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Foodborne transmission of *Toxoplasma gondii*: a laboratory and literature based assessment

Coordinatore: Ch.mo Prof. Alessandro Zotti

Supervisore: Dott. Antonia Ricci

Dottorando: Simone Belluco

Abstract (English)

Toxoplasma gondii is a zoonotic parasite with a wide diffusion, a high human seroprevalence and the potential for causing severe harm when infection occurs in at-risk individuals such as pregnant women, HIV-positive individuals, recipients of organ transplants or other immunocompromised subjects. The consumption of contaminated food is estimated to account for about half of all infections worldwide, with variations across years and countries. Among food, meat is of high concern, as it is consumed in large amounts, not always well cooked, by a large part of the population.

The aim of the present project was to gain insight into the role of food as a source of human toxoplasmosis. The first step in this path was to assess the prevalence of *T. gondii* in livestock species commonly used as sources of meat for human consumption. To obtain relevant scientific evidence, a double-sided approach was applied. Firstly, a systematic review of all published studies dealing with the direct detection of *T. gondii* in meat belonging to relevant livestock species was carried out. This work allowed the estimation of the worldwide *T. gondii* prevalence in each species (cattle, pigs and sheep), and also the evaluation of differences due to the geographical origins and to the laboratory methods applied for diagnostic purposes. Two species were selected for a biomolecular investigation of prevalence at local level: cattle and pigs. Different reasons supported the choice to focus on these species, such as the wide consumption, the inconsistent epidemiological evidence (cattle) or the primary importance as a source of meatborne toxoplasmosis (pigs). Samples were collected from retail or ready to retail processing stages.

To support the outcomes of different activities, both from the systematic review and from experimental results, the best available epidemiological evidence about the role of different food consumption habits in human infection was systematically collected.

Finally, a quantitative risk assessment model was built, thanks to all the previously collected information, to estimate the yearly probability of infection due to bovine meat and pork consumption, in Italy.

The investigations carried out within the present research project allowed us to conclude that *T. gondii* prevalence in meat animals worldwide, as well as in Europe and Italy, is not negligible. Sheep meat displayed the highest prevalence rate, followed by pork and beef. However, our survey confirmed that meat preparation habits make beef a relevant risk factor for *T. gondii* infection in humans, as confirmed by the epidemiological evidence from the literature. In addition, the model allowed us to observe that, In Italy, bovine meat contributes more to the annual toxoplasmosis incidence than does pork.

Abstract (Italian)

Toxoplasma gondii è un parassita di interesse zoonosico con una notevole diffusione su scala mondiale, un'elevata sieroprevalenza nell'uomo ed è in grado di causare sintomatologia grave in soggetti a rischio come le donne in gravidanza e tutti i soggetti immunocompromessi. Si stima che il consumo di alimenti contaminati sia responsabile di circa la metà dei casi totali di infezione nell'uomo, con alcune differenze tra paesi. La carne, in particolare, è di notevole interesse epidemiologico in quanto viene consumata in grandi quantità dalla maggior parte della popolazione e non sempre viene cotta adeguatamente prima del consumo. L'obiettivo di questo progetto era quello di approfondire le conoscenze circa il ruolo degli alimenti come causa di toxoplasmosi umana tenendo in considerazione l'intera filiera alimentare.

Il primo passo è stato quello di stimare la prevalenza di *T. gondii* negli animali da reddito più comunemente utilizzati per la produzione di carne utilizzando un duplice approccio. In primis, sono stati sistematicamente raccolti gli studi pubblicati nella letteratura scientifica e riguardanti la ricerca di *T. gondii* nella carne di specie animali da reddito comunemente consumate. Questi studi hanno consentito di ottenere una stima di prevalenza per ogni specie selezionata (bovini, maiali e pecore) e di valutare le differenze dovute alla diversa origine geografica e al metodo diagnostico applicato. Successivamente sono state selezionate due specie (bovini, maiali) per un'indagine molecolare in grado di stimare la prevalenza su carni locali. Le specie sono state selezionate per ragioni diverse: la grande diffusione del loro consumo, evidenze epidemiologiche contrastanti (bovini), o il ruolo epidemiologico riconosciuto nella trasmissione di *T. gondii* (maiali). I campioni analizzati erano tutti idonei alla vendita. Per sostenere i risultati ottenuti dalle diverse attività di questo progetto, sia sul fronte della sintesi delle ricerche già pubblicate sia per quanto riguarda le attività sperimentali, una seconda *systematic review* è stata realizzata. In questo caso sono stati raccolti tutti gli studi epidemiologici (caso-controllo) pubblicati, indirizzati all'identificazione degli alimenti responsabili delle infezioni alimentare da *T. gondii*. Infine, è stato realizzato un modello di valutazione del rischio quantitativo, in grado di considerare tutti i dati e le evidenze precedentemente ottenute, con l'obiettivo di stimare la probabilità annua di contrarre toxoplasmosi a seguito del consumo di carne bovina o suina, in Italia.

Le analisi effettuate nel contesto di questo progetto di ricerca consentono di concludere che la prevalenza di *T. gondii* in specie animali comunemente consumate, nel mondo e in Europa, non è trascurabile. La carne di pecora ha la prevalenza più elevata, seguita dalla carne suina e bovina. Tuttavia l'abitudine di consumare la carne bovina cruda o poco cotta aumenta il rischio derivante da essa. Tale osservazione è confermata dagli studi epidemiologici ottenuti con la ricerca sistematica della letteratura cosi come dal modello statistico realizzato, che identifica il consumo di carne bovina come più probabile causa di toxoplasmosi nella popolazione italiana rispetto al consumo di carne suina.

