around 30%. In particular, in Sardinia, Masala *et al.* (2003) investigated serum samples from sheep and goats by IFAT, finding a seroprevalence values of 28.4% (2048/7194) and 12.3% (302/2445), respectively. In Campania and in Tuscany, among investigated sheep, 28.8% (333/1170) (Fusco *et al.* 2007) and 34% (214/630) tested positive by IFAT, respectively. We found that seroprevalence significantly increased with farm size (from 25.4% in flocks <50 animals to 53.3% in flocks) and that dairy sheep were more positive than meat producing sheep ($p<0.000$). Also Vesco *et al.* (2007) reported an increase in seroprevalence in larger flocks, whereas Cenci-Goga *et al.* (2013) found significantly higher seroprevalence values in smaller flocks. In Sicily sheep farm management is very uniform with all flocks are permanently kept outside, whereas in Tuscany small flocks are generally kept more extensively (Cenci-Goga *et al.* 2013). In the E-R region intensive management is reported to be more widespread (65%; 2161/3331; http://www.izs.it/IZS). However, the major parts of intensive managed small-medium size flocks usually have access to fenced pastures located in close proximity to the farms (semi-intensive conditions) whereas farms with higher numbers of sheep tend to be more intensive (IZSLER technical group, personal communication). Various risk assessment studies demonstrated that extensive, intensive and/or semi-intensive conditions were statistically associated with the likelihood of *T. gondii* infection. Extensively managed flocks because animals may have had more exposure to cats in the environment or to contaminated stagnant pools (Tenter *et al.* 2000; Cenci-Goga *et al.* 2013); intensive or semi-intensive herds because farm facilities may provide shelter to various hosts of *T. gondii* (such as cats and rodents) which may be involved in the spread of the infection (Tzanidakis *et al*. 2012). Since we found that dairy sheep were more seropositive than meat producing ones ($p<0.000$) and we know that dairy flocks are usually larger and more intensively managed, we can suppose that animals kept under intensive rearing systems are more likely to acquire the infection from *T. gondii* oocysts accumulated in farm facilities.

Although these are preliminary results that cannot be extended to all regional districts, we can affirm that *T. gondii* infection is widely distributed in sheep reared in Forli-Cesena district (41.9% of overall seroprevalence). Considering that in previous studies seropositive sheep have been found to carry tissue cysts (Opsteegh *et al.* 2010a), the high seroprevalence found in meat producing sheep (38.7%) in this study could indicate that mutton may be an important source of human infection. In particular in Forli-Cesena district, the most consumed

mutton meat is the so called "castrato", i.e. a neutered ram of 5-12 months that is usually eaten grilled but not well cooked to maintain its tender texture. Since sheep tested in this study are >6 months old, we can make an inference about the risk of consumption of undercooked "castrato" meat. In fact, it has been reported that in areas with high seroprevalence values, the infection may occur also in young animals due to high levels of environmental contamination by oocysts (Vesco *et al*. 2007). More detailed information on farms management is needed in order to assess risk factors associated with *T. gondii* infection in sheep flocks. In conclusion, when this serological survey will be completed and serum samples from all regional districts have been tested, it will possible to draw a detailed risk's map for consumers and to design strategies for reducing the prevalence of infection in ovicaprine flocks.

The serological tool developed and proposed by this study, if used in a National Surveillance Program could provide reliable epidemiological data on *T. gondii* infection in small ruminants and provide missing information highlighted frequently by EFSA in their scientific reports (EFSA, 2007; EFSA, 2014).

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PCR DETECTION METHODS OF *Toxoplasma gondii* IN RAW AND FINISHED DRINKING WATER SAMPLES FROM SCOTLAND

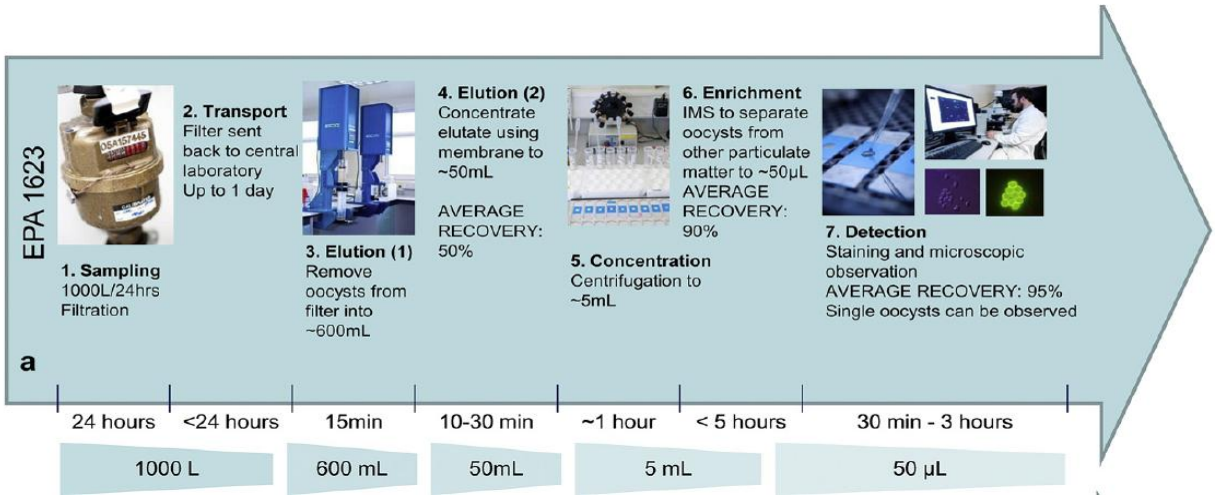
PREFACE

The waterborne transmission route of *T.gondii* to humans via the dissemination of oocysts through surface water and its epidemiological impact is now thought to be more significant than previously believed (Jones and Dubey 2010; Karanis *et al.* 2013).
Detection of *T. gondii* oocysts in environmental samples (soil, water) is technically difficult and rapid and standardized methods for *Toxoplasma* oocysts separation from water are not available.
Differently, standardized methods for detecting the presence of oocysts and/or cysts of other waterborne pathogens, such as *Cryptosporidium* sp. and *Giardia* sp., have been available for several years (U.S. Environmental Protection Agency, 2001). EPA Method 1623 is a performance-based method applicable to the determination of *Cryptosporidium* in aqueous matrices. This method is used in many EU Countries for monitoring of water supplies for the presence of *Cryptosporidium*, which is one of the most widespread protozoa that causes waterborne diarrheal disease outbreaks worldwide. In the UK, *Cryptosporidium* represents the commonest protozoal cause of acute gastroenteritis, with 3000-6000 laboratory confirmed cases annually (Bridle *et al*., 2012). EPA Method 1623 requires filtration, immunomagnetic separation of the oocysts from the material captured, and an immunofluorescence assay for determination of oocyst concentrations, with confirmation through vital dye staining and differential interference contrast microscopy (Fig.1).

In the absence of commercially available techniques for IMS of *T. gondii* oocysts, PCR is becoming a favored technique for the detection of *T. gondii* oocysts in water (Kourenti and Karanis, 2004, 2006; Schwab and McDevitt, 2003; Sroka *et al*., 2006; Villena *et al*., 2004) over the conventional mouse bioassay (Isaac-Renton *et al*., 1998; Villena *et al*., 2004). Since no specific PCR protocols have been developed for detecting *T. gondii* DNA from water samples, PCR protocols for the detection of *T. gondii* DNA in clinical specimens have been adapted to this matrix (Yang *et al.* 2009; Jones and Dubey 2010).

The aim of this study was to investigate the presence of *T. gondii* DNA in raw and finished water samples collected in Scotland. Scottish Water is a statutory corporation that provides water and sewerage services in Scotland and that, as expected by the UK monitoring program, monitors water supplies for the presence of *Cryptosporidium* utilizing the EPA method 1623.
Since *T. gondii* oocysts (10 to 12 µm in diameter) are larger than *Cryptosporidium* oocysts (3 to 5 µm) and they can be recovered following the EPA Method 1623, we decided to collect the remainder of raw and finished water samples after the IMS removal of *Cryptosporidium* oocysts by Scottish Water and to detect the presence of *T.gondii* DNA by PCR methods.

Fig. 1 EPA methods1623



MATERIALS AND METHODS

COMPARISON OF DNA ISOLATION METHODS

Nine samples of 15 ml sterile deionized water (DW) have been seeded in duplicate with a known number of *T. gondii* oocysts (M4 strain), i.e. 2x 3 samples with 10^3 oocysts; 2x 3 samples with 10^2 oocysts; 2x 3 samples with 10 oocysts). All samples were centrifuged at 1,250 x g at 4°C for 10 min in order to obtain a pellet for DNA isolation. The volume of 15 ml was chosen because it simulates the maximum volume of remainder environmental water samples after the IMS procedure supplied by Scottish Water. To aid rupturing *Toxoplasma* oocysts and to increase DNA yields, the pellet (obtained after centrifugation) was subjected to five freeze-thaw cycles.

We tested two commercial kits for DNA isolation, in particular the Wizard ® SV Genomic DNA Purification System (Promega) and NucleoSpin ® Tissue kit (Macherey-Nagel).
The first kit is designed for the isolation of DNA from tissues and cell cultures only, whereas the second is designed for a broader variety of starting materials like yeasts, bacteria, viruses or stool samples, in addition to tissues and cell cultures. Detailed descriptions of each protocol are shown in Table 1.
The two methods were compared using an ITS1 n-PCR that targets the multi-copy 18S-5·8S rRNA internal transcribed spacer (ITS1) region of the parasite. The methodology was carried out as reported by (Burrells *et al.* 2013). Briefly, each 20 µL reaction contained

- 2µL 10× custom PCRmix – SM0005 (45mM Tris-HCl 11mM (NH4)2SO4, 4·5mM MgCl2, 0·113 mg mL−1 BSA, 4·4 µM EDTA and 1·0mM dATP, dATC, dGTC, dTTP – ABgene, Epsom, Surrey, UK);
- 0·75 units BioTaq (Bioline, London, UK);
- 0·05 µM of forward primer and reverse primers NN1, NN2, first round (Table 2);
- 2 µL DNA.

The reaction was made to a final volume of 20 µL with DNase/RNase free water. To improve the sensitivity of the technique, each reaction was carried out in triplicate at these cycling conditions:

- 5min at 95 °C;
- 35 cycles of 1min at 95 °C, 1min at 55 °C, 1min at 72 °C;
- A final extension period of 5min at 72 °C.

To reduce unused primers from the primary PCR, the first round PCR products were diluted 1:5 with dH_2O, 2 µL of the diluted product was used in the second round reaction. The conditions for each second round were identical to the first round, however, contained 0·05 µM in internal forward and reverse primers NP1, NP2 (Table 2).
Second round PCR products (10 µL) were visualized by gel electrophoresis on 2% (w/v) agarose gel, stained with Gel Red (Biotium, Hayward, CA, USA) and visualized under UV light. A sample was regarded positive if an amplicon of 227 bp was present in any triplicate reaction.

Multiple negative controls (dH_2O) were included in each PCR run as well as DNA extraction controls; results were only accepted if these reactions were all negative.

Table 1 Detailed description of the two DNA extraction methods tested in this study.

Method 1: Wizard ® SV Genomic DNA Purification System (Promega)	Method 2: NucleoSpin ® Tissue kit (Macherey-Nagel)
• Spin down sample at 1.250*g* (2750 rpm) for 10 min. • Resuspend pellet in 1000 µl of Nuclei Lysis Solution and transfer in a 2ml tube. • Place tube into liquid nitrogen for 10 seconds. • Place in water bath at 50 °C until thawed, repeat 5x vortexing between stages. • Transfer 400 µl in a new 2ml tube. • Add 900 µl of the Nuclei Lysis Solution. • Incubate at 56 °C for 1 h and check • Cool to room temperature. • Add 300µl of the Protein Precipitation Solution. • Vortex for 20 seconds. • Incubate on ice for 5 minutes. • Spin at 13000g for 5 minutes. • Transfer the supernatant into a fresh 2ml tube with 900µl of isopropanol. • Mix gently by inversion. • Leave overnight at -20°C. • Centrifuge at 13000g for 5 min. • Remove supernatant. • Add 600 µl of 70% ethanol. • Centrifuge at 13000 g for 2 minutes (pellet visible). • Remove supernatant. • Centrifuge at 13000 g for 1 minute and remove the rest of ethanol with one fine tip. • Allow the pellet to dry: it is dry when the edges become clear, it takes about 15 minutes, not more; over-drying will make difficult to resuspend the pellet. • Add 200µl of dH_20 (adjust the volume of water; if you want more concentrated DNA 100 µl of dH_20) and dislodge the pellet. • Leave the pellet to dissolve overnight at 4 °C.	• Spin down sample at 1.250*g* (2750 rpm) for 10 min and discard supernatant. • Resuspend the pellet in 400µl of Buffer T1 and transfer in a new tube. • Place tube into liquid nitrogen for 10 seconds. • Place in water bath at 50 ºC until thawed, repeat 5x vortexing between stages. • Transfer 200 µl in a new 1.5ml tube, add 25µl of Proteinase K and vortex. • Incubate 1h at 56 °C. • Vortex. • Add 200 µl of Buffer 3, vortex and incubate at 70 °C for 10 min. • * If insoluble particles are present, spin down the samples at maximum speed for 5 min and transfer the supernatant in a new 1.5ml tube. Discard the pellet. • Add 210 µl of EtOH (100%) and vortex. • Place a spin column in a collection tube and add the whole sample (610 µl). • Centrifuge for 1 min at 11,000g; discard the flow and replace the column. • Add 500 µl of Buffer BW and centrifuge at 11,000 for 1 min. Discard the flow and replace the column. • Add 600 µl of Buffer B5, centrifuge at 11,000g for 1 min. Discard the flow and replace the column. • Centrifuge column at 11,000 g for 1 min to dry the membrane (remove the residue of ethanol). • Place the column into a 1.5 ml tube and add 100 µl prewarmed Buffer BE 70 °C. • Incubate at room temperature for 1minute. Centrifuge 1 min at 11,000 g. • Transfer the eluted DNA in a new tube

Table 2 Oligonucleotide sequences of primers used in the ITS1 n-PCR

NAME	SEQUENCE 5'-3'	REFERENCE
NN1	TCAACCTTTGAATCCAAA	(Buxton *et al.* 2001)
NN2	CGAGCCAAGACATCCATT	
NP1	GTGATAGTATCGAAAGGTAT	(Hurtado *et al.* 2001)
NP2	ACTCTCTCTCAAATGTTCCT	

EVALUATION OF PCR INHIBITORS IN POOLS OF RAW (RW) AND FINISHED WATER (FW) WITH DIFFERENT LEVELS OF TURBIDITY

Spiked environmental samples of raw (RW) and finished (FW) water have been analyzed in order to find out whether these samples contain PCR inhibitors, which have been found very frequently in other studies investigating the presence of *T. gondii* DNA in water samples (Villena *et al*., 2004; Aubert and Villena 2009). For showing whether inhibitors were more likely to be present in turbid samples than in clean ones, samples have been pooled into 3 groups characterized by different turbidity level. The turbidity level (low, medium, high) has been evaluated for each sample on the basis of its colour (no colour, yellow, dark brown), its density (liquid or muddy) and the presence or absence of debris (Table 3).

Table 3 Evaluation of turbidity level of investigating samples

N° SAMPLES	COLOR	PRESENCE OF DEBRIS	DENSITY	LEVEL OF TURBIDITY	GROUP
15	NO COLOR	NO	LIQUID	LOW	1
12	YELLOW	YES	LIQUID	MEDIUM	2
34	BROWN	YES	MUDDY	HIGH	3

From each group, a 15 ml sample was seeded in duplicate with 1000, 100 and 10 *T. gondii* oocysts (M4 strain). All samples were centrifuged at 1,250 x g at 4°C for 10 min to isolate the pellet and DNA extraction was performed using Method 2.
All samples were analyzed by the ITS1 n-PCR described above and by a quantitative real-time PCR based on the 529-bp element, in triplicate. Spiked samples that tested negative in the ITS1 n-PCR were considered to be inhibited.

The real-time PCR targets the highly conserved 529 bp fragment that is repeated 200- to 300-times in the genome of *T .gondii* (Homan *et al.* 2000). The reaction was adapted from Opsteegh *et al.* (2010a). The 529 bp element was amplified using Tox-9F and Toxo-11R, forward and reverse primers, and detected by TOX-TP1 probe; the probe was 5 'end labelled with 6-FAM and 3' end labelled with Black Hole Quencher (BHQ1). Table 4 reports the oligonucleotide sequence of primers and probes used.

A competitive internal amplification control (CIAC) was included to enable identification of false negative PCR results. This control was kindly provided by the National Institute for Public Health and Environment (RIVM, Bilthoven, The Netherlands) where it has been developed amplifying a *Yersinia pestis* Caf1 DNA construct (Janse *et al.* 2010) using overhanging primers constructed adding the binding sites for Tox-9F and Toxo-11R -primers to the 5' and 3' end of the *Y. pestis* Caf1 primer sites.
Once this PCR product arrived in our laboratory, it was re-amplified using a 20 µL reaction mixture containing:

- 0.07 µM Tox-9F and Tox-11R primers (see Table 2);
- 0.15 units BioTaq (Bioline, London, UK);
- 2 µL 10× custom PCR mix – SM0005 (Burrells *et al.* 2013);
- 2 µL DNA.

The reaction was made to a final volume of 20 µL adding DNase/RNase free water and carried out at these cycling conditions:

- 9 min at 95 °C;
- 38 cycles of 30'' at 95 °C, 30'' at 58 °C, 45'' at 72 °C;
- final extension period of 10 min at 72 °C.

The amplified product was run on a 1.5% agarose gel, stained with Gel Red (Biotium, Hayward, CA, USA) and visualized under UV light. The 188 bp long product was purified (QIAquick Gel Extraction kit, Qiagen, Venlo, The Netherlands) and its concentration determined by NanoDrop-1000 spectrophotometer (Thermo Scientific, Waltham, MA).
In the 529 bp real-time PCR, CIAC was amplified using the Tox-primers (Table 2) and detected by the CIAC-probe, which was 5' end labeled with JOE and 3' end labeled 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.
The addition of 0.0075 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.

Table 4 Oligonucleotide sequences of primers and probed using in the real-time PCR

NAME	SEQUENCE 5'-3'	5'LABEL	3' LABEL
Tox-9F	AGGAGAGATA TCAGGACTGT AG		
Tox-11R	GCGTCGTCTC GTCTAGATCG		
ToxTP1	CCGGCTTGGC TGCTTTTCCT	6-FAM	BHQ1
CIAC-probe	AGCGTACCAA CAAGTAATTC TGTATCGATG	JOE	BHQ1

PCR amplification was performed in 96-wells plates using a LightCycler480 II 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; Table 4);
- 0.05 µM of Tox-TP1 (Table 4);
- 0.2 µM of CIAC-probe (Table 4);

- 0.0075 fg of CIAC;
- 10 µl of template DNA.

The reaction mixture was made to final volume with DNase/RNase free water (Roche).

The PCR protocol consisted of these conditions:

- initial incubation at 95 °C for 10 min to activate FastStart DNA polymerase.
- 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
- 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 10-fold dilutions series of *T. gondii* DNA was included in each run for calculation of the standard curve. 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). 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 as negative. Samples without a Cp value and with a negative CIAC-PCR were considered inhibited.

Inhibited samples were retested by a modified real-time quantitative PCR on 529- bp repetitive element where the volume of template DNA was reduced from 10 µl to 2 µl and 8 µg of Bovine Serum Albumine (BSA) has been added to the reaction mixture, as reported by Villena *et al.* (2004).

No variations were made to the cycling conditions. Thus, the new 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);
- 0.05 µM of Tox-TP1;
- 0.2 µM of CIAC-probe;
- 0.0075 fg of CIAC;
- 2 µl of template DNA;
- 8 µg of BSA.