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Contents

1. Introduction

Toxoplasma gondii is a zoonotic coccidian parasite with a striking diffusion across species and countries. Its evolutionary success made scientists define it as one of the more polyxenous parasites (Flegr, 2013; Tenter et al., 2000). *T. gondii* is able to infect nearly any nucleated cell in any warm-blooded animal (Harker et al., 2013). Seroprevalence in the human population, despite its decrease over time, is still remarkably high, being up to 30%, on average (Flegr, 2013).

In most aspects, *T. gondii* seems to be almost the ideal parasite thanks to set of features making it extremely widespread and highly prevalent:

-it does not affect host survival, at least in the short term;

-it can infect almost all warm blooded animals;

-it can be transmitted between different hosts in different ways (horizontally and vertically);

-it has three infective forms (sporozoites, tachyzoites, bradyzoites);

-it can rely both on the sexual and asexual life cycle for transmission.

Most of these features, and in particular the asymptomatic infection that the parasite triggers in the majority of subjects, probably explain why the fight against *T. gondii* has never been a priority for the food risk manager. In some cases, symptoms can be manifested but the real danger is for pregnant women and immunocompromised individuals. As regards the latent infection, it seems to cause no worries among practitioners and infected people despite the fact that the consequences of the cysts' presence in human tissues are largely unknown.

1.1 History of *T. gondii* research

One hundred years ago, the French physiologist Charles Richet (1850-1925), later awarded the Nobel Prize for physiology thanks to his research on anaphylaxis, decided to commercialize a new product, at the boundary between food and drugs: Zomine.

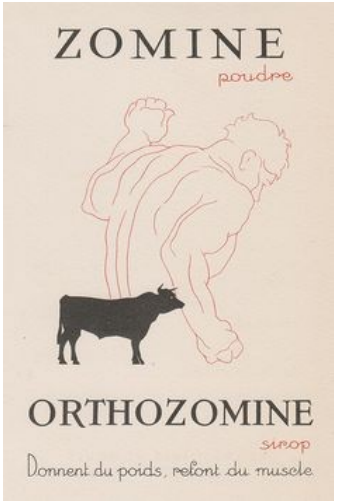

Figure 1: Zomine Advertisement

Zomine was an industrial lyophilized raw meat juice that, according to Richet, was necessary to widen the application of zoomotherapy. This therapy, based on the daily consumption of large

amounts of raw meat juice, was firstly used in experiments on dogs and subsequently, on humans. According to Richet, zoomotherapy was able to heal tuberculosis, or at least to halt the development of the disease. Despite several attempts, Richet was never able to prove the efficacy of the therapy, while remaining strongly convinced of its benefits. On the other hand, most peers attributed the health restoration to the nutritional benefits delivered from the protein-rich diet supplied to the treated patients (Lowy, 2010). Efficient or not, in the 1960s, the therapy was still being applied to children hospitalised in the pulmonary tuberculosis unit of Brevanne Hospital in Paris. In this hospital, the history of zomine crossed paths with the history of toxoplasmosis.

Toxoplasmosis is a disease caused by the protozoan *T. gondii*, first isolated in 1908 from *Ctenodactilus gondii*, and so called because of its curved shape. Even as late as 1937, *T. gondii* was not fully recognized as a human pathogen (Anonymous, 1937).

The first recorded case of human toxoplasmosis dates back to 1928, the existence of tissue cysts as the persistent parasite stage within tissues was unknown until 1928 and the potential for vertical transmission in humans was disclosed only in 1942.

In the fifties, the possibility of transmission through meat consumption had been hypothesized and from 1960, it was clear that the high seroprevalence of *T. gondii* in humans could hardly be explained by a single transmission route.

The theory of foodborne transmission to humans, already recognized for animals, was supported by the following data, as described by Desmonts and colleagues:

- *T. gondii* had been isolated from pork, mutton and beef (Jacobs et al., 1960), and its diffusion in animals was supported by seroprevalence data;
- the parasite’s virulence was preserved after treatment with pepsin in chloride media and with trypsin, and thus, was unaffected by gastric digestion (Jacobs et al., 1960);
- animals became infected after ingestion of cysts (Desmonts et al., 1965).

However, the final proof of meat’s involvement in human infection was obtained only when research about toxoplasmosis met zoomotherapy. Desmonts and colleagues (1965) noticed a higher seroprevalence in children hospitalised at Brevanne than in the general French population. This finding was thought to be linked to the special diet supplied to these children. From six months of age onward, the diet at the hospital was based on a high frequency of raw meat consumption (horse, beef and meat juice) in line with Richet’s beliefs. To better investigate this first observation, Desmonts and colleagues, starting from the belief that toxoplasmosis was a benign infection, decided to increase the daily intake of raw meat, supplying additional portions of raw mutton to a group of child patients. As a result, a corresponding increase in the infection rate was observed (Desmonts et al., 1965). Other relevant milestones in the study of *T. gondii* biology were the 1970 descriptions of the protozoan’s sexual reproduction in the small intestine of cats (Dubey et al., 1970; Frenkel et al., 1970) , with the recognition of the centrality of felines’ epidemiological role. In the 90s, the largest outbreak of acute toxoplasmosis in humans (100 cases) due to municipal drinking water was described.