The reaction mixture was made to final volume with DNase/RNase free water (Roche). Samples were scored as positive, negative or inhibited as described above.

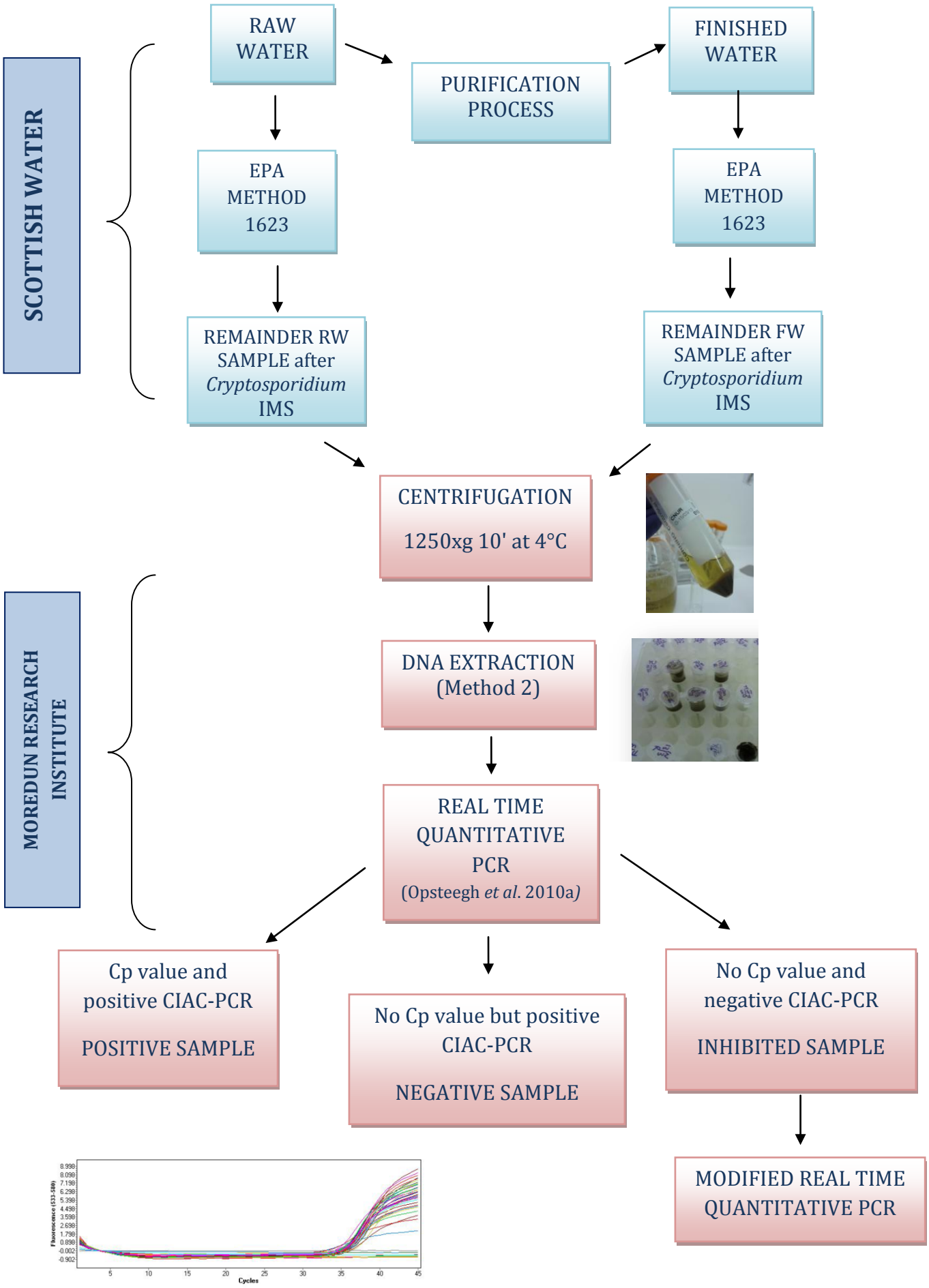
ENVIRONMENTAL MATCHED SAMPLES OF RW AND FW

From February to June 2013, 179 matched samples of raw and finished water (358 in total) were collected by Scottish Water from 68 water plants located throughout Scotland and were analyzed for the presence of *T. gondii* DNA.
With "matching samples", we indicate one sample of raw and one sample of finished water collected on the same day from the same water plant before and after the purification process, respectively. Prior to DNA extraction, the turbidity level of each sample was evaluated, and recorded, as high, medium, low, according to its colour, the presence of debris and its density (as described above).

From each sample, the DNA was extracted using Method 2 and the presence of *T. gondii* DNA was investigated using the original real-time quantitative PCR based on the 529- bp repetitive element, adapted from Opsteegh *et al.* (2010a). Samples were scored as positive, negative or inhibited and inhibited samples were retested using the modified real-time quantitative PCR protocol as previously in the above section.

DATA ANALYSIS

The data were analyzed by χ^2 test using SPSS v.17.0 statistical package.



Graph 1 Schematization of the analysis of FW and RW environmental samples by real-time PCRs

RESULTS AND DISCUSSIONS

COMPARISON OF DNA ISOLATION METHODS

Samples of sterile deionized water (DW) were seeded with a known number of purified *T. gondii* oocysts (M4 strain) in order to test the efficiency of two different commercial DNA extraction kits.

DNA extraction from *T. gondii* oocysts is critical, as the oocyst wall is very robust. Different methods have been proposed, including freeze-thawing, grinding with glass beads or sonication and lengthy proteinase K digestion (Dumètre & Dardé 2003). However, since there is no standard protocol and no optimal conditions have been identified to break open the oocyst wall, we chose to resuspend each sample in the lysis buffer supplied from each kit and to proceed with 5 freeze-thaw cycles.

The NucleoSpin ® Tissue kit (Macherey-Nagel; Method 2) proved to be more efficient showing a PCR success rate of 100% for the detection of 10^2 and 10^3 oocysts (Table 5). On the contrary, only one sample spiked with 10^3 oocysts tested positive in n-PCR using the Wizard SV Genomic DNA Purification System (Promega; Method 1). The higher efficiency of Method 2 is probably due to intrinsic characteristics of the kit as it is designed for isolation of DNA from various starting materials (including stool). Furthermore, it utilizes proteinase K digestion that has been shown to be very efficient by other authors (Villena *et al.* 2004; Aubert and Villena 2009); nevertheless it ensures appropriate conditions for binding of DNA to the silica membrane by addition of large amounts of chaotropic ions (Binding Buffer B3) and ethanol (Macherey-Nagel, 2012). However, even with Method 2, no positive results were obtained for DW samples seeded with 10 oocysts. To our knowledge, this is the first time that the The NucleoSpin ® Tissue kit (Macherey-Nagel) has been used for the DNA extraction from water samples. In other studies investigating the presence of *T.gondii* in water samples, the two methods more frequently used were the phenol-chloroform method or QIAamp mini kit (Kourenti and Karanis 2004, 2006; Dumétre and Dardè 2007; Jones and Dubey 2010). Interestingly, Yang *et al.* (2009) reported a high efficacy of the FastDNA spin kit for soil in extracting *Toxoplasma* DNA directly from spiked water concentrates.

The evaluation of the most efficient method for recovering oocysts from experimentally spiked water samples is a preliminary step necessary before starting to analyze environmental water samples. The concentration of oocysts can be done through various procedures, such as filtration, centrifugation and flocculation (Karanis *et al.,* 2013).

Filtration is utilized by the U.S. EPA Method 1623 and requires the use of specific equipment, i.e. capsules (Envirochek ™, Pall Corporation, Ann Arbor, MI) or filters (Filta-Max® foam filter) suitable for filtration of large volumes of water (U.S. Environmental Protection Agency, 2001). Villena *et al.* (2004) using Envirochek™ membrane filters obtained PCR success of 100% (10/10) and 60% (6/10) in recovering 10 and 1 oocyst per litre in spiked DW samples, respectively.

Flocculation is the concentration of oocysts with the aid of flocculants, e.g. ferric sulfate and aluminium sulfate; it is easy to perform, inexpensive and can be used on large volumes of water (Kourenti and Karanis 2004, 2006; Karanis *et al.* 2013). Kourenti *et al.* (2003) evaluated the recovery rate of two flocculation procedures and one centrifugation on 50 ml DW samples seeded with 10^4-10^5 oocysts; the flocculation was generally more successful than the centrifugation, with a recovery rate that approached 100%. However, filtration is considered more robust than chemical flocculation for turbid water processing and for field investigations (Dumètre and Dardé 2003).

We did not focus on testing different methods for concentrating oocysts from experimentally infected DW samples because the environmental samples analyzed later in this study had already been concentrated by filtration at Scottish Water's laboratories, according to the U.S. EPA Mehod 1623.
As we were more interested in the evaluation of the most efficient DNA extraction method, the recovery of the seeded oocysts was performed by the most rapid and easiest method, i.e. the centrifugation of the samples. In absence of a visible pellet, this process led to the lose of oocysts, in particular in samples seeded with a low number of parasites and this loss affected the detection limit of the ITS1 n-PCR.

Table 5 Comparison of DNA extraction methods by ITS1 n-PCR

N°.OOCYSTS / 15 ml OF DW	NO. OF POSITIVE SAMPLES IN ITS1 N-PCR / TOTAL TESTED (%POSITIVE)	
	DNA EXTRACTION METHOD 1	DNA EXTRACTION METHOD 2
10	0/3 (0%)	0/3 (0%)
10	0/3 (0%)	0/3 (0%)
10	0/3 (0%)	0/3 (0%)
100	0/3 (0%)	3/3 (100%)
100	0/3 (0%)	3/3 (100%)
100	0/3 (0%)	3/3 (100%)
1000	1/3 (33.3%)	3/3 (100%)
1000	0/3 (0%)	3/3 (100%)
1000	0/3 (0%)	3/3 (100%)

EVALUATION OF PCR INHIBITORS IN SPIKED POOLS OF RAW (RW) AND FINISHED WATER (FW) WITH DIFFERENT LEVELS OF TURBIDITY

The presence of PCR inhibitors and their relation to the grade of turbidity of water was evaluated by testing spiked environmental samples with different turbidities, using both ITS1 n-PCR and real-time quantitative PCR based on the 529-bp element, in triplicate.
The evaluation of the turbidity level was performed by visual inspection of the samples; this is a subjective method that can be affected by individual perception, especially for samples with

medium turbidity levels. The official instrument for measuring the grade of turbidity of the water is the nephelometer which quantifies the turbidity level of water in terms of nephelometric turbidity units, but it is not routinely present in bio molecular laboratories.
For samples showing low and medium turbidity level (group 1 and 2), there was no evidence of the presence of PCR inhibitors and both PCR methods (n-PCR and original real-time PCR) gave similar results, i.e. all spiked samples tested positive. All the samples with high turbidity levels tested positive by ITS1 n-PCR (18/18 replicates), whereas 14/18 replicates tested CIAC-PCR negative by the original real-time PCR (Opsteegh *et al*., 2010a), revealing the presence of PCR inhibitors (Table 6).
Thus, these samples were retested by the modified real-time PCR developed in this study and all the replicates (18/18) showed Cp- values and tested CIAC-PCR positive.
Considering the results obtained with the original real-time PCR (Opsteegh *et al.,* 2010a), samples with high turbidity level contained PCR-inhibitors. The presence of PCR- inhibitors in water represents a common problem reported in various water studies (Rochelle *et al.,* 1997; Villena *et al.,* 2004; Kourenti and Karanis 2006; Aubert and Villena 2009). Inhibitions have been ascribed to humic-type materials or other non-characterized substances that co-extract with the DNA (Rochelle *et al.* 1997; Karanis *et al.* 2013); such substances may interfere with restriction endonucleases and polymerase enzyme activity such as Taq DNA polymerase (Tebbe and Vahjen 1993; Watson and Blackwell 2000; Tsai and Olson 1992), or lead to non-specific binding of primers that reduce the sensitivity of detection (Tsai and Olson 1992). It is more likely that these substances are present in water with high turbidities (Rochelle *et al.,* 1997). In order to overcome this problem, it has been suggested that the DNA extracted from water with higher turbidities will need extra rounds of purification prior to PCR and various methods have been described for this purpose, as preparative agarose gel electrophoresis or absorption to glass particles (Rochelle *et al.* 1997). To date, the addition of BSA to the reaction mix is considered the most effective and easiest strategy for overcoming inhibitions and is widely used by researchers working with water samples (Villena *et al.* 2004; Kourenti and Karanis, 2006). The BSA has the ability to prevent the uncoupling of oxidative phosphorylation by phenols (Weinbach and Garbus 1966a, b) and prevent inhibition from samples that contain plasmin or endogenous protease activity. It also has the capacity to bind to lipids by hydrophobic forces and to bind anions (Loomis 1974). The effectiveness of the BSA was also confirmed in this study, as it overcame the inhibition problem in 100% of cases. Also the reduction of DNA from 10 µl to 2 µl positively affected the outcome because the smaller the volume of template added the lower concentration PCR- inhibitors, coextracted with the DNA, in the final PCR.

Although the results obtained by the ITS n-PCR and the real-time PCR were similar, we decided to test the environmental RW and FW samples with the 529 bp- real-time PCR. First of all, because by using a real-time PCR it is possible to obtain quantitative results, as the concentration of DNA present in the sample is calculated according to the standard curve. Secondly, the 529 bp-real-time PCR protocol had previously been shown to be highly effective in detecting *T. gondii* DNA in spiked water samples (Yang *et al.* 2009) and it significantly improved the performances of detection of *T. gondii* in clinical specimens (Switaj *et al.* 2005; Karanis *et al*. 2013). These authors compared, three real-time PCR assyas based on the B1

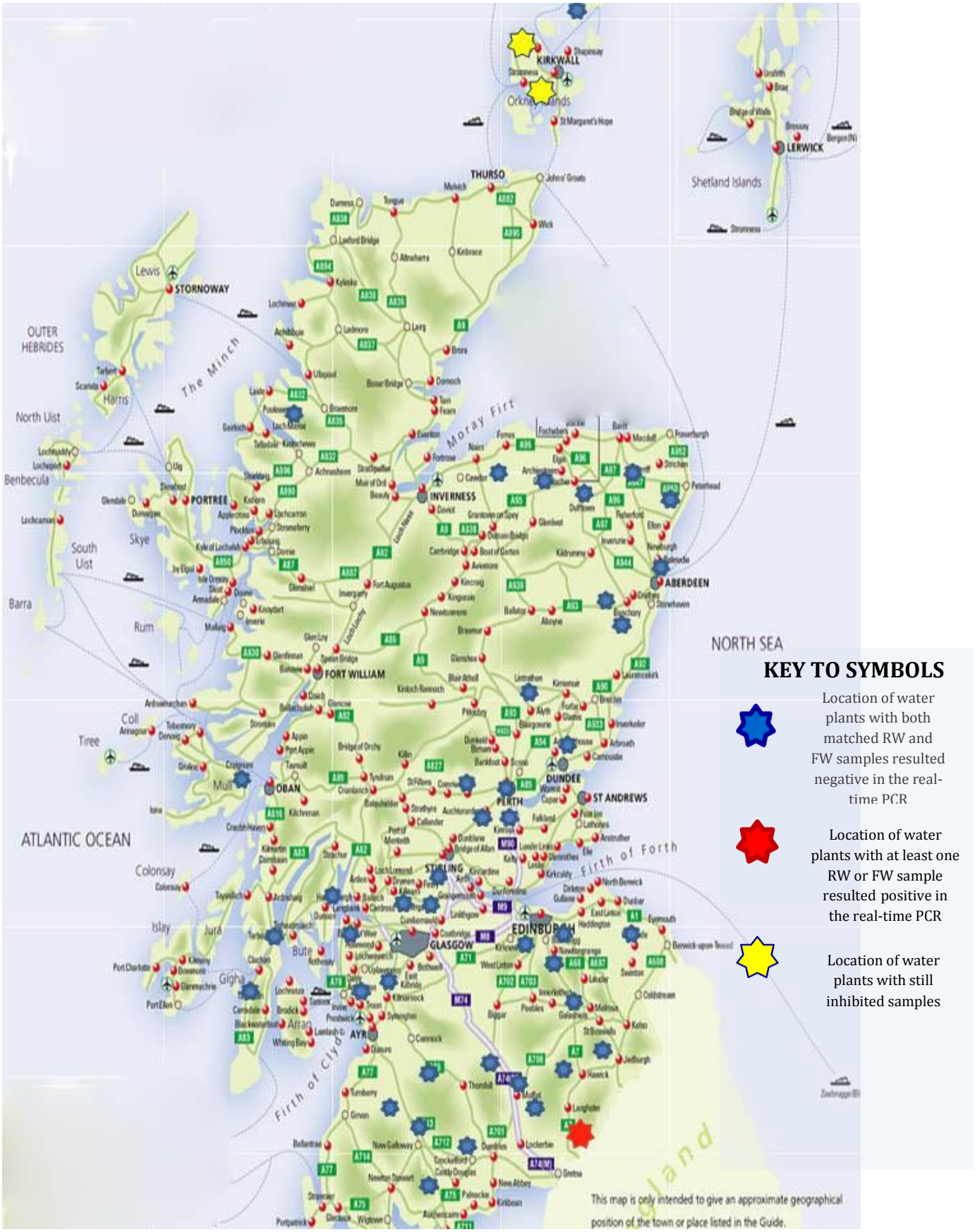
gene and a 529 bp for the detection of *T. gondii* tachyzoites and oocysts in water; all reaction mixtures included 2 µl of template and a high concentration of BSA (250 µg/ µl) for overcoming inhibitors. The authors reported that the 529-bp PCR method was not only the most effective in detecting *T. gondii* oocysts seeded in concentrates of stream water samples but also that it was the most sensitive, with a detection limit of 0.06 to 0.3 tachyzoites/PCR. Finally, because the inclusion of a competitive internal amplification control (CIAC) into the reaction mix enabled the immediate identification of false negative PCR results; this saved performing a second PCR with the addition of spiked *T. gondii* to the reaction mix or the addition of DNA from a mimetic plasmid insert to DNA samples, as reported by Kourenti and Karanis (2006) and Villena *et al.* (2004), respectively.

Table 6 Results of spiked environmental samples with different level of turbidity

GROUP	TURBIDITY LEVEL	NO. OF OOCYSTS / 15 ml	N° OF *T. gondii* POSITIVE SAMPLES / TOTAL N°. REPLICATES		N° OF SAMPLES WITH INHIBITION / TOTAL N° REPLICATES		
			ITS1	ORIGINAL REAL TIME PCR	ITS 1	ORIGINAL REAL TIME PCR	MODIFIED REAL TIME PCR
1	LOW	10	3/3	3/3	0/3	0/3	N.D*
1	LOW	10	3/3	3/3	0/3	0/3	N.D
1	LOW	100	3/3	3/3	0/3	0/3	N.D
1	LOW	100	3/3	3/3	0/3	0/3	N.D
1	LOW	1000	3/3	3/3	0/3	0/3	N.D
1	LOW	1000	3/3	3/3	0/3	0/3	N.D
2	MEDIUM	10	3/3	3/3	0/3	0/3	N.D
2	MEDIUM	10	3/3	3/3	0/3	0/3	N.D
2	MEDIUM	100	3/3	3/3	0/3	0/3	N.D
2	MEDIUM	100	3/3	3/3	0/3	0/3	N.D
2	MEDIUM	1000	3/3	3/3	0/3	0/3	N.D
2	MEDIUM	1000	3/3	3/3	0/3	0/3	N.D
3	HIGH	10	3/3	0/3	0/3	3/3	0/3
3	HIGH	10	3/3	1/3	0/3	2/3	0/3
3	HIGH	100	3/3	0/3	0/3	3/3	0/3
3	HIGH	100	3/3	0/3	0/3	3/3	0/3
3	HIGH	1000	3/3	3/3	0/3	0/3	0/3
3	HIGH	1000	3/3	0/3	0/3	3/3	0/3

*n.d: not done

Fig. 2 Geographical distribution of the water plants examined in this study



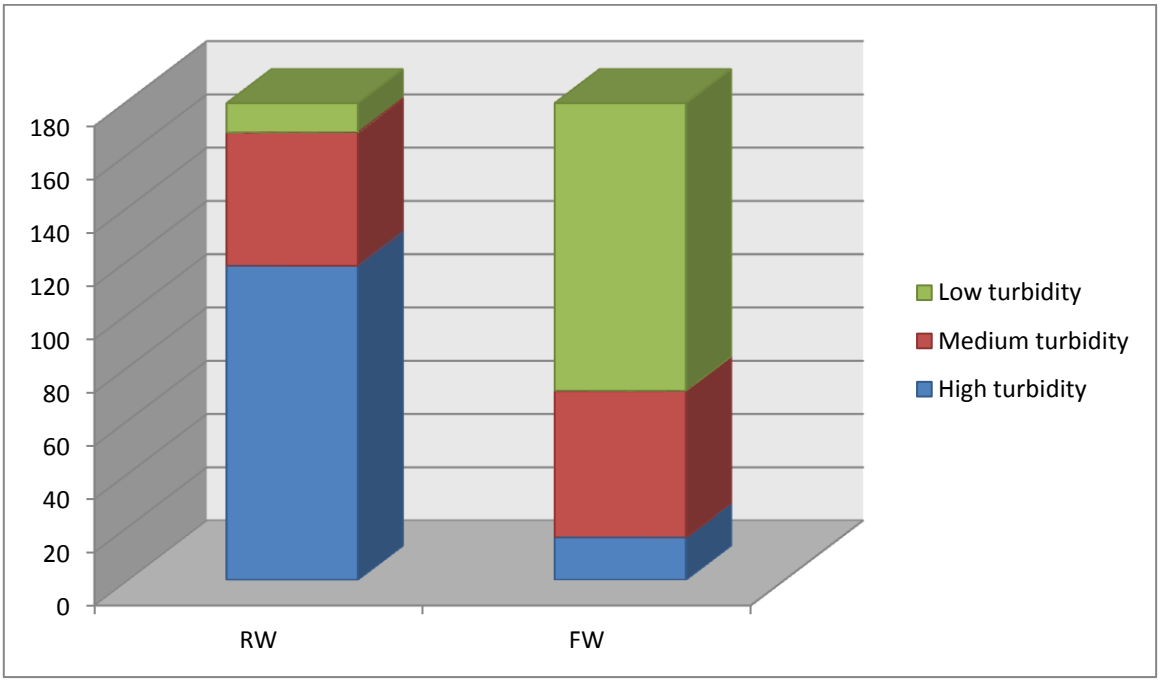
ENVIRONMENTAL MATCHED RAW AND FINISHED WATER SAMPLES ANALYSED BY REAL-TIME PCR

The 358 samples tested in this study were collected from February to June 2013 from 68 water plants under the *Cryptosporidium* monitoring plan.

Since the filtration process was already performed by Scottish Water (U.S. Environmental Protection Agency, 2001), we decided to centrifugate the samples, in order to obtain a pellet to subject directly to DNA extraction. DNA was extracted using the The NucleoSpin ® Tissue kit (Macherey-Nagel; Method 2) and parasite DNA detection was performed by a quantitative real-time PCR targeting the highly conserved 529 bp fragment and the rections included a competitive internal amplification control (CIAC) (Opsteegh *et al*.2010a). In case of negative CIAC-PCRs, the inhibited samples were retested by the modified real-time PCR proposed in this study, which included the addition of BSA to the reaction mix and a decreased volume of template. The evaluation of the turbidity level (low, medium, high) of each sample was performed by visual inspection, as described above.

The overall frequency of the turbidity level of the samples is shown in Graph 2. Raw water samples showed higher turbidity level than finished water, 65% (118/179) vs 9% (16/179); this finding was also reported by Villena *et al.* (2004), which measured the turbidity level of samples using a nephelometer and this concordance validates our method of grading turbidity.

Graph 2 Turbidity level of environmental samples examined in this study.



Fifty (50/358; 13.9%) samples contained PCR inhibitors, i.e. tested CIAC-PCR negative in the original real-time PCR (Opsteegh *et al*. 2010a). The presence of PCR-inhibitors was higher in samples with high turbidity level (29.1%; 39/134) than in samples with low-medium turbidity level (4.9%; 11/224) and also in RW samples (25%; 45/179) than in FW samples (2.8%; 5/179). These differences are statistically significant (p value < 0.0000). No statistical

significant differences in terms of presence of inhibitors were found between different water plants and different months of sampling (*p* value >0.005) (Fig.3). According to Rochelle *et al.* (1997), this finding confirms that inhibitory substances are more likely to be present in untreated water with high turbidities. Nevertheless, the fact that, among inhibited samples, 3 were matched RW and FW samples indicates that the amount of inhibitors can also depend on the water source.
The 50 inhibited samples were retested by the modified real-time PCR and the reduction of the volume of template plus the addition of BSA overcame the inhibitions in the 94% of cases but they all tested *T. gondii* negative. Three samples (6%; 3/50) still tested CIAC-PCR negative remaining uninterpretable; all these samples had a high level of turbidity and belonged to two water plants located in Mainland, Orkney Islands. This highlights once more that the presence of inhibitors in water is strictly related to the substances present in the soil that are washed out in the water.

Only one raw water sample (0.3%), out of the 358 examined, tested *T. gondii* positive a with a Cp-value of 34.32 corresponding to 822 fg of DNA, calculated according to the standard curve. However, when the sample was retested one month later, it was *T. gondii* negative.
False-positive results may occur either due to laboratory contamination or by detection of free DNA. In our experiments, negative controls were included in each examination and were all constantly negative. As additional explanation, it is likely that *T. gondii* DNA was present in the sample but in a very low concentration; this will limit the reproducibility of the result. This fact could be further enhanced by an inappropriate mixing of the extracted sample prior to the collection of the 2 µl of DNA destined for the reaction mix. A continuation of the study by another researcher within the group has obtained further *T. gondii* DNA positive samples from the same water treatment plant, giving support that this was a true positive result.
Considering this positive result as a true positive and comparing this detection rate of 0.3% (1/358) with the others published in literature in the last decade, this result is the lowest. In fact, in France, Villena *et al.* (2004) detected *Toxoplasma* DNA in 10 samples out the 125 analyzed (8%): positive samples were underground water (6/10), raw surface water (3/10) and one sample of public drinking water. In order to confirm PCR results and to determine the infectivity of recovered oocysts, all the samples were also tested by bioassay in mice, but none of the *T. gondii* PCR-positive resulted positive by bioassay. Still in France but few years later, Aubert and Villena (2009) detected *Toxoplasma* in 7.7% (37/482) samples of underground, raw surface and public drinking water but again none of the PCR positive samples resulted positive by bioassay in mice. The main reason of this failure could be the detection of DNA from non-infective oocysts present in the environment or DNA contamination in PCR. Also Kourenti and Karanis (2006) did not obtain positive results when they retested their 4 (4/60) water samples that tested positive in the first PCR examination; the positive samples consisted of: one sample of river water, one of well water, one of tap water and one of sewage water. Sroka *et al.* 2006 analyzed by PCR a total of 114 drinking water samples collected from wells (shallow and deep) and water supplies located on Polish farms; all the *Toxoplasma*-positive samples (27.2%; 31/114) were taken from wells and the majority of water samples originated from shallow wells. Sotiriadou and Karanis (2008) reported positive samples in 48% (25/52) of water samples (river, lake and tap waters), collected in Bulgaria and Russia,

using a LAMP-specific protocol targeting the *Tg*OWP and B1 *Toxoplasma* genes; most of positive samples were river waters (28.8%; 15/52) but *T. gondii* DNA was also found in 2 (2/52; 3.8%) samples of tap water. Through the same LAMP-protocol, Gallas-Lindermann *et al*. (2013) found 8 (8/95; 8.4%) positive water samples collected from the Lower Rhine area in Germany; all the positive samples were influent and effluent samples obtained from wastewater treatment plants. Koloren and Demirel (2013) detected, again by LAMP, *T. gondii* DNA in 20 (20/56; 35.7%) river water samples.

Considering the origin of the *T. gondii* positive water samples found in these studies, it is not surprising that the majority were raw surface water, underground water, river and sewage water samples. In fact raw surface water can be easily contaminated by soil after peaks in rainfall (Bowie *et al.* 1997) and also underground water is considered a vulnerable resource in relation to its frequent contamination by other waterborne pathogens suchas *Cryptosporidium* and *Giardia* (Aubert and Villena 2009); the contamination of wastewater with *Toxoplasma* oocysts may be due to the disposal of waste from cat litter boxes into toilettes (Jones and Dubey 2010; Gallas-Lindermann *et al.* 2013).

The fact that some authors found *T. gondii* DNA in public drinking water is more surprising, but, as reported above, DNA amplification may be due to the detection of dead oocysts (Villena *et al.* 2004; Aubert and Villena 2009) and, in that case, water does not pose a risk for consumers. However, as oocysts are highly resistant to the various inactivation procedures based on chemical reagents and disinfection processes used by water utilities (Dubey *et al.,* 1970b, 1998), a PCR positive finding in public drinking water samples should be always confirmed by further investigation of the infectivity of the detected oocysts. To date, mouse bioassay is still considered the reference method to detect the viability of *Toxoplasma* oocysts in meat (Opsteegh *et al.* 2010a) and water (Karanis *et al.* 2013); this method is not only time consuming, as 7 days is required for sporulation before the inoculation and an additional 4 weeks is required to obtain the immunological results, but it is also expensive (Fayer 2004; Shapiro *et al.* 2010), unavailable in many laboratories (Fayer *et al.* 2004) and uses many animals in order to determine viability. Furthermore, bioassay results using environmental water samples can be disappointing, as reported by various authors (Dubey *et al.* 1996; Isaac-Renton *et al.* 1998; Villena *et al.* 2004; Aubert and Villena 2009). However, the loss of infectivity of oocysts may not be a true representation but could be caused by vigorous shaking for consistent elution of oocysts from filters (Simmons *et al.* 2001), the low pathogenicity of *Toxoplasma* strains or the low infective dose (Karanis *et al.* 2013). For public health purposes, it could be beneficial to develop a quantitative reverse transcription-PCR method to differentiate between viable and nonviable oocysts detected in environmental samples in order to substitute the mouse bioassays (Villena *et al.* 2004).

Based on detection rates of *T. gondii* DNA in water samples collected in different parts of Europe, the scenario that comes to a light is highly heterogeneous. Similarly to what has been reported for worldwide seroprevalence values (Tenter *et al.* 2000), these detection rates are difficult to compare because different methods were utilized for the concentration of the water samples and different PCRs protocols were used for the detection of *T. gondii* DNA. In the most recent studies (Sotiriadou and Karanis, 2008; Gallas-Lindermann *et al*. 2013; Koloren and Demirel 2013), a new molecular assay is used for the detection of *Toxoplasma* in

environmental samples, i.e. a loop-mediated isothermal amplification (LAMP); this was first developed by Notomi *et al.* (2000). This method is reported to be highly specific, efficient, simple and rapid as the amplification runs under isothermal conditions (Karanis *et al.* 2013) and, by adding a fluorescent metal indicator to the reaction solution, results can be determined by visual inspection without the need of specialized equipment (Notomi *et al*., 2000; Tomita *et al.* 2008; Karanis and Ongerth, 2009; Fu *et al*. 2011). Furthermore, the LAMP assay is more sensitive than PCR because inhibitors do not affect the LAMP reaction, as described by Bakheit *et al*. (2008) in fecal samples and by Koloren *et al*. (2011) in surface water samples.

Despite the detection methods used, differences in detection rates of *Toxoplasma* in water samples are also the result of three combined environmental factors, i.e. oocyst load, oocyst survival and oocyst transport (VanWormer *et al.* 2013). Oocyst load depends on the distribution of wild and domestic felids and on the prevalence, the frequency, the duration and the quantity of oocysts being shed. The survival of oocysts depends on terrestrial conditions (soil chemistry and moisture, air temperature and humidity) and aquatic conditions. Finally, the transport and accumulation of oocysts in water is determined by felid behavior, terrestrial conditions (watershed, permeability of the substrate), precipitation patterns (quantity, intensity, frequency and duration) and aquatic conditions (water chemistry, water quality, flow rate) (VanWormer *et al.* 2013).

In light of what has been reported until now, some considerations on our results can be drawn, as the *T. gondii* prevalence found in this study is close to 0%.

First of all, this can reflect a poor recovery of oocysts from concentrated water samples. We performed DNA exctraction directly from concentrated pellet. Although this procedure provided good results in spiked water samples, as also reported by Yang *et al.*(2009), it is likely that further experiments are needed to improve the recovery step and to find out if these results are realistic. In order to improve the oocysts recovery rate, some authors used sucrose flotation method (Villena *et al.* 2004; Sroka *et al.* 2006; Aubert and Villena 2009) or purification by discontinuous Sheather's sugar gradient solution (Kourenti *et al.* 2003; Kourenti ans Karanis 2006; Gallas-Lindemann *et al.* 2013) reporting to recover 78-100% of sporulated or unsporulated *T. gondii* oocysts from spiked water concentrates; however, the recovery was also influenced by the amounts of contaminating debris (Kourenti and Karanis 2006). In addition, Dumètre and Dardé (2003) demonstrated that recovery rates of oocysts from concentrated water samples was affected by the "age" of oocysts, where older oocysts are recovered with more difficulty than fresh oocyts due to their fragility and density. The turning point will be the developing of a standardized protocolusing Immunomagnetic Separation (IMS) techniques together with the commercialization of immunofluorescence staining reagents that specifically detect *Toxoplasma* oocysts from concentrated water samples. These techniques are used for *Cryptosporidium* and *Giardia* detection in environmental samples with good recovery rates (U.S. Environmental Protection Agency,, 2001; Aubert and Villena 2009). Preliminary studies on the IMS has been performed by Dumètre and Dardé in 2005 and 2007. In the first study, the authors used a monoclonal antibody directed against the oocysts wall, recovering 45 to 83% of oocysts under experimental conditions; in 2007, they tested another monoclonal antibody targeting the

sporocyst wall. However, due to cross-reactions with multiple organisms related to *Toxoplasma*, PCR was still necessary to characterize detected sporocysts, so its effectiveness in environmental waters remains to be evaluated.

Additionally, our results can be considered realistic and the prevalence of *T. gondii* in water samples collected between late winter and early summer is really close to 0%. This statement can be dawn considering the three factors influencing the level of oocysts environmental contamination, i.e. oocysts shedding, surviving and transport. Scotland is generally not affected by the problem of stray cat population either in urbanized areas and in areas where water reservoirs are located, i.e. mainly in Central and Southern of Scotland. The exception is represented by the Scottish Highlands where the last remaining large predators of Britain, i.e. Scottish Wildcats (*Felis silvestris grampia)*, are confined and where there are thought to live at least 100,000 feral hybrid cats, results of the mating between Scottish Wildcats and domestic cats (www.scottishwildcats.co.uk). In relation to the high predation attitude of the Scottish Wildcats, mainly on rabbits and other small to medium size prey species, they are likely to become infected and shed *T. gondii* oocysts. Wild cats, included *Felis silvestris*, can shed oocysts (Jones and Dubey, 2010). It is well known that young cats, and in particular feral kittens, may shed higher number of oocysts following primary infection compared to adults (Dubey and Beattie 1988). In light of this, we can suppose that kittens of Scottish Wildcats can play the key role in *T. gondii* shedding. In relation to their biology, this hypothetically happens when they are around 5 or 6 months of age when they head out independently and start their hunting actively (www.scottishwildcats.co.uk). As kittens are born in early spring, the supposed shedding period could be dated to between August and September, i.e. after we finished analyzing water samples in this study. Furthermore, most rainfall in Scotland is seen in September to November (www.weather-and-climate.com) and, as previously reported, transport and accumulation of oocysts in water is determined not only by terrestrial conditions (watershed, permeability of the substrate), but also by precipitation patterns (quantity, intensity, frequency and duration) (VanWormer *et al*. 2013).

Evidence that the above speculations are correct and directly relevant for Scottish water samples came from a continuation of the study, by scientists at Moredun. They confirmed that there was an increase in the detection of *T. gondii* positive water samples later within the year, where 8.2% of samples collected during July to October tested positive for *T. gondii* DNA. Even during this sampling period an increase in the detection rate was observed during October, which coincided with an increase in rain fall. This could be further substantiated by looking at localised rainfall events in the catchments of specific water treatment plants where detection peaks were directly linked to heavy rain fall events (Katzer *et al*, personal communications).

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REFERENCES

Abu-Dalbouh, M. A., M. M. Ababneh, N. D. Giadinis and S. Q. Lafi (2012). "Ovine and caprine toxoplasmosis (Toxoplasma gondii) in aborted animals in Jordanian goat and sheep flocks." Trop Anim Health Prod **44**(1): 49-54.

AFSSA (2005). "Toxoplasmose: état des connaissances et évaluation du risque lié à l'alimentation – rapport du groupe de travail "Toxoplasma gondii" de l'Afssa." 328.

Ahmed, Y. F., S. M. Sokkar, H. M. Desouky and A. H. Soror (2008). "Abortion Due to Toxoplasmosis in Small Ruminants." Global Veterinaria **2**(6): 337-342.

Ajzenberg, D. (2010). "Type I strains in human toxoplasmosis: myth or reality?" Future Microbiol **5**(6): 841-843.

Ajzenberg, D. (2011). "Unresolved questions about the most successful known parasite." Expert Rev Anti Infect Ther **9**(2): 169-171.

Al-Khalidi, N. W. and J. P. Dubey (1979). "Prevalence of Toxoplasma gondii infection in horses." J Parasitol **65**(2): 331-334.

Aleandri, M., E. Lillini, S. Piragino, E. Adorisio and O. Zardi (1978). "Isolamento di Toxoplasma gondii da feto equino." Proc Societá Italiana Science Veterinarie **32**: 364.

Alfonso, Y., J. Fraga, N. Jimenez, C. Fonseca, A. J. Dorta-Contreras, R. Cox, . . . D. Ginorio (2009). "Detection of Toxoplasma gondii in cerebrospinal fluid from AIDS patients by nested PCR and rapid identification of type I allele at B1 gene by RFLP analysis." Exp Parasitol **122**(3): 203-207.

Alvarado-Esquivel, C., S. Rodriguez-Pena, I. Villena and J. P. Dubey (2012). "Seroprevalence of Toxoplasma gondii infection in domestic horses in Durango State, Mexico." J Parasitol **98**(5): 944-945.

Arkush, K. D., M. A. Miller, C. M. Leutenegger, I. A. Gardner, A. E. Packham, A. R. Heckeroth, . . . P. A. Conrad (2003). "Molecular and bioassay-based detection of Toxoplasma gondii oocyst uptake by mussels (Mytilus galloprovincialis)." Int J Parasitol **33**(10): 1087-1097.

Aspinall, T. V., D. Marlee, J. E. Hyde and P. F. Sims (2002). "Prevalence of Toxoplasma gondii in commercial meat products as monitored by polymerase chain reaction--food for thought?" Int J Parasitol **32**(9): 1193-1199.

Asthana, S. P., C. N. Macpherson, S. H. Weiss, R. Stephens, T. N. Denny, R. N. Sharma and J. P. Dubey (2006). "Seroprevalence of Toxoplasma gondii in pregnant women and cats in Grenada, West Indies." J Parasitol **92**(3): 644-645.