Year	Event	Reference
1908	Description of *T. gondii* merozoites in *Ctenodactilus gondii*	(Nicolle and Manceaux, 1909)
1909	Introduction of the genus *Toxoplasma*	(Nicolle and Manceaux, 1909)
1923	First recorded case of toxoplasmosis in humans, in an 11-month-old infant	
1928	First description of a tissue cyst as a persistent stage	(Levaditi et al., 1928)
1942	Vertical transmission recognized in humans	(Paige et al., 1942)
1954-56	Hypothesis about meat's role in *T. gondii* transmission	
1965	Epidemiological evidence of transmission to humans through undercooked meat	(Desmonts et al., 1965)
1970	Description of the sexual phase of the life cycle in the small intestine of cats	(Dubey et al., 1970; Frenkel et al., 1970; Hutchison et al., 1970)
1969-72	1969-72 Recognition of epidemiological role of cats	(Munday, 1972; Wallace, 1969)
1995-99	Largest recorded outbreak of toxoplasmosis due to water consumption	(Bowie et al., 1997)
2005	*T. gondii* genome annotated	(Khan et al., 2005)
2010	Development of a Magnetic Capture RealTime PCR method for *T. gondii* detection	(Opsteegh et al., 2010)

Table 1: milestones in *Toxoplasma gondii* research, from the first isolation to recent years

1.2 Biology

T. gondii is a ubiquitous parasite with an extremely complex life cycle. Definitive hosts are members of the family *Felidae*, whereas almost all warm blooded animals can be intermediate hosts of the parasite (Tenter et al., 2000).

In intermediate hosts, *T. gondii* can complete its asexual life cycle where tachyzoites are able to actively penetrate in nucleated cells and to establish a non-fusogenic vacuole where it multiplies, originating in two offspring (endodiogeny). After that, tachyzoites enter the bloodstream, disseminate to other tissues and form cysts. During this phase, a strong inflammatory response takes place with the potential to control the infection and reduce parasite burden. In tissue cysts, tachyzoites differentiate to bradyzoites, another *T. gondii* developmental stage, which multiply slowly, increasing the dimension and the parasite content of the cysts up to 500/1000 parasites and setting up the chronic infection. These cysts can persist for the entire life of the host and are mainly located in brain, eye, skeletal and cardiac muscles. During strong immunosuppression events, cysts can break down and potentiate other tissue invasions during the host life (Harker et al., 2015).

In the definitive host, the parasite can behave in the same way as in intermediate hosts, but in addition to endodyogeny, it can also carry out endopolygeny in intestinal cells, a reproductive process characterized by the genesis of several parasites in a single event.

In addition to endopolygeny, the other peculiarity of the life cycle in the definitive host is the sexual propagation that initiates after gamete formation. The sexual reproduction produces millions of oocysts in the intestinal lumen, which are subsequently delivered into the environment through faeces and are able to cause huge environmental contamination with the potential for infecting other hosts.

According to the previously described life cycle, there are three infectious stages in the *T. gondii* life cycle:

-sporozoites, included in oocysts delivered into the environment by cat faeces. Oocysts become infective after a few days of maturation and can be horizontally transmitted through the ingestion of contaminated matrices (e.g. water, soil, vegetables);
-bradyzoites, contained in tissue cysts and able to be transmitted horizontally by ingestion of meat from infected animals;
-tachyzoites, available in body fluids during acute infection or during reactivation of latent infections and able to be transmitted vertically during gestation or through milk during lactation.