Aubert, D., D. Ajzenberg, C. Richomme, E. Gilot-Fromont, M. E. Terrier, C. de Gevigney, . . . I. Villena (2010). "Molecular and biological characteristics of Toxoplasma gondii isolates from wildlife in France." Vet Parasitol **171**(3-4): 346-349.

Aubert, D. and I. Villena (2009). "Detection of Toxoplasma gondii oocysts in water: proposition of a strategy and evaluation in Champagne-Ardenne Region, France." Mem Inst Oswaldo Cruz **104**(2): 290-295.

Bakheit, M. A., D. Torra, L. A. Palomino, O. M. Thekisoe, P. A. Mbati, J. Ongerth and P. Karanis (2008). "Sensitive and specific detection of Cryptosporidium species in PCR-negative samples by loop-mediated isothermal DNA amplification and confirmation of generated LAMP products by sequencing." Vet Parasitol **158**(1-2): 11-22.

Baril, L., T. Ancelle, V. Goulet, P. Thulliez, V. Tirard-Fleury and B. Carme (1999). "Risk factors for Toxoplasma infection in pregnancy: a case-control study in France." Scand J Infect Dis **31**(3): 305-309.

Barnes, T. S., A. W. Goldizen, J. M. Morton and G. T. Coleman (2010). "Parasites of the brush-tailed rock-wallaby (Petrogale penicillata)." J Wildl Dis **46**(1): 218-228.

Barragan, A. and L. D. Sibley (2002). "Transepithelial migration of Toxoplasma gondii is linked to parasite motility and virulence." J Exp Med **195**(12): 1625-1633.

Bellini, S., L. Alborali, I. Massirio and C. S. (2010). Evoluzione del comparto suinicolo in Italia: criticità e fattori di rischio. L'Osservatorio. Brescia, Istituto Zooprofilattico Sperimentale della Lombardia ed Emilia Romagna "Bruno Ubertini". **2:** 1-8.

Benard, A., E. Petersen, R. Salamon, G. Chene, R. Gilbert and L. R. Salmi (2008). "Survey of European programmes for the epidemiological surveillance of congenital toxoplasmosis." Euro Surveill **13**(15).

Benenson, M. W., E. T. Takafuji, S. M. Lemon, R. L. Greenup and A. J. Sulzer (1982). "Oocyst-transmitted toxoplasmosis associated with ingestion of contaminated water." N Engl J Med **307**(11): 666-669.

Beral, M., S. Rossi, D. Aubert, P. Gasqui, M. E. Terrier, F. Klein, . . . E. Jourdain (2012). "Environmental factors associated with the seroprevalence of Toxoplasma gondii in Wild Boars (Sus scrofa), France." Ecohealth **9**(3): 303-309.

Berdoy, M., J. P. Webster and D. W. Macdonald (1995). "Parasite-altered behaviour: is the effect of Toxoplasma gondii on Rattus norvegicus specific?" Parasitology **111 (Pt 4)**: 403-409.

Berdoy, M., J. P. Webster and D. W. Macdonald (2000). "Fatal attraction in rats infected with Toxoplasma gondii." Proc Biol Sci **267**(1452): 1591-1594.

Berger, F., V. Goulet, Y. Le Strat and J. C. Desenclos (2009). "Toxoplasmosis among pregnant women in France: risk factors and change of prevalence between 1995 and 2003." Rev Epidemiol Sante Publique **57**(4): 241-248.

Berger-Schoch, A. E., D. Bernet, M. G. Doherr, B. Gottstein and C. F. Frey (2011). "Toxoplasma gondii in Switzerland: a serosurvey based on meat juice analysis of slaughtered pigs, wild boar, sheep and cattle." Zoonoses Public Health **58**(7): 472-478.

Bezerra, M. J., P. C. Kim, E. P. Moraes, S. G. Sa, P. P. Albuquerque, J. G. Silva, B. H. Alves and R. A. Mota (2013). "Detection of Toxoplasma gondii in the milk of naturally infected goats in the Northeast of Brazil." Transbound Emerg Dis.

Blewett, D. A. and A. J. Trees (1987). "The epidemiology of ovine toxoplasmosis with especial respect to control." Br Vet J **143**(2): 128-135.

Boia, M. N., F. A. Carvalho-Costa, F. C. Sodre, G. M. Pinto and M. R. Amendoeira (2008). "Seroprevalence of Toxoplasma gondii infection among indian people living in Iauarete, Sao Gabriel da Cachoeira, Amazonas, Brazil." Rev Inst Med Trop Sao Paulo **50**(1): 17-20.

Bonhomme, A., L. Pingret and J. M. Pinon (1992). "Review: Toxoplasma gondii cellular invasion." Parassitologia **34**(1-3): 31-43.

Boyer, K., D. Hill, E. Mui, K. Wroblewski, T. Karrison, J. P. Dubey, . . . R. McLeod (2011). "Unrecognized ingestion of Toxoplasma gondii oocysts leads to congenital toxoplasmosis and causes epidemics in North America." Clin Infect Dis **53**(11): 1081-1089.

Bowie, W. R., A. S. King, D. H. Werker, J. L. Isaac-Renton, A. Bell, S. B. Eng and S. A. Marion (1997). "Outbreak of toxoplasmosis associated with municipal drinking water. The BC Toxoplasma Investigation Team." Lancet **350**(9072): 173-177.

Brezin, A. P., C. E. Egwuagu, M. Burnier, Jr., C. Silveira, R. M. Mahdi, R. T. Gazzinelli, . . . R. B. Nussenblatt (1990). "Identification of Toxoplasma gondii in paraffin-embedded sections by the polymerase chain reaction." Am J Ophthalmol **110**(6): 599-604.

Bridle, H., M. Kersaudy-Kerhoas, B. Miller, D. Gavriilidou, F. Katzer, E. A. Innes and M. P. Desmulliez (2012). "Detection of Cryptosporidium in miniaturised fluidic devices." Water Res **46**(6): 1641-1661.

Buffolano, W., R. E. Gilbert, F. J. Holland, D. Fratta, F. Palumbo and A. E. Ades (1996). "Risk factors for recent toxoplasma infection in pregnant women in Naples." Epidemiol Infect **116**(3): 347-351.

Burg, J. L., C. M. Grover, P. Pouletty and J. C. Boothroyd (1989). "Direct and sensitive detection of a pathogenic protozoan, Toxoplasma gondii, by polymerase chain reaction." J Clin Microbiol **27**(8): 1787-1792.

Burnett, A. J., S. G. Shortt, J. Isaac-Renton, A. King, D. Werker and W. R. Bowie (1998). "Multiple cases of acquired toxoplasmosis retinitis presenting in an outbreak." Ophthalmology **105**(6): 1032-1037.

Burrells, A., P. M. Bartley, I. A. Zimmer, S. Roy, A. C. Kitchener, A. Meredith, . . . F. Katzer (2013). "Evidence of the three main clonal Toxoplasma gondii lineages from wild mammalian carnivores in the UK." Parasitology **140**(14): 1768-1776.

Buxton, D. (1990). "Ovine toxoplasmosis: a review." J R Soc Med **83**(8): 509-511.

Buxton, D. (1998). "Protozoan infections (Toxoplasma gondii, Neospora caninum and Sarcocystis spp.) in sheep and goats: recent advances." Vet Res **29**(3-4): 289-310.

Buxton, D. and E. A. Innes (1995). "A commercial vaccine for ovine toxoplasmosis." Parasitology **110 Suppl**: S11-16.

Buxton, D., S. W. Maley, P. P. Pastoret, B. Brochier and E. A. Innes (1997). "Examination of red foxes (Vulpes vulpes) from Belgium for antibody to Neospora caninum and Toxoplasma gondii." Vet Rec **141**(12): 308-309.

Buxton, D., S. W. Maley, S. E. Wright, S. Rodger, P. Bartley and E. A. Innes (2007). "Toxoplasma gondii and ovine toxoplasmosis: new aspects of an old story." Vet Parasitol **149**(1-2): 25-28.

Buxton, D., S. M. Rodger, S. W. Maley and S. E. Wright (2006). "Toxoplasmosis: The possibility of vertical transmission." Small Ruminant Research **62**: 43-46.

Buxton, D., S. Wright, S. W. Maley, A. G. Rae, A. Lunden and E. A. Innes (2001). "Immunity to experimental neosporosis in pregnant sheep." Parasite Immunol **23**(2): 85-91.

Camossi, L. G., H. Greca-Junior, A. P. Correa, V. B. Richini-Pereira, R. C. Silva, A. V. Da Silva and H. Langoni (2011). "Detection of Toxoplasma gondii DNA in the milk of naturally infected ewes." Vet Parasitol **177**(3-4): 256-261.

Canfield, P. J., W. J. Hartley and J. P. Dubey (1990). "Lesions of toxoplasmosis in Australian marsupials." J Comp Pathol **103**(2): 159-167.

Carme, B., F. Bissuel, D. Ajzenberg, R. Bouyne, C. Aznar, M. Demar, S. Bichat, D. Louvel, A. M. Bourbigot, C. Peneau, P. Neron and M. L. Darde (2002). "Severe acquired toxoplasmosis in immunocompetent adult patients in French Guiana." J Clin Microbiol **40**(11): 4037-4044.

Cavalcante, G. T., D. M. Aguilar, L. M. Camargo, M. B. Labruna, H. F. de Andrade, L. R. Meireles, . . . S. M. Gennari (2006). "Seroprevalence of Toxoplasma gondii antibodies in humans from rural Western Amazon, Brazil." J Parasitol **92**(3): 647-649.

Cenci-Goga, B. T., A. Ciampelli, P. Sechi, F. Veronesi, I. Moretta, V. Cambiotti and P. N. Thompson (2013). "Seroprevalence and risk factors for Toxoplasma gondii in sheep in Grosseto district, Tuscany, Italy." BMC Vet Res **9**: 25.

Cenci-Goga, B. T., P. V. Rossitto, P. Sechi, C. M. McCrindle and J. S. Cullor (2011). "Toxoplasma in animals, food, and humans: an old parasite of new concern." Foodborne Pathog Dis **8**(7): 751-762.

Chanton-Greutmann, H., R. Thoma, L. Corboz, N. Borel and A. Pospischil (2002). "[Abortion in small ruminants in Switzerland: investigations during two lambing seasons (1996-1998) with special regard to chlamydial abortions]." Schweiz Arch Tierheilkd **144**(9): 483-492.

Chartier, C. and M. P. Mallereau (2001). "Efficacite vaccinale de la souche S48 de Toxoplasma gondii vis-a-vis d'une infection experimentale chez le chevre." Ann Méd Vét **145**: 202-209.

Chessa, G., V. Chisu, R. Porcu and G. Masala (2014). "Molecular characterization of Toxoplasma gondii Type II in sheep abortion in Sardinia, Italy." Parasite **21**: 6.

Chiari Cde, A. and D. P. Neves (1984). "[Human toxoplasmosis acquired by ingestion of goat's milk]." Mem Inst Oswaldo Cruz **79**(3): 337-340.

Chikweto, A., S. Kumthekar, K. Tiwari, B. Nyack, M. S. Deokar, G. Stratton, . . . J. P. Dubey (2011). "Seroprevalence of Toxoplasma gondii in pigs, sheep, goats, and cattle from Grenada and Carriacou, West Indies." J Parasitol **97**(5): 950-951.

Chinchilla, M., O. M. Guerrero, et al. (1993). "Natural and induced blood dissemination of Toxoplasma gondii: experimental model in white mice and hamsters." Rev Biol Trop **41**(2): 197-202.

Choi, W. Y., H. W. Nam, N. H. Kwak, W. Huh, Y. R. Kim, M. W. Kang, . . . J. P. Dubey (1997). "Foodborne outbreaks of human toxoplasmosis." J Infect Dis **175**(5): 1280-1282.

Conrad, P. A., M. A. Miller, C. Kreuder, E. R. James, J. Mazet, H. Dabritz, D. A. Jessup, F. Gulland and M. E. Grigg (2005). "Transmission of Toxoplasma: clues from the study of sea otters as sentinels of Toxoplasma gondii flow into the marine environment." Int J Parasitol **35**(11-12): 1155-1168.

Cook, A. J., R. E. Gilbert, W. Buffolano, J. Zufferey, E. Petersen, P. A. Jenum, . . . D. T. Dunn (2000). "Sources of toxoplasma infection in pregnant women: European multicentre case-control study. European Research Network on Congenital Toxoplasmosis." BMJ **321**(7254): 142-147.

Cortini, M. (1999). L'allevamento degli ovini nel Forlivese e nel Cesenate. Agricoltura**:** 27-29.

da Silva, A. V. and H. Langoni (2001). "The detection of Toxoplasma gondii by comparing cytology, histopathology, bioassay in mice, and the polymerase chain reaction (PCR)." Vet Parasitol **97**(3): 191-198.

Dabritz, H. A., E. R. Atwill, I. A. Gardner, M. A. Miller and P. A. Conrad (2006). "Outdoor fecal deposition by free-roaming cats and attitudes of cat owners and nonowners toward stray pets, wildlife, and water pollution." J Am Vet Med Assoc **229**(1): 74-81.

Dabritz, H. A. and P. A. Conrad (2010). "Cats and Toxoplasma: implications for public health." Zoonoses Public Health **57**(1): 34-52.

Dalimi, A. and A. Abdoli (2012). "Latent toxoplasmosis and human." Iran J Parasitol **7**(1): 1-17.

Darde, M. L., B. Bouteille and M. Pestre-Alexandre (1992). "Isoenzyme analysis of 35 Toxoplasma gondii isolates and the biological and epidemiological implications." J Parasitol **78**(5): 786-794.

de Camps, S., J. P. Dubey and W. J. Saville (2008). "Seroepidemiology of Toxoplasma gondii in zoo animals in selected zoos in the midwestern United States." J Parasitol **94**(3): 648-653.

De Capraris, D. and E. Gravino (1981). "Diffusione della toxoplasmosi nella specie caprina: indagine sieroepidemiologica su un campione di animali del basso Lazio. ." Acta Med Vet **27**: 261-261.

De Craeye, S., N. Speybroeck, D. Ajzenberg, M. L. Darde, F. Collinet, P. Tavernier, . . . K. Dierick (2011). "Toxoplasma gondii and Neospora caninum in wildlife: common parasites in Belgian foxes and Cervidae?" Vet Parasitol **178**(1-2): 64-69.

De Luca, G. (2004). L'allevamento della capra: gestione, produzione e trasformazione del latte, EdAgricole.

de Macedo, M. F., C. A. de Macedo, M. P. Ewald, G. F. Martins, D. L. Zulpo, I. A. da Cunha, A. Taroda, S. T. Cardim, C. Su and J. L. Garcia (2012). "Isolation and genotyping of Toxoplasma gondii from pregnant dairy cows (Bos taurus) slaughtered." Rev Bras Parasitol Vet **21**(1): 74-77.

de Moraes, E. P., M. M. da Costa, A. F. Dantas, J. C. da Silva and R. A. Mota (2011). "Toxoplasma gondii diagnosis in ovine aborted fetuses and stillborns in the State of Pernambuco, Brazil." Vet Parasitol **183**(1-2): 152-155.

de Moura, L., L. M. Bahia-Oliveira, M. Y. Wada, J. L. Jones, S. H. Tuboi, E. H. Carmo, . . . D. O. Garrett (2006). "Waterborne toxoplasmosis, Brazil, from field to gene." Emerg Infect Dis **12**(2): 326-329.

de Oliveira Mendonca, A., P. F. Domingues, A. V. da Silva and S. B. Pezerico (2004). "Detection of Toxoplasma gondii in swine sausages." Parasitol. Latinoam. **59**: 42-45.

De Paschale, M., C. Agrappi, P. Clerici, P. Mirri, M. T. Manco, S. Cavallari and E. F. Viganò (2006). "Prevalence of Toxoplasma gondii infection in the Italian and foreign female population living in the area of Legnano (Milan)." Microbiol Med **21**: 322-327.

De Paschale, M., C. Agrappi, P. Clerici, P. Mirri, M. T. Manco, S. Cavallari and E. F. Vigano (2008). "Seroprevalence and incidence of Toxoplasma gondii infection in the Legnano area of Italy." Clin Microbiol Infect **14**(2): 186-189.

Decoster, A., B. Slizewicz, J. Simon, C. Bazin, F. Darcy, G. Vittu, . . . et al. (1991). "Platelia-Toxo IgA, a new kit for early diagnosis of congenital toxoplasmosis by detection of anti-P30 immunoglobulin A antibodies." J Clin Microbiol **29**(10): 2291-2295.

Dehkordi, F. S., M. R. Borujeni, E. Rahimi and R. Abdizadeh (2013). "Detection of Toxoplasma gondii in raw caprine, ovine, buffalo, bovine, and camel milk using cell cultivation, cat bioassay, capture ELISA, and PCR methods in Iran." Foodborne Pathog Dis **10**(2): 120-125.

Delair, E., D. Monnet, S. Grabar, J. Dupouy-Camet, H. Yera and A. P. Brezin (2008). "Respective roles of acquired and congenital infections in presumed ocular toxoplasmosis." Am J Ophthalmol **146**(6): 851-855.

Deliberazione della Giunta Regionale 27/06/2011, n. (2011). PIANO REGIONALE di CONTROLLO DELLA BRUCELLOSI OVINA E CAPRINA (Num. Reg. Proposta: GPG/2011/798).

Demar, M., D. Ajzenberg, D. Maubon, F. Djossou, D. Panchoe, W. Punwasi, N. Valery, C. Peneau, J. L. Daigre, C. Aznar, B. Cottrelle, L. Terzan, M. L. Darde and B. Carme (2007). "Fatal outbreak of human toxoplasmosis along the Maroni River: epidemiological, clinical, and parasitological aspects." Clin Infect Dis **45**(7): e88-95.

Desmonts, G., J. Couvreur, F. Alison, J. Baudelot, J. Gerbeaux and M. Lelong (1965). "[Epidemiological study on toxoplasmosis: the influence of cooking slaughter-animal meat on the incidence of human infection]." Rev Fr Etud Clin Biol **10**(9): 952-958.

Desmonts, G., Y. Naot and J. S. Remington (1981). "Immunoglobulin M-immunosorbent agglutination assay for diagnosis of infectious diseases: diagnosis of acute congenital and acquired Toxoplasma infections." J Clin Microbiol **14**(5): 486-491.

Diza, E., F. Frantzidou, E. Souliou, M. Arvanitidou, G. Gioula and A. Antoniadis (2005). "Seroprevalence of Toxoplasma gondii in northern Greece during the last 20 years." Clin Microbiol Infect **11**(9): 719-723.

Dohoo, I., W. Martin and H. Stryhn (2003). Veterinary Epidemiologic Research. Charlottetown, AVC Inc.

Dubey, J. P. (1980a). "Mouse pathogenicity of Toxoplasma gondii isolated from a goat." Am J Vet Res **41**(3): 427-429.
Dubey, J. P. (1980b). "Persistence of encysted Toxoplasma gondii in caprine livers and public health significance of toxoplasmosis in goats." J Am Vet Med Assoc **177**(12): 1203-1207.
Dubey, J. P. (1982). "Repeat transplacental transfer of Toxoplasma gondii in dairy goats." J Am Vet Med Assoc **180**(10): 1220-1221.
Dubey, J. P. (1984). "Experimental toxoplasmsosis in sheep fed Toxoplasma gondii oocysts." Int Goat Sheep Res **2**: 93-104.
Dubey, J. P. (1986). "A review of toxoplasmosis in cattle." Vet Parasitol **22**(3-4): 177-202.
Dubey, J. P. (1994). "Toxoplasmosis." J Am Vet Med Assoc **205**(11): 1593-1598.
Dubey, J. P. (1995). "Duration of immunity to shedding of Toxoplasma gondii oocysts by cats." J Parasitol **81**(3): 410-415.
Dubey, J. P. (1996a). "Strategies to reduce transmission of Toxoplasma gondii to animals and humans." Vet Parasitol **64**(1-2): 65-70.
Dubey, J. P. (1996b). "Infectivity and pathogenicity of Toxoplasma gondii oocysts for cats." J Parasitol **82**(6): 957-961.
Dubey, J. P. (1997a). "Survival of Toxoplasma gondii tissue cysts in 0.85-6% NaCl solutions at 4-20 C." J Parasitol **83**(5): 946-949.
Dubey, J. P. (1997b). "Validation of the specificity of the modified agglutination test for toxoplasmosis in pigs." Vet Parasitol **71**(4): 307-310.
Dubey, J. P. (1998a). "Advances in the life cycle of Toxoplasma gondii." Int J Parasitol **28**(7): 1019-1024.
Dubey, J. P. (1998b). "Re-examination of resistance of Toxoplasma gondii tachyzoites and bradyzoites to pepsin and trypsin digestion." Parasitology **116 (Pt 1)**: 43-50.
Dubey, J. P. (1998c). "Toxoplasma gondii oocyst survival under defined temperatures." J Parasitol **84**(4): 862-865.
Dubey, J. P. (2004). "Toxoplasmosis - a waterborne zoonosis." Vet Parasitol **126**(1-2): 57-72.
Dubey, J. P. (2008). "The History of Toxoplasma gondii—The First 100 Years." J. Eukaryot. Microbiol **55**(6): 467-475.
Dubey, J. P. (2009a). "Toxoplasmosis in pigs--the last 20 years." Vet Parasitol **164**(2-4): 89-103.
Dubey, J. P. (2009b). "Toxoplasmosis in sheep--the last 20 years." Vet Parasitol **163**(1-2): 1-14.
Dubey, J. P. (2010). "Toxoplasma gondii infections in chickens (Gallus domesticus): prevalence, clinical disease, diagnosis and public health significance." Zoonoses Public Health **57**(1): 60-73.
Dubey, J. P. and C. P. Beattie (1988). Toxoplasmosis of Animals and Man. Boca Raton, FL, CRC Press.
Dubey, J. P. and G. Desmonts (1987). "Serological responses of equids fed Toxoplasma gondii oocysts." Equine Vet J **19**(4): 337-339.
Dubey, J. P., L. R. Ferreira, J. Martins and J. L. Jones (2011). "Sporulation and survival of Toxoplasma gondii oocysts in different types of commercial cat litter." J Parasitol **97**(5): 751-754.
Dubey, J. P. and J. K. Frenkel (1972). "Cyst-induced toxoplasmosis in cats." J Protozool **19**(1): 155-177.
Dubey, J. P., D. E. Hill, J. L. Jones, A. W. Hightower, E. Kirkland, J. M. Roberts, P. L. Marcet, T. Lehmann, M. C. Vianna, K. Miska, C. Sreekumar, O. C. Kwok, S. K. Shen and H. R. Gamble (2005). "Prevalence of viable Toxoplasma gondii in beef, chicken, and pork from retail

meat stores in the United States: risk assessment to consumers." J Parasitol **91**(5): 1082-1093.
Dubey, J. P., D. E. Hill, D. W. Rozeboom, C. Rajendran, S. Choudhary, L. R. Ferreira, O. C. Kwok and C. Su (2012). "High prevalence and genotypes of Toxoplasma gondii isolated from organic pigs in northern USA." Vet Parasitol **188**(1-2): 14-18.
Dubey, J. P. and J. L. Jones (2008). "Toxoplasma gondii infection in humans and animals in the United States." Int J Parasitol **38**(11): 1257-1278.
Dubey, J. P. and C. A. Kirkbride (1989). "Enzootic toxoplasmosis in sheep in north-central United States." J Parasitol **75**(5): 673-676.
Dubey, J. P., A. W. Kotula, A. Sharar, C. D. Andrews and D. S. Lindsay (1990a). "Effect of high temperature on infectivity of Toxoplasma gondii tissue cysts in pork." J Parasitol **76**(2): 201-204.
Dubey, J. P., L. W. Kramer and S. E. Weisbrode (1985). "Acute death associated with Toxoplasma gondii in ring-tailed lemurs." J Am Vet Med Assoc **187**(11): 1272-1273.
Dubey, J. P., D. S. Lindsay and C. A. Speer (1998a). "Structures of Toxoplasma gondii tachyzoites, bradyzoites, and sporozoites and biology and development of tissue cysts." Clin Microbiol Rev **11**(2): 267-299.
Dubey, J. P., N. L. Miller and J. K. Frenkel (1970a). "The Toxoplasma gondii oocyst from cat feces." J Exp Med **132**(4): 636-662.
Dubey, J.P., N.L. Miller and J.K. Frenkel (1970b). "Characterization of the new fecal form of *Toxoplasma gondii*" . J Parasitol **56**: 447-456.
Dubey, J. P., C. Rajendran, L. R. Ferreira, J. Martins, O. C. Kwok, D. E. Hill, I. Villena, H. Zhou, C. Su and J. L. Jones (2011). "High prevalence and genotypes of Toxoplasma gondii isolated from goats, from a retail meat store, destined for human consumption in the USA." Int J Parasitol **41**(8): 827-833.
Dubey, J. P., E. A. Rollor, K. Smith, O. C. Kwok and P. Thulliez (1997). "Low seroprevalence of Toxoplasma gondii in feral pigs from a remote island lacking cats." J Parasitol **83**(5): 839-841.
Dubey, J. P. and S. P. Sharma (1980). "Parasitemia and tissue infection in sheep fed Toxoplasma gondii oocysts." J Parasitol **66**(1): 111-114.
Dubey, J. P., S. P. Sharma, C. W. Lopes, J. F. Williams, C. S. Williams and S. E. Weisbrode (1980). "Caprine toxoplasmosis: abortion, clinical signs, and distribution of Toxoplasma in tissues of goats fed Toxoplasma gondii oocysts." Am J Vet Res **41**(7): 1072-1076.
Dubey, J. P., R. J. Sonn, O. Hedstrom, S. P. Snyder and E. D. Lassen (1990b). "Serologic and histologic diagnosis of toxoplasmic abortions in sheep in Oregon." J Am Vet Med Assoc **196**(2): 291-294.
Dubey, J. P. and C. Su (2009). "Population biology of Toxoplasma gondii: what's out and where did they come from." Mem Inst Oswaldo Cruz **104**(2): 190-195.
Dubey, J. P., N. Sundar, S. M. Gennari, A. H. Minervino, N. A. Farias, J. L. Ruas, T. R. dos Santos, G. T. Cavalcante, O. C. Kwok and C. Su (2007). "Biologic and genetic comparison of Toxoplasma gondii isolates in free-range chickens from the northern Para state and the southern state Rio Grande do Sul, Brazil revealed highly diverse and distinct parasite populations." Vet Parasitol **143**(2): 182-188.
Dubey, J. P., N. Sundar, D. Hill, G. V. Velmurugan, L. A. Bandini, O. C. Kwok, . . . C. Su (2008). "High prevalence and abundant atypical genotypes of Toxoplasma gondii isolated from lambs destined for human consumption in the USA." Int J Parasitol **38**(8-9): 999-1006.
Dubey, J.P., D.W. Thayer, C.A. Speer, S.K. Shen (1998b)." Effect of gamma irradiation on unsporulated and sporulated *Toxoplasma gondii* oocysts". Int J Parasitol **28**: 369-75.
Dubey, J. P. and P. Thulliez (1993). "Persistence of tissue cysts in edible tissues of cattle fed Toxoplasma gondii oocysts." Am J Vet Res **54**(2): 270-273.

Dubey, J. P., P. Thulliez, R. M. Weigel, C. D. Andrews, P. Lind and E. C. Powell (1995). "Sensitivity and specificity of various serologic tests for detection of Toxoplasma gondii infection in naturally infected sows." Am J Vet Res **56**(8): 1030-1036.

Dubey, J. P., D. M. Webb, N. Sundar, G. V. Velmurugan, L. A. Bandini, O. C. Kwok and C. Su (2007). "Endemic avian toxoplasmosis on a farm in Illinois: clinical disease, diagnosis, biologic and genetic characteristics of Toxoplasma gondii isolates from chickens (Gallus domesticus), and a goose (Anser anser)." Vet Parasitol **148** (3-4): 207-212.

Dumetre, A. and M. L. Darde (2003). "How to detect Toxoplasma gondii oocysts in environmental samples?" FEMS Microbiol Rev **27**(5): 651-661.

Dumetre, A. and M.L. Darde (2004). "Purification of Toxoplasma gondii oocysts by cesium chloride gradient. " J Microbiol Methods **56**: 427–430.

Dumetre, A. and M. L. Darde (2005). "Immunomagnetic separation of Toxoplasma gondii oocysts using a monoclonal antibody directed against the oocyst wall." J Microbiol Methods **61**(2): 209-217.

Dumetre, A. and M. L. Darde (2007). "Detection of Toxoplasma gondii in water by an immunomagnetic separation method targeting the sporocysts." Parasitol Res **101**(4): 989-996.

Dupouy-Camet, J., S. L. de Souza, C. Maslo, A. Paugam, A. G. Saimot, R. Benarous, . . . F. Derouin (1993). "Detection of Toxoplasma gondii in venous blood from AIDS patients by polymerase chain reaction." J Clin Microbiol **31**(7): 1866-1869.

Edwards, J. F. and J. P. Dubey (2013). "Toxoplasma gondii abortion storm in sheep on a Texas farm and isolation of mouse virulent atypical genotype T. gondii from an aborted lamb from a chronically infected ewe." Vet Parasitol **192**(1-3): 129-136.

EFSA (2007). "Surveillance and monitoring of Toxoplasma in humans, food and animals. Scientific Opinion of the Panel on Biological Hazards." EFSA Journal(583): 1-64.

EFSA (2013). "Technical specifications on harmonised epidemiological indicators for biological hazards to be covered by meat inspection of domestic sheep and goats." EFSA Journal **11**(6): 3277.

EFSA (2014). "The European Union Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents and Food-borne Outbreaks in 2012." The EFSA Journal **12**(2): 3547 (3312 pp.).

Ellis, J. T. (1998). "Polymerase chain reaction approaches for the detection of Neospora caninum and Toxoplasma gondii." Int J Parasitol **28**(7): 1053-1060.

El-Tras, W. F., A. A. Tayel and N. N. El-Kady (2011). "Source diversity of toxoplasma gondii infection during meal preparation." Journal of Food Safety **32**(1): 1-5.

Ertug, S., P. Okyay, M. Turkmen and H. Yuksel (2005). "Seroprevalence and risk factors for toxoplasma infection among pregnant women in Aydin province, Turkey." BMC Public Health **5**: 66.

Esteban-Redondo, I. and E. A. Innes (1998). "Detection of Toxoplasma gondii in tissues of sheep orally challenged with different doses of oocysts." Int J Parasitol **28**(9): 1459-1466.

Esteban-Redondo, I., S. W. Maley, K. Thomson, S. Nicoll, S. Wright, D. Buxton and E. A. Innes (1999). "Detection of T. gondii in tissues of sheep and cattle following oral infection." Vet Parasitol **86**(3): 155-171.

Evers, F., J. L. Garcia, I. T. Navarro, D. L. Zulpo, S. Nino Bde, M. P. Ewald, S. Pagliari, J. C. Almeida and R. L. Freire (2013). "Diagnosis and isolation of Toxoplasma gondii in horses from Brazilian slaughterhouses." Rev Bras Parasitol Vet **22**(1): 58-63.

Farrell, R. L., F. L. Docton, D. M. Chamberlain and C. R. Cole (1952). "Toxoplasmosis. I. Toxoplasma isolated from swine." Am J Vet Res **13**(47): 181-185.

Fayer, R. (2004). "Cryptosporidium: a water-borne zoonotic parasite." Vet Parasitol **126**(1-2): 37-56.
Fayer, R., J. P. Dubey and D. S. Lindsay (2004). "Zoonotic protozoa: from land to sea." Trends Parasitol **20**(11): 531-536.
Ferguson, D. J. (2009). "Toxoplasma gondii: 1908-2008, homage to Nicolle, Manceaux and Splendore." Mem Inst Oswaldo Cruz **104**(2): 133-148.
Ferguson, D. J., A. Birch-Andersen, J. C. Siim and W. M. Hutchison (1979). "Ultrastructural studies on the sporulation of oocysts of Toxoplasma gondii. III. Formation of the sporozoites within the sporocysts." Acta Pathol Microbiol Scand B **87**(4): 253-260.
Ferguson, D. J. and W. M. Hutchison (1987). "An ultrastructural study of the early development and tissue cyst formation of Toxoplasma gondii in the brains of mice." Parasitol Res **73**(6): 483-491.
Ferreira Ade, M., R. W. Vitor, R. T. Gazzinelli and M. N. Melo (2006). "Genetic analysis of natural recombinant Brazilian Toxoplasma gondii strains by multilocus PCR-RFLP." Infect Genet Evol **6**(1): 22-31.
Flegr, J. (2013). "How and why Toxoplasma makes us crazy." Trends Parasitol **29**(4): 156-163.
Flegr, J., J. Havlicek, P. Kodym, M. Maly and Z. Smahel (2002). "Increased risk of traffic accidents in subjects with latent toxoplasmosis: a retrospective case-control study." BMC Infect Dis **2**: 11.
Flegr, J., J. Klose, M. Novotna, M. Berenreitterova and J. Havlicek (2009). "Increased incidence of traffic accidents in Toxoplasma-infected military drivers and protective effect RhD molecule revealed by a large-scale prospective cohort study." BMC Infect Dis **9**: 72.
Frenkel, J. K. (1973). "Toxoplasma in and around us." BioScience **23**: 343-352.
Frenkel, J. K. and J. P. Dubey (1972). "Toxoplasmosis and its prevention in cats and man." J Infect Dis **126**(6): 664-673.
Frenkel, J. K. and J. P. Dubey (1973). "Effects of freezing on the viability of toxoplasma oocysts." J Parasitol **59**(3): 587-588.
Frenkel, J. K., J. P. Dubey and N. L. Miller (1969). "Toxoplasma gondii: fecal forms separated from eggs of the nematode Toxocara cati." Science **164**(3878): 432-433.
Frenkel, J. K. and L. Jacobs (1958). "Ocular toxoplasmosis; pathogenesis, diagnosis and treatment." AMA Arch Ophthalmol **59**(2): 260-279.
Frenkel, J. K. and B. B. Parker (1996). "An apparent role of dogs in the transmission of Toxoplasma gondii. The probable importance of xenosmophilia." Ann N Y Acad Sci **791**: 402-407.
Frenkel, J. K., E. R. Pfefferkorn, D. D. Smith and J. L. Fishback (1991). "Prospective vaccine prepared from a new mutant of Toxoplasma gondii for use in cats." Am J Vet Res **52**(5): 759-763.
Frenkel, J. K., A. Ruiz and M. Chinchilla (1975). "Soil survival of toxoplasma oocysts in Kansas and Costa Rica." Am J Trop Med Hyg **24**(3): 439-443.
Fu, S., G. Qu, S. Guo, L. Ma, N. Zhang, S. Zhang, . . . Z. Shen (2011). "Applications of loop-mediated isothermal DNA amplification." Appl Biochem Biotechnol **163**(7): 845-850.
Fusco, G., L. Rinaldi, A. Guarino, Y. T. Proroga, A. Pesce, M. Giuseppina de and G. Cringoli (2007). "Toxoplasma gondii in sheep from the Campania region (Italy)." Vet Parasitol **149**(3-4): 271-274.
Gallas-Lindemann, C., I. Sotiriadou, M. R. Mahmoodi and P. Karanis (2013). "Detection of Toxoplasma gondii oocysts in different water resources by Loop Mediated Isothermal Amplification (LAMP)." Acta Trop **125**(2): 231-236.
Gamble, H. R., R. C. Brady and J. P. Dubey (1999). "Prevalence of Toxoplasma gondii infection in domestic pigs in the New England states." Vet Parasitol **82**(2): 129-136.

Garcia-Bocanegra, I., O. Cabezon, A. Arenas-Montes, A. Carbonero, J. P. Dubey, A. Perea and S. Almeria (2012). "Seroprevalence of Toxoplasma gondii in equids from Southern Spain." Parasitol Int **61**(3): 421-424.

Garcia-Bocanegra, I., O. Cabezon, E. Hernandez, M. S. Martinez-Cruz, A. Martinez-Moreno and J. Martinez-Moreno (2013). "Toxoplasma gondii in ruminant species (cattle, sheep, and goats) from southern Spain." J Parasitol **99**(3): 438-440.

Garcia, J. L., I. T. Navarro, L. Biazzono, R. L. Freire, J. da Silva Guimaraes Junior, A. L. Cryssafidis, . . . R. C. Dias (2007). "Protective activity against oocyst shedding in cats vaccinated with crude rhoptry proteins of the Toxoplasma gondii by the intranasal route." Vet Parasitol **145**(3-4): 197-206.

Garcia, J. L., I. T. Navarro, O. Vidotto, S. M. Gennari, R. Z. Machado, A. B. da Luz Pereira and I. L. Sinhorini (2006). "Toxoplasma gondii: comparison of a rhoptry-ELISA with IFAT and MAT for antibody detection in sera of experimentally infected pigs." Exp Parasitol **113**(2): 100-105.

Gaskell, E. A., J. E. Smith, J. W. Pinney, D. R. Westhead and G. A. McConkey (2009). "A unique dual activity amino acid hydroxylase in Toxoplasma gondii." PLoS One **4**(3): e4801.

Gebremedhin, E. Z., A. Agonafir, T. S. Tessema, G. Tilahun, G. Medhin, M. Vitale and V. Di Marco (2013). "Some risk factors for reproductive failures and contribution of Toxoplasma gondii infection in sheep and goats of Central Ethiopia: A cross-sectional study." Res Vet Sci **95**(3): 894-900.

Gelmetti, D., G. Sironi, M. Finazzi, L. Gelmini, C. Rosignoli, P. Cordioli and A. Lavazza (1999). "Diagnostic investigations of toxoplasmosis in four swine herds." J Vet Diagn Invest **11**(1): 87-90.

Giacomini, E., M. Lazzaro, C. Bacci, S. Giovannini, P. Razzini, E. Pozio, P. Pasquali and L. G. Alborali (2013). Anti- Toxoplasma gondii antibodies IgG and IgM in heavy pigs reared in Northern Italy. LXVII Convegno Nazionale S.I.S.Vet. , Centro Congressi Camera di Commercio di Brescia

Gilbert, R. E. and M. R. Stanford (2000). "Is ocular toxoplasmosis caused by prenatal or postnatal infection?" Br J Ophthalmol **84**(2): 224-226.

Gill, C. O. (2005). "Safety and storage stability of horse meat for human consumption." Meat Sci **71**(3): 506-513.

Greiner, M., C. R. Franke, D. Bohning and P. Schlattmann (1994). "Construction of an intrinsic cut-off value for the sero-epidemiological study of Trypanosoma evansi infections in a canine population in Brazil: a new approach towards an unbiased estimation of prevalence." Acta Trop **56**(1): 97-109.

Greiner, M., D. Pfeiffer and R. D. Smith (2000). "Principles and practical application of the receiver-operating characteristic analysis for diagnostic tests." Prev Vet Med **45**(1-2): 23-41.

Grover, C. M., P. Thulliez, J. S. Remington and J. C. Boothroyd (1990). "Rapid prenatal diagnosis of congenital Toxoplasma infection by using polymerase chain reaction and amniotic fluid." J Clin Microbiol **28**(10): 2297-2301.

Guimaraes, L. A., R. A. Bezerra, S. Rocha Dde and G. R. Albuquerque (2013). "Prevalence and risk factors associated with anti-Toxoplasma gondii antibodies in sheep from Bahia state, Brazil." Rev Bras Parasitol Vet **22**(2): 220-224.