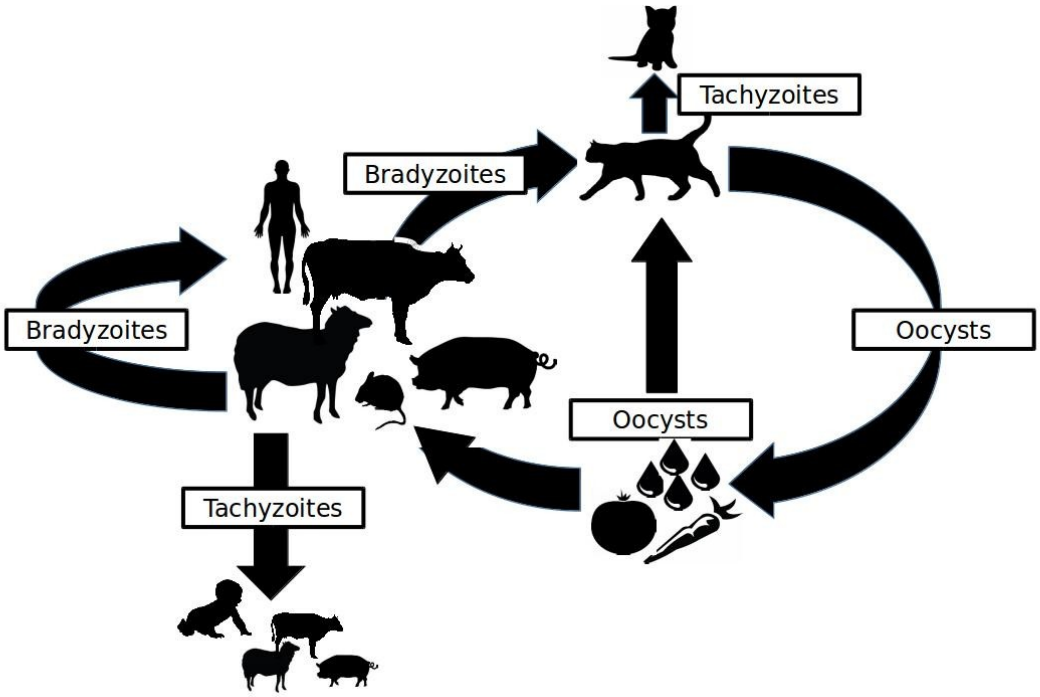

Figure 2: *Toxoplasma gondii* life cycle

1.2.1 Evolution and phylogenesis

The scientific knowledge about *T. gondii* is quite recent, as previously described, but the history of this protozoan is far more ancient. It has been argued that *T. gondii* evolutionary success is linked to the acquisition of the ability to circumvent sexual reproduction and to rely on oral transmission between intermediate hosts. This ability not only differentiate *T. gondii* from its closely related parasites, such as *Neospora*, *Sarcocystis*, and *Hammondia*, but also is probably the characteristic that gave *T. gondii* the possibility to infect a wide range of hosts. Interestingly, this ability probably originated from a recent single genetic cross (1 million years ago) that differentiate the three most common serotype with exotic ones, the last not always showing oral infectivity. This meiotic event not only gave *T. gondii* a selective advantage, but was also a mean of fixing the entire genotype via the hitchhiking effect. According to epidemiological data there are three main strains of the parasite circulating worldwide with some additional genotypes categorised as “wild” or exotic. The wide epidemiological pattern

and the predominance of a sexual life cycle probably would hardly explain this population feature. The estimated origin of direct oral transmission, in *T. gondii,* is concurrent with the time of human agricultural expansion and adaptation of the cat as a companion animal, developments that created an unprecedented concentration of hosts and opportunities for new routes of transmission (Su, 2003)

With the acquisition of oral infectivity, *T. gondii* became able to transmit from definitive to intermediate hosts and vice versa, between definitive hosts and between intermediate hosts. This unique transmission pathway is of capital importance for the parasite success, however it is still unclear which route of transmission is more important epidemiologically (Tenter et al., 2000).

T. gondii has a complex population structure comprising several strains. Existing genotypes belong to three major groups, I, II or III. However, a variety of genotypes not included in these major groups have been isolated worldwide and are defined as "atypical", "wild" or "ancient" strains. The importance of the genomic study of *T. gondii* is not limited to taxonomical investigations or epidemiological concerns but has a direct link to the severity of the disease. Genotypes I, II and III are similar on a genetic basis but they differ in terms of virulence. Genotype I shows the highest virulence and migratory capacity, whereas atypical genotypes are more different to each other. The majority of strains isolated in Europe and North America belong to types I, II or III, whereas a fourth strain is commonly found in wildlife in North America. In South America, these major lineages are rarely isolated and a variety of atypical strains exists (Xiao and Yolken, 2015). A link between human disease and *T. gondii* strains has been recently highlighted for the most common pathological conditions caused by the parasite: ocular infection in immunocompetent adults, congenital infection in the foetus or newborn, infection in adults with AIDS or other immunocompromised states, and severe diseases (e.g. disseminated, pulmonary toxoplasmosis and psychosis) in immunocompetent patients. Despite the incidence of different genotypes reflecting the geographical origins of cases, a significant association has been observed between type I and ocular disease, congenital toxoplasmosis and some neurological disorders (Saeij et al., 2005; Xiao and Yolken, 2015). The different pathogenicity among strains can be explained by the different load reached by parasites belonging to different genotypes in affected human tissues. In immunocompromised individuals, genotype I, reaching the highest burden, causes the most severe diseases. Atypical strains show the highest virulence in immunocompetent individuals, probably because of the lack of parasite-human co-evolution with host adaptation, as in the case of typical strains. Differences among strains, however, do not completely explain the variability of the effects that *T. gondii* can cause in humans, as other factors can play important roles, such as the characteristics of the host and co-infections (Saeij et al., 2005; Xiao and Yolken, 2015).

1.2.2 Distribution in animals

Among *T. gondii* transmission routes, the horizontal one, between intermediate hosts, is of high concern. It is well-known that meat from infected animals can lead to human infection but the relative role of different animal species as well as the cysts distribution within animals is still

unclear. Recently, the presence of *T. gondii* in different animal tissues has been extensively reviewed with the identification of a tissue ranking system based on the number of positives samples from different tissues described in the relevant literature (Opsteegh et al., 2016) The most important tissues are listed in Table 2. Brain, heart and meat/muscle are the highest-ranking tissues with higher frequency of *T. gondii*.

	Swine	Cattle	Ovine	Caprine	Chickens	Turkeys	Horses
Brain	1	-	1	3	2	2	4
Heart	2	-	2	4	1	1	1
Meat/muscle	3	4	3	1	3	4	-
Tongue	4	-	-	-	-	-	2
Diaphragm	-	-	4	-	-	-	-
Lymph nodes	-	1	-	-	-	-	-
Small intestine	-	2	-	-	-	-	2
Liver	-	3	-	-	-	3	-
Kidneys	-	-	-	2	-	-	-
Ovary duct	-	-	-	-	4	-	-

Table 2: ranking of tissues most commonly infected by *T. gondii* in different species (Opsteegh et al., 2016)

Quantitative data describing parasite concentration in animal tissues are available for pork (Juránková et al., 2014), goats (Juránková et al., 2013) and sheep (Opsteegh et al., 2010). Brain was confirmed as the favoured site in pigs by quantitative techniques as well. After experimental infection, the median (range) number of parasites per gram was estimated to be: brain 553.7 (3858-122), lungs 0.3 (61-0.02), heart 2.6 (7.32-0.37), dorsal muscle 0.6 (2.81-0.3), forelimb, hindlimb, kidney and liver 0.2 (Juránková et al., 2014).
In goats, brain and lungs were the tissues with the highest concentrations of parasites, whereas lower numbers, between 10 and 100 parasites/gram were detected in heart and muscles (forelimbs, hindlimbs and dorsal muscle) (Juránková et al., 2013).
As regards sheep heart, a distribution with a mean value of 3.6 parasites/gram was used by Opsteegh and colleagues (2011) after the elaboration by PCR results from a previous study (Opsteegh et al., 2011).