Gutierrez, J., J. O'Donovan, A. Proctor, C. Brady, P. X. Marques, S. Worrall, . . . B. K. Markey (2012). "Application of quantitative real-time polymerase chain reaction for the diagnosis of toxoplasmosis and enzootic abortion of ewes." J Vet Diagn Invest **24**(5): 846-854.

Gutierrez, J., J. O'Donovan, E. Williams, A. Proctor, C. Brady, P. X. Marques, . . . B. K. Markey (2010). "Detection and quantification of Toxoplasma gondii in ovine maternal and

foetal tissues from experimentally infected pregnant ewes using real-time PCR." Vet Parasitol **172**(1-2): 8-15.
Haroon, F., U. Handel, F. Angenstein, J. Goldschmidt, P. Kreutzmann, H. Lison, . . . E. Budinger (2012). "Toxoplasma gondii actively inhibits neuronal function in chronically infected mice." PLoS One **7**(4): e35516.
Hall, S. M., A. Pandit, A. Golwilkar and T. S. Williams (1999). "How do Jains get toxoplasma infection?" Lancet **354**(9177): 486-487.
Halos, L., A. Thebault, D. Aubert, M. Thomas, C. Perret, R. Geers, . . . I. Villena (2010). "An innovative survey underlining the significant level of contamination by Toxoplasma gondii of ovine meat consumed in France." Int J Parasitol **40**(2): 193-200.
Halova, D., G. Mulcahy, P. Rafter, L. Turcekova, T. Grant and T. de Waal (2013). "Toxoplasma gondii in Ireland: seroprevalence and novel molecular detection method in sheep, pigs, deer and chickens." Zoonoses Public Health **60**(2): 168-173.
Hamilton, C. M., R. Gray, S. E. Wright, B. Gangadharan, K. Laurenson and E. A. Innes (2005). "Prevalence of antibodies to Toxoplasmagondii and Neosporacaninum in red foxes (Vulpesvulpes) from around the UK." Vet Parasitol **130**(1-2): 169-173.
Hay, J., P. P. Aitken, D. M. Hair, W. M. Hutchison and D. I. Graham (1984). "The effect of congenital Toxoplasma infection on mouse activity and relative preference for exposed areas over a series of trials." Ann Trop Med Parasitol **78**(6): 611-618.
Hedman, K., M. Lappalainen, I. Seppaia and O. Makela (1989). "Recent primary toxoplasma infection indicated by a low avidity of specific IgG." J Infect Dis **159**(4): 736-740.
Herrmann, D. C., P. Maksimov, A. Maksimov, A. Sutor, S. Schwarz, W. Jaschke, . . . G. Schares (2012). "Toxoplasma gondii in foxes and rodents from the German Federal States of Brandenburg and Saxony-Anhalt: seroprevalence and genotypes." Vet Parasitol **185**(2-4): 78-85.
Heukelbach, J., V. Meyer-Cirkel, R. C. Moura, M. Gomide, J. A. Queiroz, P. Saweljew and O. Liesenfeld (2007). "Waterborne toxoplasmosis, northeastern Brazil." Emerg Infect Dis **13**(2): 287-289.
Hill, D. E. and J. P. Dubey (2013). "Toxoplasma gondii prevalence in farm animals in the United States." Int J Parasitol **43**(2): 107-113.
Hill, A. E., S. M. C. Benedetto, C. Coss, J. L. McCrary, V. M. Fournet and D. JP (2006). "Effects of time and temperature on the viability of Toxoplasma gondii tissue cysts in enhanced pork loin." J. Food Prot. **69**: 1961-1965.
Hill, D., C. Coss, J. P. Dubey, K. Wroblewski, M. Sautter, T. Hosten, . . . R. McLeod (2011). "Identification of a sporozoite-specific antigen from Toxoplasma gondii." J Parasitol **97**(2): 328-337.
Hill, D. E., C. Haley, B. Wagner, H. R. Gamble and J. P. Dubey (2010). "Seroprevalence of and risk factors for Toxoplasma gondii in the US swine herd using sera collected during the National Animal Health Monitoring Survey (Swine 2006)." Zoonoses Public Health **57**(1): 53-59.
Hill, D., C. Sreekumar, H. R. Gamble and J. P. Dubey (2004). "Effect of commonly used enhancement solutions on the viability of Toxoplasma gondii tissue cysts in pork loin." J. Food Prot. **67**: 2230-2233.
Hinze-Selch, D., W. Daubener, S. Erdag and S. Wilms (2010). "The diagnosis of a personality disorder increases the likelihood for seropositivity to Toxoplasma gondii in psychiatric patients." Folia Parasitol (Praha) **57**(2): 129-135.
Hofhuis, A., W. van Pelt, Y. T. van Duynhoven, C. D. Nijhuis, L. Mollema, F. R. van der Klis, . . . L. M. Kortbeek (2011). "Decreased prevalence and age-specific risk factors for Toxoplasma gondii IgG antibodies in The Netherlands between 1995/1996 and 2006/2007." Epidemiol Infect **139**(4): 530-538.

Homan, W. L., M. Vercammen, J. De Braekeleer and H. Verschueren (2000). "Identification of a 200- to 300-fold repetitive 529 bp DNA fragment in Toxoplasma gondii, and its use for diagnostic and quantitative PCR." Int J Parasitol **30**(1): 69-75.

Horacek, J., J. Flegr, J. Tintera, K. Verebova, F. Spaniel, T. Novak, M. Brunovsky, V. Bubenikova-Valesova, D. Holub, T. Palenicek and C. Hoschl (2012). "Latent toxoplasmosis reduces gray matter density in schizophrenia but not in controls: voxel-based-morphometry (VBM) study." World J Biol Psychiatry **13**(7): 501-509.

Hosseininejad, M., H. R. Azizi, F. Hosseini and G. Schares (2009). "Development of an indirect ELISA test using a purified tachyzoite surface antigen SAG1 for sero-diagnosis of canine Toxoplasma gondii infection." Vet Parasitol **164**(2-4): 315-319.

Howe, D. K., S. Honore, F. Derouin and L. D. Sibley (1997). "Determination of genotypes of Toxoplasma gondii strains isolated from patients with toxoplasmosis." J Clin Microbiol **35**(6): 1411-1414.

Howe, D. K. and L. D. Sibley (1995). "Toxoplasma gondii comprises three clonal lineages: correlation of parasite genotype with human disease." J Infect Dis **172**(6): 1561-1566.

http://www.istat.it

http://www.izs.it/IZS. (2013). "Anagrafe Nazionale Zootecnica."

http://www.scottishwildcats.co.uk. "Scottish Wildcat Association."

http://www.weather-and-climate.com "World weather and climate information."

Hung, C. C., C. K. Fan, K. E. Su, F. C. Sung, H. Y. Chiou, V. Gil, . . . S. H. Chou (2007). "Serological screening and toxoplasmosis exposure factors among pregnant women in the Democratic Republic of Sao Tome and Principe." Trans R Soc Trop Med Hyg **101**(2): 134-139.

Hunter, C. A. and L. D. Sibley (2012). "Modulation of innate immunity by Toxoplasma gondii virulence effectors." Nat Rev Microbiol **10**(11): 766-778.

Hurtado, A., G. Aduriz, B. Moreno, J. Barandika and A. L. Garcia-Perez (2001). "Single tube nested PCR for the detection of Toxoplasma gondii in fetal tissues from naturally aborted ewes." Vet Parasitol **102**(1-2): 17-27.

Hutchison, W. M. (1965). "Experimental transmission of Toxoplasma gondii." Nature **206**(987): 961-962.

Hutchison, W. M. (1967). "The nematode transmission of Toxoplasma gondii." Trans R Soc Trop Med Hyg **61**(1): 80-89.

Hutchison, W. M., J. F. Dunachie, J. C. Siim and K. Work (1969). "Life cycle of toxoplasma gondii." Br Med J **4**(5686): 806.

Hutchison, W. M., J. F. Dunachie, J. C. Siim and K. Work (1970). "Coccidian-like nature of Toxoplasma gondii." Br Med J **1**(5689): 142-144.

Hutchison, W. M., J. F. Dunachie, K. Work and J. C. Siim (1971). "The life cycle of the coccidian parasite, Toxoplasma gondii, in the domestic cat." Trans R Soc Trop Med Hyg **65**(3): 380-399.

Hutchinson, J. P., A. R. Wear, S. L. Lambton, R. P. Smith and G. C. Pritchard (2011). "Survey to determine the seroprevalence of Toxoplasma gondii infection in British sheep flocks." Vet Rec **169**(22): 582.

Idda, L., R. Furesi and P. Pulina (2010). Economia dell'allevamento ovino da latte. Milano, Italy, Franco Angeli s.r.l.

Innes, E. A. (1997). "Toxoplasmosis: comparative species susceptibility and host immune response." Comp Immunol Microbiol Infect Dis **20**(2): 131-138.

Innes, E. A., P. M. Bartley, D. Buxton and F. Katzer (2009). "Ovine toxoplasmosis." Parasitology **136**(14): 1887-1894.

Innes, E. A., P. M. Bartley, M. Rocchi, J. Benavidas-Silvan, A. Burrells, E. Hotchkiss, F. Chianini, G. Canton and F. Katzer (2011). "Developing vaccines to control protozoan parasites in ruminants: dead or alive?" Vet Parasitol **180**(1-2): 155-163.

Innes, E. A. and A. N. Vermeulen (2006). "Vaccination as a control strategy against the coccidial parasites Eimeria, Toxoplasma and Neospora." Parasitology **133 Suppl**: S145-168.

Isaac-Renton, J., W. R. Bowie, A. King, G. S. Irwin, C. S. Ong, C. P. Fung, . . . J. P. Dubey (1998). "Detection of Toxoplasma gondii oocysts in drinking water." Appl Environ Microbiol **64**(6): 2278-2280.

Jacobs, L., J. S. Remington and M. L. Melton (1960). "The resistance of the encysted form of Toxoplasma gondii." J Parasitol **46**: 11-21.

Jacobson, R. H. (1998). "Validation of serological assays for diagnosis of infectious diseases." Rev Sci Tech **17**(2): 469-526.

Jakubek, E. B., R. Farkas, V. Palfi and J. G. Mattsson (2007). "Prevalence of antibodies against Toxoplasma gondii and Neospora caninum in Hungarian red foxes (Vulpes vulpes)." Vet Parasitol **144**(1-2): 39-44.

Janse, I., R. A. Hamidjaja, J. M. Bok and B. J. van Rotterdam (2010). "Reliable detection of Bacillus anthracis, Francisella tularensis and Yersinia pestis by using multiplex qPCR including internal controls for nucleic acid extraction and amplification." BMC Microbiol **10**: 314.

Jerome, M. E., J. R. Radke, W. Bohne, D. S. Roos and M. W. White (1998). "Toxoplasma gondii bradyzoites form spontaneously during sporozoite-initiated development." Infect Immun **66**(10): 4838-4844.

Jokelainen, P., O. Simola, E. Rantanen, A. Nareaho, H. Lohi and A. Sukura (2012). "Feline toxoplasmosis in Finland: cross-sectional epidemiological study and case series study." J Vet Diagn Invest **24**(6): 1115-1124.

Jones, J. L. and J. P. Dubey (2010). "Waterborne toxoplasmosis--recent developments." Exp Parasitol **124**(1): 10-25.

Jones, J. L. and J. P. Dubey (2012). "Foodborne toxoplasmosis." Clin Infect Dis **55**(6): 845-851.

Jones, J. L., V. Dargelas, J. Roberts, C. Press, J. S. Remington and J. G. Montoya (2009). "Risk factors for Toxoplasma gondii infection in the United States." Clin Infect Dis **49**(6): 878-884.

Jones, J. L., D. Kruszon-Moran, K. Sanders-Lewis and M. Wilson (2007). "Toxoplasma gondii infection in the United States, 1999 2004, decline from the prior decade." Am J Trop Med Hyg **77**(3): 405-410.

Jurankova, J., M. Opsteegh, H. Neumayerova, K. Kovarcik, A. Frencova, V. Balaz, J. Volf and B. Koudela (2013). "Quantification of Toxoplasma gondii in tissue samples of experimentally infected goats by magnetic capture and real-time PCR." Vet Parasitol **193**(1-3): 95-99.

Kantzoura, V., A. Diakou, M. K. Kouam, H. Feidas, H. Theodoropoulou and G. Theodoropoulos (2013). "Seroprevalence and risk factors associated with zoonotic parasitic infections in small ruminants in the Greek temperate environment." Parasitol Int **62**(6): 554-560.

Kapperud, G., P. A. Jenum, B. Stray-Pedersen, K. K. Melby, A. Eskild and J. Eng (1996). "Risk factors for Toxoplasma gondii infection in pregnancy. Results of a prospective case-control study in Norway." Am J Epidemiol **144**(4): 405-412.

Karanis, P., H. M. Aldeyarbi, M. E. Mirhashemi and K. M. Khalil (2013). "The impact of the waterborne transmission of Toxoplasma gondii and analysis efforts for water detection: an overview and update." Environ Sci Pollut Res Int **20**(1): 86-99.

Karanis, P. and J. Ongerth (2009). "LAMP--a powerful and flexible tool for monitoring microbial pathogens." Trends Parasitol **25**(11): 498-499.

Karol, R., M. Reichlin and R. W. Noble (1978). "Idiotypic cross-reactivity between antibodies of different specificities." J Exp Med **148**(6): 1488-1497.

Katzer, F., F. Brulisauer, E. Collantes-Fernandez, P. M. Bartley, A. Burrells, G. Gunn, S. W. Maley, C. Cousens and E. A. Innes (2011). "Increased Toxoplasma gondii positivity relative to age in 125 Scottish sheep flocks; evidence of frequent acquired infection." Vet Res **42**: 121.

Kenny, D. E., M. R. Lappin, F. Knightly, J. Baler, M. Brewer and D. M. Getzy (2002). "Toxoplasmosis in Pallas' cats (Otocolobus felis manul) at the Denver Zoological Gardens." J Zoo Wildl Med **33**(2): 131-138.

Khan, A., C. Jordan, C. Muccioli, A. L. Vallochi, L. V. Rizzo, R. Belfort, Jr., R. W. Vitor, C. Silveira and L. D. Sibley (2006). "Genetic divergence of Toxoplasma gondii strains associated with ocular toxoplasmosis, Brazil." Emerg Infect Dis **12**(6): 942-949.

Khan, A., C. Su, M. German, G. A. Storch, D. B. Clifford and L. D. Sibley (2005). "Genotyping of Toxoplasma gondii strains from immunocompromised patients reveals high prevalence of type I strains." J Clin Microbiol **43**(12): 5881-5887.

Kijlstra, A., O. A. Eissen, J. Cornelissen, K. Munniksma, I. Eijck and T. Kortbeek (2004). "Toxoplasma gondii infection in animal-friendly pig production systems." Invest Ophthalmol Vis Sci **45**(9): 3165-3169.

Kijlstra, A. and E. Jongert (2008). "Control of the risk of human toxoplasmosis transmitted by meat." Int J Parasitol **38**(12): 1359-1370.

Kijlstra, A., B. Meerburg, J. Cornelissen, S. De Craeye, P. Vereijken and E. Jongert (2008). "The role of rodents and shrews in the transmission of Toxoplasma gondii to pigs." Vet Parasitol **156**(3-4): 183-190.

Kijlstra, A. and E. Petersen (2013). "Epidemiology, Pathophysiology, and the Future of Ocular Toxoplasmosis." Ocul Immunol Inflamm.

Koloren, Z. and E. Demirel (2013). "Detection of Toxoplasma gondii in Turkish River and Drinking Water Samples by Different PCR and LAMP Methods." CLEAN – Soil, Air, Water **41**(10): 963-968.

Koloren, Z., I. Sotiriadou and P. Karanis (2011). "Investigations and comparative detection of Cryptosporidium species by microscopy, nested PCR and LAMP in water supplies of Ordu, Middle Black Sea, Turkey." Ann Trop Med Parasitol **105**(8): 607-615.

Kotula, A. W., d. JP, A. Sharar, C. D. Andrews, S. K. Shen and D. S. Lindsay (1991). "Effect of freezing on infectivity of Toxoplasa gondii tissue cysts in pork." J. Food Prot **54**: 687-690.

Kouam, M. K., A. Diakou, V. Kanzoura, E. Papadopoulos, A. A. Gajadhar and G. Theodoropoulos (2010). "A seroepidemiological study of exposure to Toxoplasma, Leishmania, Echinococcus and Trichinella in equids in Greece and analysis of risk factors." Vet Parasitol **170**(1-2): 170-175.

Kourenti, C., A. Heckeroth, A. Tenter and P. Karanis (2003). "Development and application of different methods for the detection of Toxoplasma gondii in water." Appl Environ Microbiol **69**(1): 102-106.

Kourenti, C. and P. Karanis (2004). "Development of a sensitive polymerase chain reaction method for the detection of Toxoplasma gondii in water." Water Sci Technol **50**(1): 287-291.

Kourenti, C. and P. Karanis (2006). "Evaluation and applicability of a purification method coupled with nested PCR for the detection of Toxoplasma oocysts in water. " Lett Appl Microbiol **43** (5): 475-81.

KPL (2013). Troubleshooting ELISA guide.

Kusbeci, O. Y., O. Miman, M. Yaman, O. C. Aktepe and S. Yazar (2011). "Could Toxoplasma gondii have any role in Alzheimer disease?" Alzheimer Dis Assoc Disord **25**(1): 1-3.

Kuticic, V. and T. Wikerhauser (1996). Studies of the Effect of Various Treatments on the Viability of Toxoplasma gondii Tissue Cysts and Oocysts. Toxoplasma gondii. P. D. U. Gross, Springer Berlin Heidelberg**:** pp 261-265.

Langoni, H., H. Greca, Jr., F. F. Guimaraes, L. S. Ullmann, F. C. Gaio, R. S. Uehara, . . . R. C. Da Silva (2011). "Serological profile of Toxoplasma gondii and Neospora caninum infection in commercial sheep from Sao Paulo State, Brazil." Vet Parasitol **177**(1-2): 50-54.

Lappin, M. R. (2010). "Update on the Diagnosis and Management of Toxoplasma gondii Infection in Cats." Topics in Companion Animal Medicine **25**(3): 136-141.

Lappin, M. R., J. W. George, N. C. Pedersen, J. E. Barlough, C. J. Murphy and L. S. Morse (1996). "Primary and secondary Toxoplasma gondii infection in normal and feline immunodeficiency virus-infected cats." J Parasitol **82**(5): 733-742.

Le Potier, M. F., A. Fournier, C. Houdayer, E. Hutet, V. Auvigne, D. Hery, . . . B. Toma (1998). "Use of muscle exudates for the detection of anti-gE antibodies to Aujeszky's disease virus." Vet Rec **143**(14): 385-387.

Lehmann, T., D. H. Graham, E. Dahl, C. Sreekumar, F. Launer, J. L. Corn, H. R. Gamble and J. P. Dubey (2003). "Transmission dynamics of Toxoplasma gondii on a pig farm." Infect Genet Evol **3**(2): 135-141.

Lehmann, T., P. L. Marcet, D. H. Graham, E. R. Dahl and J. P. Dubey (2006). "Globalization and the population structure of Toxoplasma gondii." Proc Natl Acad Sci U S A **103**(30): 11423-11428.

Leroy, V., A. Raeber, E. Petersen, L. R. Salmi, M. Kaminski, I. Villena, . . . R. Gilbert. (2005). "National public Health policies and routine programs to prevent congenital toxoplasmosis in Europe, 2005.".

Li, X., Y. Wang, F. Yu, T. Li and D. Zhang (2010). "An outbreak of lethal toxoplasmosis in pigs in the Gansu province of China." J Vet Diagn Invest **22**(3): 442-444.

Lin, Y. L., Y. S. Liao, L. R. Liao, F. N. Chen, H. M. Kuo and S. He (2008). "Seroprevalence and sources of Toxoplasma infection among indigenous and immigrant pregnant women in Taiwan." Parasitol Res **103**(1): 67-74.

Lind, P., J. Haugegaard, A. Wingstrand and S. A. Henriksen (1997). "The time course of the specific antibody response by various ELISAs in pigs experimentally infected with Toxoplasma gondii." Vet Parasitol **71**(1): 1-15.

Lindsay, D. S., M. V. Collins, S. M. Mitchell, C. N. Wetch, A. C. Rosypal, G. J. Flick, . . . J. P. Dubey (2004). "Survival of Toxoplasma gondii oocysts in Eastern oysters (Crassostrea virginica)." J Parasitol **90**(5): 1054-1057.

Lindsay, D. S. and J. P. Dubey (2009). "Long-term survival of Toxoplasma gondii sporulated oocysts in seawater." J Parasitol **95**(4): 1019-1020.

Lindstrom, I., N. Sundar, J. Lindh, F. Kironde, J. D. Kabasa, O. C. Kwok, J. P. Dubey and J. E. Smith (2008). "Isolation and genotyping of Toxoplasma gondii from Ugandan chickens reveals frequent multiple infections." Parasitology **135**(Pt 1): 39-45.

Loomis, W. D. (1974). Overcoming problems of phenolics and quinones in the isolation of plant enzymes and organelles. Methods in Enzymology, vol. XXXI (A) S. Fleischer and L. Packer**:** 528-544.

Lopes, A. P., J. P. Dubey, F. Neto, A. Rodrigues, T. Martins, M. Rodrigues and L. Cardoso (2013). "Seroprevalence of Toxoplasma gondii infection in cattle, sheep, goats and pigs from the North of Portugal for human consumption." Vet Parasitol **193**(1-3): 266-269.

Lopez, A., V. J. Dietz, M. Wilson, T. R. Navin and J. L. Jones (2000). "Preventing congenital toxoplasmosis." MMWR Recomm Rep **49**(RR-2): 59-68.

Lunden, A., P. Lind, E. O. Engvall, K. Gustavsson, A. Uggla and I. Vagsholm (2002). "Serological survey of Toxoplasma gondii infection in pigs slaughtered in Sweden." Scand J Infect Dis **34**(5): 362-365.

Lunden, A., A. Nasholm and A. Uggla (1994). "Long-term study of Toxoplasma gondii infection in a Swedish sheep flock." Acta Vet Scand **35**(3): 273-281.
Lunden, A. and A. Uggla (1992). "Infectivity of Toxoplasma gondii in mutton following curing, smoking, freezing or microwave cooking." Int J Food Microbiol **15**(3-4): 357-363.
Lyons, R. E., R. McLeod and C. W. Roberts (2002). "Toxoplasma gondii tachyzoite-bradyzoite interconversion." Trends Parasitol **18**(5): 198-201.
Macri, G., M. Sala, A. M. Linder, N. Pettirossi and M. Scarpulla (2009). "Comparison of indirect fluorescent antibody test and modified agglutination test for detecting Toxoplasma gondii immunoglobulin G antibodies in dog and cat." Parasitol Res **105**(1): 35-40.
Mainar-Jaime, R. C. and M. Barberan (2007). "Evaluation of the diagnostic accuracy of the modified agglutination test (MAT) and an indirect ELISA for the detection of serum antibodies against Toxoplasma gondii in sheep through Bayesian approaches." Vet Parasitol **148**(2): 122-129.
Mancianti, F., S. Nardoni, G. Ariti, D. Parlanti, G. Giuliani and R. A. Papini (2010). "Cross-sectional survey of Toxoplasma gondii infection in colony cats from urban Florence (Italy)." J Feline Med Surg **12**(4): 351-354.
Mancianti, F., S. Nardoni, C. D'Ascenzi, F. Pedonese, L. Mugnaini, F. Franco and R. Papini (2013). "Seroprevalence, Detection of DNA in Blood and Milk, and Genotyping of Toxoplasma gondii in a Goat Population in Italy." Biomed Res Int **2013**: 905326.
Masala, G., R. Porcu, C. Daga, S. Denti, G. Canu, C. Patta and S. Tola (2007). "Detection of pathogens in ovine and caprine abortion samples from Sardinia, Italy, by PCR." J Vet Diagn Invest **19**(1): 96-98.
Masala, G., R. Porcu, L. Madau, A. Tanda, B. Ibba, G. Satta and S. Tola (2003). "Survey of ovine and caprine toxoplasmosis by IFAT and PCR assays in Sardinia, Italy." Vet Parasitol **117**(1-2): 15-21.
Mateus-Pinilla, N. E., J. P. Dubey, L. Choromanski and R. M. Weigel (1999). "A field trial of the effectiveness of a feline Toxoplasma gondii vaccine in reducing T. gondii exposure for swine." J Parasitol **85**(5): 855-860.
Mecca, J. N., L. R. Meireles and H. F. de Andrade, Jr. (2011). "Quality control of Toxoplasma gondii in meat packages: standardization of an ELISA test and its use for detection in rabbit meat cuts." Meat Sci **88**(3): 584-589.
Meerburg, B. G., J. W. Van Riel, J. B. Cornelissen, A. Kijlstra and M. F. Mul (2006). "Cats and goat whey associated with Toxoplasma gondii infection in pigs." Vector Borne Zoonotic Dis **6**(3): 266-274
Mehlhorn, H. and J. K. Frenkel (1980). "Ultrastructural comparison of cysts and zoites of Toxoplasma gondii, Sarcocystis muris, and Hammondia hammondi in skeletal muscle of mice." J Parasitol **66**(1): 59-67.
Mele, A., P. J. Paterson, H. G. Prentice, P. Leoni and C. C. Kibbler (2002). "Toxoplasmosis in bone marrow transplantation: a report of two cases and systematic review of the literature." Bone Marrow Transplant **29**(8): 691-698.
Mendonca, C. E., S. L. Barros, V. A. Guimaraes, A. S. Ferraudo and A. D. Munhoz (2013). "Prevalence and risk factors associated to ovine toxoplasmosis in northeastern Brazil." Rev Bras Parasitol Vet **22**(2): 230-234.
Mercier, A., S. Devillard, B. Ngoubangoye, H. Bonnabau, A. L. Banuls, P. Durand, B. Salle, D. Ajzenberg and M. L. Darde (2010). "Additional haplogroups of Toxoplasma gondii out of Africa: population structure and mouse-virulence of strains from Gabon." PLoS Negl Trop Dis **4**(11): e876.
Mercier, A., S. Devillard, B. Ngoubangoye, H. Bonnabau, A. L. Bañuls, P. Durand, B. Salle, D. Ajzenberg and M. L. Dardé (2011). "Additional haplogroups of Toxoplasma gondii out

of Africa: population structure and mouse-virulence of strains from Gabon." PLoS Negl. Trop. Dis. **4**(11): e876.

Mishima, M., X. Xuan, N. Yokoyama, I. Igarashi, K. Fujisaki, H. Nagasawa and T. Mikami (2002). "Recombinant feline herpesvirus type 1 expressing Toxoplasma gondi ROP2 antigen inducible protective immunity in cats." Parasitol Res **88**(2): 144-149.

Miman, O., O. Y. Kusbeci, O. C. Aktepe and Z. Cetinkaya (2010a). "The probable relation between Toxoplasma gondii and Parkinson's disease." Neurosci Lett **475**(3): 129-131.

Miman, O., E. A. Mutlu, O. Ozcan, M. Atambay, R. Karlidag and S. Unal (2010b). "Is there any role of Toxoplasma gondii in the etiology of obsessive-compulsive disorder?" Psychiatry Res **177**(1-2): 263-265.

Minho, A. P., I. T. Navarro, R. L. Freire, O. Vidotto, S. M. Gennari, E. M. Marana and J. L. Garcia (2004). "Evaluation of the indirect fluorescent antibody test and modified agglutination test for detection of antibodies against Toxoplasma gondii in experimentally infected pigs." Pesq. Vet. Bras. **24**(4): 199-202.

Moeller, R. B., Jr. (2001). "Causes of caprine abortion: diagnostic assessment of 211 cases (1991-1998)." J Vet Diagn Invest **13**(3): 265-270.

Montoya, J. G. (2002). "Laboratory diagnosis of Toxoplasma gondii infection and toxoplasmosis." J Infect Dis **185 Suppl 1**: S73-82.

Montoya, J. G. and O. Liesenfeld (2004). "Toxoplasmosis." Lancet **363**(9425): 1965-1976.

Montoya, J. G., S. Parmley, O. Liesenfeld, G. J. Jaffe and J. S. Remington (1999). "Use of the polymerase chain reaction for diagnosis of ocular toxoplasmosis." Ophthalmology **106**: 1554-1563.

More, G., W. Basso, D. Bacigalupe, M. C. Venturini and L. Venturini (2008). "Diagnosis of Sarcocystis cruzi, Neospora caninum, and Toxoplasma gondii infections in cattle." Parasitol Res **102**(4): 671-675.

Moreno, B., E. Collantes-Fernandez, A. Villa, A. Navarro, J. Regidor-Cerrillo and L. M. Ortega-Mora (2012). "Occurrence of Neospora caninum and Toxoplasma gondii infections in ovine and caprine abortions." Vet Parasitol **187**(1-2): 312-318.

Morisaki, J. H., J. E. Heuser and L. D. Sibley (1995). "Invasion of Toxoplasma gondii occurs by active penetration of the host cell." J Cell Sci **108 (Pt 6)**: 2457-2464.

Morley, E. K., R. H. Williams, J. M. Hughes, R. S. Terry, P. Duncanson, J. E. Smith and G. Hide (2005). "Significant familial differences in the frequency of abortion and Toxoplasma gondii infection within a flock of Charollais sheep." Parasitology **131**(Pt 2): 181-185.

Morley, E. K., R. H. Williams, J. M. Hughes, D. Thomasson, R. S. Terry, P. Duncanson, J. E. Smith and G. Hide (2008). "Evidence that primary infection of Charollais sheep with Toxoplasma gondii may not prevent foetal infection and abortion in subsequent lambings." Parasitology **135**(2): 169-173.

Mosti, M., B. Pinto, A. Giromella, S. Fabiani, R. Cristofani, M. Panichi and F. Bruschi (2013). "A 4-year evaluation of toxoplasmosis seroprevalence in the general population and in women of reproductive age in central Italy." Epidemiol Infect **141**(10): 2192-2195.

Mousing, J., P. T. Jensen, C. Halgaard, F. Bager, N. Feld, B. Nielsen, . . . S. Bech-Nielsen (1997). "Nation-wide Salmonella enterica surveillance and control in Danish slaughter swine herds." Prev Vet Med **29**(4): 247-261.

Natale, A., M. Porqueddu, G. Capelli, G. Mocci, A. Marras, G. Sanna-Coccone, . . . A. Scala (2006). Sero-epidemiological update on sheep toxoplasmosis in Sardinia (Italy). Toxo&Food.

Nicolle, C. and L. H. Manceaux (2009). "On a leishman body infection (or related organisms) of the gondi. 1908." Int J Parasitol **39**(8): 863-864.

Nockler, K., F. J. Serrano, P. Boireau, C. M. Kapel and E. Pozio (2005). "Experimental studies in pigs on Trichinella detection in different diagnostic matrices." Vet Parasitol **132**(1-2): 85-90.

Notomi, T., H. Okayama, H. Masubuchi, T. Yonekawa, K. Watanabe, N. Amino and T. Hase (2000). "Loop-mediated isothermal amplification of DNA." Nucleic Acids Res **28**(12): E63.

O'Connell, E., M. F. Wilkins and W. A. Te Punga (1988). "Toxoplasmosis in sheep. II. The ability of a live vaccine to prevent lamb losses after an intravenous challenge with Toxoplasma gondii." N Z Vet J **36**(1): 1-4.

Obendorf, D. L., P. Statham and M. Driessen (1996). "Detection of agglutinating antibodies to Toxoplasma gondii in sera from free-ranging eastern barred bandicoots (Perameles gunnii)." J Wildl Dis **32**(4): 623-626.

Obendorf, D. L., P. Statham and B. L. Munday (1990). "Resistance to Toxoplasma abortion in female goats previously exposed to Toxoplasma infection." Aust Vet J **67**(6): 233-234.

Okusaga, O., P. Langenberg, A. Sleemi, D. Vaswani, I. Giegling, A. M. Hartmann, B. Konte, M. Friedl, M. W. Groer, R. H. Yolken, D. Rujescu and T. T. Postolache (2011). "Toxoplasma gondii antibody titers and history of suicide attempts in patients with schizophrenia." Schizophr Res **133**(1-3): 150-155.

OIE (2008). Toxoplasmosis. OIE Terrestrial Manual.

Opsteegh, M., M. Langelaar, H. Sprong, L. den Hartog, S. De Craeye, G. Bokken, . . . J. van der Giessen (2010a). "Direct detection and genotyping of Toxoplasma gondii in meat samples using magnetic capture and PCR." Int J Food Microbiol **139**(3): 193-201.

Opsteegh, M., S. Prickaerts, K. Frankena and E. G. Evers (2011a). "A quantitative microbial risk assessment for meatborne Toxoplasma gondii infection in The Netherlands." Int J Food Microbiol **150**(2-3): 103-114.

Opsteegh, M., P. Teunis, M. Mensink, L. Zuchner, A. Titilincu, M. Langelaar and J. van der Giessen (2010b). "Evaluation of ELISA test characteristics and estimation of Toxoplasma gondii seroprevalence in Dutch sheep using mixture models." Prev Vet Med **96**(3-4): 232-240.

Opsteegh, M., P. Teunis, L. Zuchner, A. Koets, M. Langelaar and J. van der Giessen (2011b). "Low predictive value of seroprevalence of Toxoplasma gondii in cattle for detection of parasite DNA." Int J Parasitol **41**(3-4): 343-354.

Overdulve, J. P. (1970). "The identity of Toxoplasma Nicolle and Manceaux, 1909 with Isospora Schneider, 1881. II." Proc K Ned Akad Wet C **73**(1): 142-151.

Palanisamy, M., B. Madhavan, M. B. Balasundaram, R. Andavar and N. Venkatapathy (2006). "Outbreak of ocular toxoplasmosis in Coimbatore, India." Indian J Ophthalmol **54**(2): 129-131.

Pappas, G., N. Roussos and M. E. Falagas (2009). "Toxoplasmosis snapshots: global status of Toxoplasma gondii seroprevalence and implications for pregnancy and congenital toxoplasmosis." Int J Parasitol **39**(12): 1385-1394.

Parameswaran, N., R. M. O'Handley, M. E. Grigg, S. G. Fenwick and R. C. Thompson (2009). "Seroprevalence of Toxoplasma gondii in wild kangaroos using an ELISA." Parasitol Int **58**(2): 161-165.

Paul, M. (1998). "[Potential risk factors for Toxoplasma gondii infection in cases with recently acquired toxoplasmosis]." Przegl Epidemiol **52**(4): 447-454.

Pearce, B. D., D. Kruszon-Moran and J. L. Jones (2012). "The relationship between Toxoplasma gondii infection and mood disorders in the third National Health and Nutrition Survey." Biol Psychiatry **72**(4): 290-295.

Pena, H. F., S. M. Gennari, J. P. Dubey and C. Su (2008). "Population structure and mouse-virulence of Toxoplasma gondii in Brazil." Int J Parasitol **38**(5): 561-569.

Pereira-Bueno, J., A. Quintanilla-Gozalo, V. Perez-Perez, G. Alvarez-Garcia, E. Collantes-Fernandez and L. M. Ortega-Mora (2004). "Evaluation of ovine abortion associated

with Toxoplasma gondii in Spain by different diagnostic techniques." Vet Parasitol **121**(1-2): 33-43.
Petersen, E., A. Kijlstra and M. Stanford (2012). "Epidemiology of ocular toxoplasmosis." Ocul Immunol Inflamm **20**(2): 68-75.
Petersen, E., G. Vesco, S. Villari and W. Buffolano (2010). "What do we know about risk factors for infection in humans with Toxoplasma gondii and how can we prevent infections?" Zoonoses Public Health **57**(1): 8-17.
Pfaff, A. W., A. de-la-Torre, E. Rochet, J. Brunet, M. Sabou, A. Sauer, T. Bourcier, J. E. Gomez-Marin and E. Candolfi (2013). "New clinical and experimental insights into Old World and neotropical ocular toxoplasmosis." Int J Parasitol.
Piergili Fioretti, D. (2004). "[Problems and limitations of conventional and innovative methods for the diagnosis of Toxoplasmosis in humans and animals]." Parassitologia **46**(1-2): 177-181.
Pinon, J. M., H. Dumon, C. Chemla, J. Franck, E. Petersen, M. Lebech, . . . E. Candolfi (2001). "Strategy for diagnosis of congenital toxoplasmosis: evaluation of methods comparing mothers and newborns and standard methods for postnatal detection of immunoglobulin G, M, and A antibodies." J Clin Microbiol **39**(6): 2267-2271.
Pinto, B., B. Castagna, R. Mattei, R. Bruzzi, L. Chiumiento, R. Cristofani, . . . F. Bruschi (2012). "Seroprevalence for toxoplasmosis in individuals living in north west Tuscany: access to Toxo-test in central Italy." Eur J Clin Microbiol Infect Dis **31**(6): 1151-1156.
Pomares, C., D. Ajzenberg, L. Bornard, G. Bernardin, L. Hasseine, M. L. Darde and P. Marty (2011). "Toxoplasmosis and horse meat, France." Emerg Infect Dis **17**(7): 1327-1328.
Powell, C. C., M. Brewer and M. R. Lappin (2001). "Detection of Toxoplasma gondii in the milk of experimentally infected lactating cats." Vet Parasitol **102**(1-2): 29-33.
Prandovszky, E., E. Gaskell, H. Martin, J. P. Dubey, J. P. Webster and G. A. McConkey (2011). "The neurotropic parasite Toxoplasma gondii increases dopamine metabolism." PLoS One **6**(9): e23866.
Puccini, V., G. Colella, M. G. Abbenate and P. Palladino (1981). La toxoplasmosi degli ovini e dei caprini in Puglia e Basilicata: indagine epizoologica. ATTI SIPAOC**:** 253-286.
Ragozo, A. M., L. E. Yai, L. N. Oliveira, R. A. Dias, H. C. Goncalves, S. S. Azevedo, J. P. Dubey and S. M. Gennari (2009). "Isolation of Toxoplasma gondii from goats from Brazil." J Parasitol **95**(2): 323-326.
Rajendran, C., C. Su and J. P. Dubey (2012). "Molecular genotyping of Toxoplasma gondii from Central and South America revealed high diversity within and between populations." Infect Genet Evol **12**(2): 359-368.
Ranucci, D., F. Veronesi, R. Branciari, D. Miraglia, I. Moretta and D. P. Fioretti (2012). "Evaluation of an immunofluorescence antibody assay for the detection of antibodies against Toxoplasma gondii in meat juice samples from finishing pigs." Foodborne Pathog Dis **9**(1): 75-78.
Rawal, B. D. (1959). "Toxoplasmosis; a dye-test survey on sera from vegetarians and meat eaters in Bombay." Trans R Soc Trop Med Hyg **53**(1): 61-63.
Razmi, G. R., K. Ghezi, A. Mahooti and Z. Naseri (2010). "A serological study and subsequent isolation of Toxoplasma gondii from aborted ovine fetuses in Mashhad area, Iran." J Parasitol **96**(4): 812-814.
Reikvam, A. and A. M. Lorentzen-Styr (1976). "Virulence of different strains of Toxoplasma gondii and host response in mice." Nature **261**(5560): 508-509.
Reinken, G. (1998). "Production and trade of game and deer meat in Europe." Zeitschrift Fur Jagdwissenschaft **44**: 167-177.
Remington, J. S. and G. Desmonts (1990). Toxoplasmosis. . Infectious diseases of the fetus and newborn infant. W. Saunders**:** 89-195.