1.3 Zoonosis potential and impact on the human population

Seroprevalence

T. gondii seroprevalence in the human population has been frequently investigated. Reported rates of infection range from 0 to 100% across years and countries (Tenter et al., 2000). As regards Italy, seroprevalence has been recently described in some studies. A survey of 13,000 individuals living in the area of Massa and Carrara (Central Italy) measured a seroprevalence of 24.4% (2010) with a decreasing trend from previous years (31% in 2007), and an increasing trend according to age (Mosti et al., 2013).

Immunocompetent individuals

Primary *T. gondii* infection in children and adults (including pregnant women) is often asymptomatic, although in certain cases a mild lymphadenopathy can be observed for 4-6 weeks. Inflammation is a rare event in this population.

Chorioretinitis can be observed after congenital, postnatally acquired infection, as a result of acute events or after reactivation of latent disease. Patients shows noticeably white focal lesions with an overlying and intense vitreal inflammatory reaction (Harker et al., 2015; Montoya and Liesenfeld, 2004; Tenter et al., 2000).

Immunocompromised individuals

The disease course is life-threatening in immunocompromised individuals with or without AIDS. In these cases, the illness is often a consequence of infection reactivation, as the seroprevalence, and thus latent/chronic infection, is high among this group, especially in developing countries and with increasing age. The central nervous system is the site most typically affected by infection, with inflammation leading to an evolving encephalitis causing an acute confusional state. Clinical manifestations include mental status changes, seizures, focal motor deficits, cranial nerve disturbances, sensory abnormalities, cerebellar signs, movement disorders, and neuropsychiatric findings. Toxoplasmosis in immunocompromised patients can also present as chorioretinitis, pneumonia, or multi organ involvement presenting with acute respiratory failure and haemodynamic abnormalities similar to septic shock. *T. gondii* pneumonia seems to be more frequent in recipients of bone-marrow transplants and in patients with AIDS (Harker et al., 2015; Montoya and Liesenfeld, 2004; Tenter et al., 2000).

Congenital toxoplasmosis

Congenital toxoplasmosis is the most severe form of infection, especially when acquired by pregnant women early in pregnancy. The mother-to-foetus transmission rate increases with gestational age from 9% in the first trimester to 59% in the last three months. The severity of symptoms shows an inverse trend. The most severe consequences of infection occur in foetuses during the first trimester, with clinically apparent disease occurring in 79% of newborns and a mortality rate up to 5% (Montoya and Liesenfeld, 2004).

Pre-natal lesions include intracranial calcifications, ventricular dilatation, hepatic enlargement, ascites, and increased placental thickness. Neonatal clinical manifestations of congenital toxoplasmosis vary widely and include hydrocephalus, microcephaly, intracranial calcifications, chorioretinitis, strabismus, blindness, epilepsy, psychomotor or mental retardation, petechia due to thrombocytopenia, and anaemia. The classic triad of chorioretinitis, hydrocephalus, and cerebral calcifications is rather rare. None of the signs described in newborns with congenital disease is pathognomonic for toxoplasmosis.

Latent infection

Latent *T. gondii* infection sets up after the acute phase in both in immunocompromised and immunocompetent individuals. Tissue cysts develop in different organs, with tissue tropism varying according to host and strength of the immune response. The worst effect caused by latent *T. gondii* is the potential for reactivation during strong immunosuppression events such as HIV infection or organ transplantation. The reactivation of an old infection can be life threatening for the host.

However, recently, the presence of tissue cysts in the host brain has become associated with neurological disorders. This is a fascinating chapter of *Toxoplasma* research. In mice, *T. gondii* has been demonstrated to have the potential to modify host behaviour, with advantages for parasite transmission, despite an uneven distribution within the brain (Berenreiterová et al.,

2011). The apparent randomness of *T. gondii* distribution within the brain makes investigation of the effect on host behaviour quiet difficult. However, evidence exists in the scientific literature supporting the behavioural manipulation hypothesis in humans as well as in mice (Flegr, 2013), and also linking *T. gondii* infection with neurological disorders (Fabiani et al., 2015; Sutterland et al., 2015).

1.3.1 Burden of disease

Another aspect that has contributed to the increased attention toward *T. gondii* in recent years is the study of disease impact on the human population. This technique is able to quantify the impact of a disease, taking into account different consequences of infection, such as death and disability, and accounting also for illness severity and duration. Different metrics can be used to go beyond the consideration of single aspects of disease consequences, but the most commonly used metric is Disability Adjusted Life Years (DALY). DALY is the sum of Years of Life Lost (YLL) and Years Lived with Disability (YLD). YLL is the number of years of life lost due to mortality of a specific disease in a specified population, calculated by summation of all fatal cases due to all health outcomes of that specific disease, each case multiplied by the expected individual life span at the age of death. YLD is the number of years lived with a disability, calculated by accumulation over all cases and all health outcomes of the product of the duration of the illness and the disability weight of a specific disease (Havelaar et al., 2012).
In a global effort, a WHO working group recently published global estimates and regional comparisons of the burden of foodborne disease in 2010 (Havelaar et al., 2015; WHO, 2014). *T. gondii* was included as a foodborne parasite and its burden was estimated with reference to a previous work targeting congenital disease (Torgerson and Mastroiacovo, 2013). *T. gondii*, together with *T. solium*, contributed significantly to the foodborne disease burden in Central and South American subregions. Prenatal infections accounted for 32% of the toxoplasmosis burden. Among all the investigated hazards, it ranked 13th considering the median DALY. *T. gondii* DALY was 829,071 with a foodborne YLD of 763,326 and a YLL of 62,899 as a results of 684 deaths and 10,280,089 cases of illness (Havelaar et al., 2015). Among foodborne parasites, *Toxoplasma* and *Ascaris* proved to be the pathogens with the highest DALYs, as reported by a recent review about helminths and toxoplasmosis, summarizing different reports (Havelaar et al., 2015).
A similar study, carried out in the Netherlands, resulted in a high DALY for both congenital and acquired toxoplasmosis. The former was the highest ranked of the foodborne pathogens (Havelaar et al., 2012).

1.3.2 Prevention strategies

In the European Union as well as elsewhere, there are no control strategies in place to identify and mitigate the risk of *T. gondii* presence in slaughtered animals.
In the recent EFSA opinions on meat inspection, the authority has carefully addressed the *T. gondii* hazard, stating that under the current system, meat inspection procedures are unable to detect *T. gondii* in meat from slaughtered animals. In the documents published by EFSA, the following conclusions have been drawn:

- *T. gondii* is one of the most relevant biological hazards for goats and sheep; however, the lack of methodologies easily applicable to detect cysts impairs the possibility of risk-based categorization. More studies are needed to evaluate the effect of processing in the reduction of parasite burden (EFSA, 2013a).
- *T. gondii* was ranked as a high priority for farmed wild boar and farmed deer, and studies defining the baseline prevalence of *T. gondii* are warranted.
- *T. gondii* was defined as an undetermined priority in bovine and horses due to the lack of data in scientific literature (EFSA, 2013b)
- *T. gondii* was defined as of medium relevance in swine due to the reduction in prevalence levels as obtained by commercial farming practices (EFSA, 2011).

1.4 Detection in food

Toxoplasmosis in food animals (e.g. sheep, goats, pigs) can be diagnosed by direct and indirect laboratory methods.

1.4.1 Direct methods

Histology and immunohistochemistry

Tachyzoites (during the acute phase of infection) and tissue cysts of *Toxoplasma* can be visualized in sections of tissues and organs of affected animals by histology (haematoxylin and eosin stain) and immunohistochemistry.

Tissues/organs eligible for this kind of analysis are: skeletal muscle, heart and brain.

Tachyzoites can also be detected in impression smears from the same organs.

In vitro culture

T. gondii can be isolated in cell culture. This technique allows the propagation of the parasite. However, cell culture is time-consuming and expensive and less sensitive than other methods.

PCR

Polymerase chain reaction (PCR) assays are widely used in the detection of parasite in food and animal tissues, and also in the detection of blood-circulating tachyzoites during acute infection. End-point PCR, Nested PCR and Real Time PCR are all described in published studies. They can be highly specific, but the small size of the sample required for the tests (usually 25 mg for commercial extraction methods) may limit their sensitivity, since the distribution of the tissue cysts is random, and the density of the parasite in affected tissues can be low (Juránková et al., 2014, 2013).

The original protocol for Nested PCR was set up by Burg et al. and was demonstrated to be highly specific for the 35-fold repetitive *T. gondii* B1 gene, despite the short target sequence. The alternative method is a Real Time protocol by Homan et al. and targets 200 to 300-fold repetitive *T. gondii* DNA fragments (Burg et al., 1989; Homan et al., 2000)

Both methods are valuable solutions to detect *T. gondii*, but they suffer from low sensitivity due to the tiny amount of tissue used for analysis. The choice of repetitive target sequence is a solution able to increase sensitivity and making detection of the 529-bp element preferable to the B1 sequence, despite the recent observation that the copy number of both is lower than was previously estimated (Costa and Bretagne, 2012). However, it was recently observed that

the 529-bp AF146527 repeat element is not present in all *T. gondii* isolates (Wahab et al., 2010).

If both techniques and targets are valuable solutions, the low amount of tissue used for analysis is still a concern. For this reason, studies have investigated potential solutions and the more interesting of them has been the recourse to a magnetic capture PCR. This technique allows the detection of parasite DNA presence in a large amount of tissue, up to 100g. The method is based on homogenization of meat sample and the capture of DNA through a magnetic system that exploits biotin-labelled capture oligonucleotides able to identify and associate with target sequences (Opsteegh et al., 2010). The DNA concentration is a valuable solution to increase sensitivity. However, it obviously does not provide a solution to the inability of PCR to distinguish between live and dead parasites.

Bioassay

The gold standard for *T. gondii* detection in animal tissue, with a high sensitivity, specificity and the ability to identify the infective status of the parasite, is cat bioassay. This *in vivo* diagnostic technique consists of feeding *Toxoplasma*-free cats with up to 500g of the tissue under investigation and subsequent evaluation of infection, usually demonstrated by oocysts excretion.