Richomme, C., E. Afonso, V. Tolon, C. Ducrot, L. Halos, A. Alliot, . . . E. Gilot-Fromont (2010). "Seroprevalence and factors associated with Toxoplasma gondii infection in wild boar (Sus scrofa) in a Mediterranean island." Epidemiol Infect **138**(9): 1257-1266.

Riemann, H. P., M. E. Meyer, J. H. Theis, G. Kelso and D. E. Behymer (1975). "Toxoplasmosis in an infant fed unpasteurized goat milk." J Pediatr **87**(4): 573-576.

Rinaldi, L. and A. Scala (2008). "Toxoplasmosis in livestock in Italy: an epidemiological update." Parassitologia **50**(1-2): 59-61.

Rochelle, P.A., R. De Leon, A. Johnson, M.H. Stewart, R.L. Wolfe (1997). "Comparison of primers and optimisation of PCR conditions for detection of *Cryptosporidium parvum* and *Giardia lamblia* in water ". Appl Environ Microbiol **63**: 106-114.

Ross, R. D., L. A. Stec, J. C. Werner, M. S. Blumenkranz, L. Glazer and G. A. Williams (2001). "Presumed acquired ocular toxoplasmosis in deer hunters." Retina **21**(3): 226-229.

Ruiz, A. and J. K. Frenkel (1980). "Toxoplasma gondii in Costa Rican cats." Am J Trop Med Hyg **29**(6): 1150-1160.

Sabin, A. B. and H. A. Feldman (1948). "Dyes as Microchemical Indicators of a New Immunity Phenomenon Affecting a Protozoon Parasite (Toxoplasma)." Science **108**(2815): 660-663.

Sabin, A. B. and P. K. Olitsky (1937). "Toxoplasma and Obligate Intracellular Parasitism." Science **85**(2205): 336-338.

Sacks, J. J., R. R. Roberto and N. F. Brooks (1982). "Toxoplasmosis infection associated with raw goat's milk." JAMA **248**(14): 1728-1732.

Saeij, J. P., J. P. Boyle and J. C. Boothroyd (2005). "Differences among the three major strains of Toxoplasma gondii and their specific interactions with the infected host." Trends Parasitol **21**(10): 476-481.

Samico Fernandes, E. F., M. F. Samico Fernandes, P. C. Kim, P. P. de Albuquerque, O. L. de Souza Neto, S. S. A. de, . . . R. A. Mota (2012). "Prevalence of Toxoplasma gondii in slaughtered pigs in the state of Pernambuco, Brazil." J Parasitol **98**(3): 690-691.

Santos, S. L., K. de Souza Costa, L. Q. Gondim, M. S. da Silva, R. S. Uzeda, K. Abe-Sandes and L. F. Gondim (2010). "Investigation of Neospora caninum, Hammondia sp., and Toxoplasma gondii in tissues from slaughtered beef cattle in Bahia, Brazil." Parasitol Res **106**(2): 457-461.

Savva, D., J. C. Morris, J. D. Johnson and R. E. Holliman (1990). "Polymerase chain reaction for detection of Toxoplasma gondii." J Med Microbiol **32**(1): 25-31.

Scala, A., M. Gobbe, P. P. Mula, A. P. Pipia, G. Sanna-Coccone, A. Firinu, A. Varcasia, R. Marrosu and G. Garippa (2008). "Toxoplasmosi dei suidi in Sardegna: indagine sieroepidemiologica." Parassitologia **50**: 235.

Schwab, K. J. and J. J. McDevitt (2003). "Development of a PCR-enzyme immunoassay oligoprobe detection method for Toxoplasma gondii oocysts, incorporating PCR controls." Appl Environ Microbiol **69**(10): 5819-5825.

Shaapan, R. M. and A. A. Ghazy (2007). "Isolation of Toxoplasma gondii from horse meat in Egypt." Pak J Biol Sci **10**(1): 174-177.

Shaapan, R. M., F. A. El-Nawawi and M. A. Tawfik (2008). "Sensitivity and specificity of various serological tests for the detection of Toxoplasma gondii infection in naturally infected sheep." Vet Parasitol **153**(3-4): 359-362.

Shapiro, K., J. A. Mazet, A. Schriewer, S. Wuertz, H. Fritz, W. A. Miller, . . . P. A. Conrad (2010). "Detection of Toxoplasma gondii oocysts and surrogate microspheres in water using ultrafiltration and capsule filtration." Water Res **44**(3): 893-903.

Sheffield, H. G. and M. L. Melton (1968). "The fine structure and reproduction of Toxoplasma gondii." J Parasitol **54**(2): 209-226.

Sheffield, H. G. and M. L. Melton (1970). "Toxoplasma gondii: the oocyst, sporozoite, and infection of cultured cells." Science **167**(3919): 892-893.

Shirley, M. W. (1997). 5.4. Working group 4, sarcocystiosis, toxoplasmosis, neosporosis, Luxembourg: Office for Official Publications of the European Communities. COST 820 - Vaccines against animal coccidioses. M. W. Shirley, EUROPEAN COMMISSION**:** 28-52.

Sibley, L. D. and J. W. Ajioka (2008). "Population structure of Toxoplasma gondii: clonal expansion driven by infrequent recombination and selective sweeps." Annu Rev Microbiol **62**: 329-351.

Silva, S. R., S. S. Meirelles and W. De Souza (1982). "Mechanism of entry of Toxoplasma gondii into vertebrate cells." J Submicrosc Cytol **14**(3): 471-482.

Simmons, O. D., 3rd, M. D. Sobsey, C. D. Heaney, F. W. Schaefer, 3rd and D. S. Francy (2001). "Concentration and detection of cryptosporidium oocysts in surface water samples by method 1622 using ultrafiltration and capsule filtration." Appl Environ Microbiol **67**(3): 1123-1127.

Skariah, S., M. K. McIntyre and D. G. Mordue (2010). "Toxoplasma gondii: determinants of tachyzoite to bradyzoite conversion." Parasitol Res **107**(2): 253-260.

Skinner, L. J., A. C. Timperley, D. Wightman, J. M. Chatterton and D. O. Ho-Yen (1990). "Simultaneous diagnosis of toxoplasmosis in goats and goatowner's family." Scand J Infect Dis **22**(3): 359-361.

Sotiriadou, I. and P. Karanis (2008). "Evaluation of loop-mediated isothermal amplification for detection of Toxoplasma gondii in water samples and comparative findings by polymerase chain reaction and immunofluorescence test (IFT)." Diagn Microbiol Infect Dis **62**(4): 357-365.

Splendor, A. (2009). "A new protozoan parasite in rabbits." Int J Parasitol **39**(8): 861-862.

Sroka, J., A. Wojcik-Fatla and J. Dutkiewicz (2006). "Occurrence of Toxoplasma gondii in water from wells located on farms." Ann Agric Environ Med **13**(1): 169-175.

Stagni, L., M. A. Romano, A. Romano, A. Magli, F. Briganti, M. A. Del Pezzo and W. Buffolano (2009). "Prenatal screening for congenital toxoplasmosis in Campania: preliminary report on activities and results." Mem Inst Oswaldo Cruz **104**(2): 374-377.

Stricker, R., R. Sitavanc, N. Liassine and F. de Marval (2009). "Toxoplasmosis during pregnancy and infancy." Swiss Med Wkly **139**(43-44): 643-644; author reply 643-644.

Szeredi, L. and A. Bacsadi (2002). "Detection of Chlamydophila (Chlamydia) abortus and Toxoplasma gondii in smears from cases of ovine and caprine abortion by the streptavidin-biotin method." J Comp Pathol **127**(4): 257-263.

Swets, J. A. (1988). "Measuring the accuracy of diagnostic systems." Science **240**(4857): 1285-1293.

Switaj, K., A. Master, M. Skrzypczak and P. Zaborowski (2005). "Recent trends in molecular diagnostics for Toxoplasma gondii infections." Clin Microbiol Infect **11**(3): 170-176.

Tebbe, C. C. and W. Vahjen (1993). "Interference of humic acids and DNA extracted directly from soil in detection and transformation of recombinant DNA from bacteria and a yeast." Appl Environ Microbiol **59**(8): 2657-2665.

Tenter, A. M. (2009). "Toxoplasma gondii in animals used for human consumption." Mem Inst Oswaldo Cruz **104**(2): 364-369.

Tenter, A. M., A. R. Heckeroth and L. M. Weiss (2000). "Toxoplasma gondii: from animals to humans." Int J Parasitol **30**(12-13): 1217-1258.

Thaller, R., F. Tammaro and H. Pentimalli (2011). "Fattori di rischio per la toxoplasmosi in gravidanza in una popolazione del centro Italia." Le Infezioni in Medicina **4**: 241-247.

Thiebaut, R., S. Leproust, G. Chene and R. Gilbert (2007). "Effectiveness of prenatal treatment for congenital toxoplasmosis: a meta-analysis of individual patients' data." Lancet **369**(9556): 115-122.

Thrusfield, M. (2005). Veterinary Epidemiology, Blackwell Publishing.
Tola, S., G. Satta, M. C. Angelici, T. Onni, C. Patta and G. Masala (2006). Epidemiology of Toxoplasma infection in small ruminants in Sardinia, Italy. Toxo & Food.
Tomita, N., Y. Mori, H. Kanda and T. Notomi (2008). "Loop-mediated isothermal amplification (LAMP) of gene sequences and simple visual detection of products." Nat Protoc **3**(5): 877-882.
Torrey, E. F., J. J. Bartko, Z. R. Lun and R. H. Yolken (2007). "Antibodies to Toxoplasma gondii in patients with schizophrenia: a meta-analysis." Schizophr Bull **33**(3): 729-736.
Torrey, E. F., J. J. Bartko and R. H. Yolken (2012). "Toxoplasma gondii and other risk factors for schizophrenia: an update." Schizophr Bull **38**(3): 642-647.
Torrey, E. F. and R. H. Yolken (2013). "Toxoplasma oocysts as a public health problem." Trends Parasitol **29**(8): 380-384.
Tsai, Y. L. and B. H. Olson (1992). "Rapid method for separation of bacterial DNA from humic substances in sediments for polymerase chain reaction." Appl Environ Microbiol **58**(7): 2292-2295.
Tzanidakis, N., P. Maksimov, F. J. Conraths, E. Kiossis, C. Brozos, S. Sotiraki and G. Schares (2012). "Toxoplasma gondii in sheep and goats: seroprevalence and potential risk factors under dairy husbandry practices." Vet Parasitol **190**(3-4): 340-348.
Uga, S., T. Minami and K. Nagata (1996). "Defecation habits of cats and dogs and contamination by Toxocara eggs in public park sandpits." Am J Trop Med Hyg **54**(2): 122-126.
Uggla, A. and D. Buxton (1990). "Immune responses against Toxoplasma and Sarcocystis infections in ruminants: diagnosis and prospects for vaccination." Rev Sci Tech **9**(2): 441-462.
U.S. Environmental Protection Agency (2001). Method 1623: Cryptosporidium and Giardia in water by filtration/IMS/FA. EPA 821-R-01-025. Washington, DC., Office of Water.
Unno, A., K. Suzuki, X. Xuan, Y. Nishikawa, K. Kitoh and Y. Takashima (2008). "Dissemination of extracellular and intracellular Toxoplasma gondii tachyzoites in the blood flow." Parasitol Int **57**(4): 515-518.
Valcavi, P. P., A. Natali, L. Soliani, S. Montali, G. Dettori and C. Cheezi (1995). "Prevalence of anti-Toxoplasma gondii antibodies in the population of the area of Parma (Italy)." Eur J Epidemiol **11**(3): 333-337.
van der Giessen, J., M. Fonville, M. Bouwknegt, M. Langelaar and A. Vollema (2007). "Seroprevalence of Trichinella spiralis and Toxoplasma gondii in pigs from different housing systems in The Netherlands." Vet Parasitol **148**(3-4): 371-374.
van Knapen, F., A. F. Kremers, J. H. Franchimont and U. Narucka (1995). "Prevalence of antibodies to Toxoplasma gondii in cattle and swine in The Netherlands: towards an integrated control of livestock production." Vet Q **17**(3): 87-91.
VanWormer, E., H. Fritz, K. Shapiro, J. A. Mazet and P. A. Conrad (2013). "Molecules to modeling: Toxoplasma gondii oocysts at the human-animal-environment interface." Comp Immunol Microbiol Infect Dis **36**(3): 217-231.
Velmurugan, G. V., J. P. Dubey and C. Su (2008). "Genotyping studies of Toxoplasma gondii isolates from Africa revealed that the archetypal clonal lineages predominate as in North America and Europe." Vet Parasitol **155**(3-4): 314-318.
Venturini, M. C., D. Bacigalupe, L. Venturini, M. Rambeaud, W. Basso, J. M. Unzaga and C. J. Perfumo (2004). "Seroprevalence of Toxoplasma gondii in sows from slaughterhouses and in pigs from an indoor and an outdoor farm in Argentina." Vet Parasitol **124**(3-4): 161-165.

Veronesi, F., D. Ranucci, R. Branciari, D. Miraglia, R. Mammoli and D. P. Fioretti (2011). "Seroprevalence and risk factors for Toxoplasma gondii infection on finishing swine reared in the Umbria region, central Italy." Zoonoses Public Health **58**(3): 178-184.

Vesco, G., W. Buffolano, S. La Chiusa, G. Mancuso, S. Caracappa, A. Chianca, S. Villari, V. Curro, F. Liga and E. Petersen (2007). "Toxoplasma gondii infections in sheep in Sicily, southern Italy." Vet Parasitol **146**(1-2): 3-8.

Vezzo, R., L. Vigorè, A. Goglio, A. Raglio, L. Vecchia, P. Terenziani and G. Fortina (2001). "Sieroprevalenza per anticorpi anti-Toxoplasma gondii in 14.186 soggetti." Microbiol Med **16**: 220.

Villari, S., G. Vesco, E. Petersen, A. Crispo and W. Buffolano (2009). "Risk factors for toxoplasmosis in pigs bred in Sicily, Southern Italy." Vet Parasitol **161**(1-2): 1-8.

Villena, I., D. Aubert, P. Gomis, H. Ferte, J. C. Inglard, H. Denis-Bisiaux, . . . J. M. Pinon (2004). "Evaluation of a strategy for Toxoplasma gondii oocyst detection in water." Appl Environ Microbiol **70**(7): 4035-4039.

Villena, I., B. Durand, D. Aubert, R. Blaga, R. Geers, M. Thomas, . . . L. Halos (2012). "New strategy for the survey of Toxoplasma gondii in meat for human consumption." Vet Parasitol **183**(3-4): 203-208.

Vyas, A., S. K. Kim, N. Giacomini, J. C. Boothroyd and R. M. Sapolsky (2007). "Behavioral changes induced by Toxoplasma infection of rodents are highly specific to aversion of cat odors." Proc Natl Acad Sci U S A **104**(15): 6442-6447.

Wallace, G. D. (1971). "Experimental transmission of Toxoplasma gondii by filth-flies." Am J Trop Med Hyg **20**(3): 411-413.

Wallace, G. D. (1972). "Experimental transmission of Toxoplasma gondii by cockroaches." J Infect Dis **126**(5): 545-547.

Wallace, G. D., L. Marshall and M. Marshall (1972). "Cats, rats, and toxoplasmosis on a small Pacific island." Am J Epidemiol **95**(5): 475-482.

Wastling, J. M., S. Nicoll and D. Buxton (1993). "Comparison of two gene amplification methods for the detection of Toxoplasma gondii in experimentally infected sheep." J Med Microbiol **38**(5): 360-365.

Watson, R. J. and B. Blackwell (2000). "Purification and characterization of a common soil component which inhibits the polymerase chain reaction." Can J Microbiol **46**(7): 633-642.

Webster, J. P. (2001). "Rats, cats, people and parasites: the impact of latent toxoplasmosis on behaviour." Microbes Infect **3**(12): 1037-1045.

Weiland, G. and D. Kuhn (1970). "[Experimental toxoplasma infections in the cat. II. Developmental stages of the parasite in the intestine]." Berl Munch Tierarztl Wochenschr **83**(7): 128-132.

Weinbach, E. C. and J. Garbus (1966a). "The rapid restoration of respiratory control to uncoupled mitochondria." J Biol Chem **241**(16): 3708-3713.

Weinbach, E. C. and J. Garbus (1966b). "Restoration by albumin of oxidative phosphorylation and related reactions." J Biol Chem **241**(1): 169-175.

Werk, R. (1985). "How does Toxoplasma gondii enter host cells?" Rev Infect Dis **7**(4): 449-457.

Wilder, H. C. (1952). "Toxoplasma chorioretinitis in adults." AMA Arch Ophthalmol **48**(2): 127-136.

Wingstrand, A., P. Lind, J. Haugegaard, S. A. Henriksen, V. Bille-Hansen and V. Sorensen (1997). "Clinical observations, pathology, bioassay in mice and serological response at slaughter in pigs experimentally infected with Toxoplasma gondii." Vet Parasitol **72**(2): 129-140.

Witte, H. M. and G. Piekarski (1970). "[Oocyst excretion in experimentally infected cats depending on the Toxoplasma strain]." Z Parasitenkd **33**(4): 358-360.
Witting, P. A. (1979). "Learning capacity and memory of normal and Toxoplasma-infected laboratory rats and mice." Z Parasitenkd **61**(1): 29-51.
Wolf, A., D. Cowen and B. Paige (1939). "Human Toxoplasmosis: Occurrence in Infants as an Encephalomyelitis Verification by Transmission to Animals." Science **89**(2306): 226-227.
Wolf, A., D. Cowen and B. H. Paige (1941). "Fetal Encephalomyelitis: Prenatal Inception of Infantile Toxoplasmosis." Science **93**(2423): 548-549.
Woods, M., R.A. McDonald and S. Harris S (2003). "Predation of wildlife by domestic cats Felis catus in Great Britain." Mammal Review **33**(2):174-88.
Worth, A. R., A. J. Lymbery and R. C. Thompson (2013). "Adaptive host manipulation by Toxoplasma gondii: fact or fiction?" Trends Parasitol **29**(4): 150-155.
Yang, W., H. D. Lindquist, V. Cama, F. W. Schaefer, 3rd, E. Villegas, R. Fayer, . . . L. Xiao (2009). "Detection of Toxoplasma gondii oocysts in water sample concentrates by real-time PCR." Appl Environ Microbiol **75**(11): 3477-3483.
Yazar, S., F. Arman, S. Yalcin, F. Demirtas, O. Yaman and I. Sahin (2003). "Investigation of probable relationship between Toxoplasma gondii and cryptogenic epilepsy." Seizure **12**(2): 107-109.
Yereli, K., I. C. Balcioglu and A. Ozbilgin (2006). "Is Toxoplasma gondii a potential risk for traffic accidents in Turkey?" Forensic Sci Int **163**(1-2): 34-37.
Yilmaz, S. M. and S. H. Hopkins (1972). "Effects of different conditions on duration of infectivity of Toxoplasma gondii oocysts." J Parasitol **58**(5): 938-939.
Zardi, O., E. Sulli, G. Venditti and G. Giorgi (1964). "Studi epidemiologici sulla toxoplasmosi. Isolamento di stipiti di Toxoplasma gondii da animali domestici. ." Nuovi Ann Ig Microbiol **15**: 545-551.
Zedda, M. T., S. Rolesu, S. Pau, I. Rosati, S. Ledda, G. Satta, C. Patta and G. Masala (2010). "Epidemiological study of Toxoplasma gondii infection in ovine breeding." Zoonoses Public Health **57**(7-8): e102-108.
Zulpo, D. L., S. A. Headley, L. Biazzono, I. A. da Cunha, M. Igarashi, L. D. de Barros, . . . J. L. Garcia (2012). "Oocyst shedding in cats vaccinated by the nasal and rectal routes with crude rhoptry proteins of Toxoplasma gondii." Exp Parasitol **131**(2): 223-230.