Cats are the laboratory animals of choice, in that they are the definitive hosts of *T. gondii* and allow isolation of oocysts for genotyping purposes. However, they are not so easily manageable under laboratory conditions and are unavailable in most facilities. Therefore, recourse to bioassay in mice has emerged has an alternative solution. The lower sensitivity of the mouse model to oral infection has been addressed with the intraperitoneal or subcutaneous inoculation of the pre-digested tissue under investigation. Usually 50-200 g of tissue is digested. Samples such as brain or peritoneal fluid are tested by microscopy or PCR to detect the infection. The acquisition of infection is usually evaluated in the brain through direct detection techniques.

The comparison between direct detection methods, recently carried out in the context of an EFSA external scientific report through a systematic review approach, showed that cat and mouse bioassays are the best available direct methods, with cat bioassay outperforming mouse bioassays when direct comparison was made. Microscopy proved to be the least sensitive method, whereas PCR was better than microscopy but never performed better than cat bioassays. Comparison of PCR with mouse bioassays was unclear in terms of performance results, but underlined the possibility that specific PCR protocols could equate with mouse bioassay performance, and also produce clear advantages in terms of costs and feasibility (Opsteegh et al., 2016).

1.4.2 Indirect methods

Serological methods

Serological methods for *T. gondii* detection are based on the detection of *T. gondii* antibodies in blood or in other bloody fluids such as thoracic fluid of aborted foetuses, milk or samples of fluid obtained by freezing and thawing portions of muscular tissue (meat juice).

The plethora of serological solutions includes:

- Immunofluorescence antibody test (IFAT)
- Enzyme-linked immunosorbent assay (ELISA)
- Carbon immunoassay (CIA)
- Modified agglutination test (MAT)
- Direct agglutination test (DAT)
- Latex agglutination test (LAT)
- Indirect haemagglutination test (IHAT)

Beyond the technical differences among tests, the main problems are linked to the scarce correlation between serological results and the effective presence of infective cysts, as observed in bovines and swine (Opsteegh et al., 2016).

This has been clearly demonstrated for cattle, where the discordance between direct and indirect techniques have demonstrated that seroprevalence cannot be used as an indicator of the number of cattle carrying infectious parasites. Parasite DNA was detected in seronegative animals, suggesting that only recent infections were detectable (Opsteegh et al., 2011). In addition to the low predictive value of seroprevalence, another circumstance is of concern, as the application of serology to food would imply the use of meat juice as the analytical matrix. The antibody titre in meat juice has been demonstrated to be lower than in serum or specific muscles. In addition, low titres in meat juice were defined as unreliable for diagnostic purposes (Wallander et al., 2015).

2. Thesis outline

Toxoplasma gondii is a zoonotic parasite with a wide diffusion, a high human seroprevalence and the potential for causing severe harm in those contracting infection, especially in at-risk individuals such as pregnant women, HIV-positive individuals, recipients of organ transplants or other immunocompromised categories. Food is estimated to account for about half of all infections worldwide, with variations across years and countries. Among food, meat is of high concern as it can harbour infectious parasites and can be consumed raw or undercooked by different parts of the population.

It is a consolidated practice, in the food safety system, to confront foodborne hazards in the context of a farm to fork approach as the strong relationships between different phases of the food chain cannot be neglected. Knowledge about the behaviour of a pathogen of interest along the food chain allows assessment of factors that are more important in the pathogen diffusion, and of which prevention measures are expected to give the best results. Knowledge of the farm-to-fork behaviour of a foodborne hazard is also essential for the risk manager and risk communicator to implement control strategies and for a communication campaign able to reduce the impact of the disease on the population. Veterinarians play a major role in this context, as they are responsible for animal health and also for the safety of derived food.

The aim of the present project was to gain insight into the role of meat as source of human toxoplasmosis by using a farm-to-fork approach. The underlying hypothesis was that meat other than pork could be important routes of transmission of the parasite due to food preparation habits rather than to high prevalence in the animals. The first step in this path was to estimate the prevalence of *T. gondii* in livestock species commonly used as sources of meat for human consumption. To obtain relevant scientific evidence, a double-sided approach was used. Firstly, a systematic review of all published literature dealing with the direct detection of *T. gondii* in commonly consumed livestock species was carried out. This process allowed the collection of data from primary research that, thanks to the meta-analytical method, were summed up to obtain average estimates of prevalence for different animal species worldwide and to evaluate differences according to the geographical origins and to the laboratory methods applied for diagnostic purposes (**Manuscript 1**). Two species were selected for a laboratory-based investigation of prevalence at local level through biomolecular techniques: cattle and pigs. Cattle and pigs were selected because of their wide consumption in Italy, as well as in the Veneto region that was targeted by these investigations (**Manuscript 2**).

In addition, to obtain the best available epidemiological evidence about the role of different food consumption habits in human infection, in order to assess the validity of other outcomes of the present project, a second systematic review was carried out addressing this topic. In particular, relevant published case-control studies were collected and their results were summed up to obtain final risk measures (**Manuscript 3**).

After that, before the building of the final risk assessment model, two important data sets were needed, addressing local consumers: data about food consumption and food preparation habits.

The first data were obtained by the last Italian survey on food consumption carried out by INRAN in 2005-06. To obtain relevant data on food consumption habits, in terms of frequency of cooking and freezing of meat, an ad hoc survey was designed and disseminated through social networks and a consumer mailing list (**Survey**).

Finally, the data from manuscript 1 and Survey were used to build a Quantitative Microbial Risk Assessment Model with the aim of estimating the yearly probability of an Italian consumer acquiring toxoplasmosis through the consumption of fresh meat from pigs or cattle. The final probability was used to predict the number of new cases of toxoplasmosis per year in the population between 5 and 65 years of age, attributable to the these meat sources. In addition, the model, properly refined, was used to predict the number of newborns with congenital toxoplasmosis, as well as the number of foetuses expected to acquire the infection through vertical transmission in the first trimester, with the worst consequences (**Manuscript 4**).

Finally, the model output was evaluated in the context of manuscript 4 and also of the available epidemiological human incidence data.

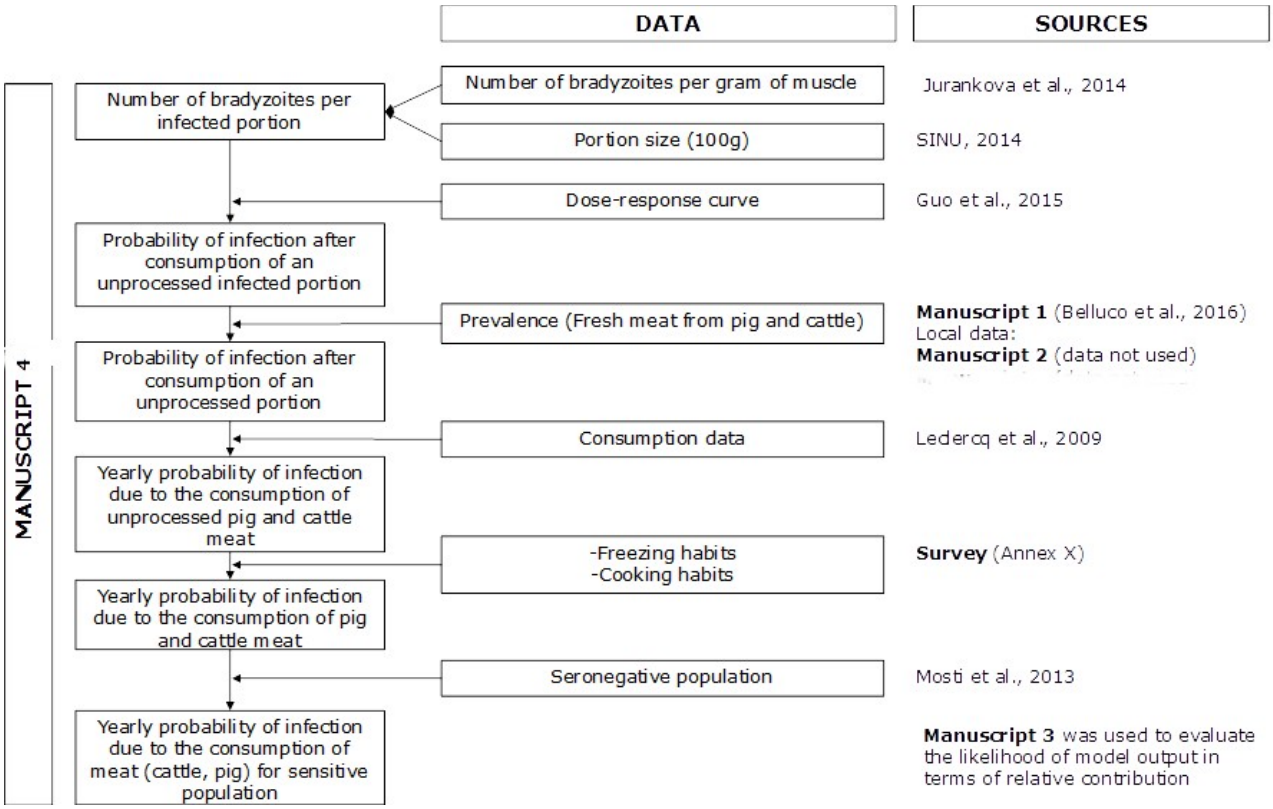

Figure 3: Flowchart showing project outline and the contribution of different activities to the final risk assessment model

3. Conclusions

- The prevalence of *Toxoplasma gondii* varies across countries and its estimate is influenced by the diagnostic method applied. However, on average, considering data obtained through direct detection techniques (bioassays, PCR) at a worldwide level, the *T. gondii* prevalence is 2.6% for cattle (2.2% in Europe), 12.3% for pigs (8.7% in Europe) and 14.7% for sheeps (9.6% in Europe). Few data are available in the scientific literature concerning horses and goats.
- The prevalence in the Veneto region, according to Nested PCR, was 8.5% for pigs, confirming systematic review results for Europe, and 20% for beef belonging to emergency slaughter. The latter result was quite unexpected, as prevalences in bovine have been shown to be low, as described in the scientific literature (see conclusion 1). The high prevalence in emergency slaughter beef can partly be explained by the particularity of sample composition in terms of age and productive category.
- The survey carried out to understand the frequency of meat consumption habits in terms of cooking and freezing, highlighted that, among the different meat species investigated (cattle, pigs, sheep, horse), meat from cattle is more likely to be consumed raw or undercooked, followed by horse meat.
- The aggregation of case-control studies collected from scientific literature and dealing with food related risk factors linked to *T. gondii* infection in humans identified the consumption of raw/undercooked beef or sheep meat as significant risk factors. However, it failed to identify consumption of raw/undercooked pork, eggs and milk as significant factors.
- The Quantitative Microbial Risk Assessment Model, informed by data from literature and from primary research carried out within this project, compared the yearly probability of infection for an Italian consumer, due to bovine meat and pork consumption. The model showed that bovine meat accounted for a higher proportion of cases than pork, due in particular to the absence of mitigation strategies at consumer levels.
-The main evidence which has emerged from this project is that bovine meat has the potential for a non-negligible role in *T. gondii* human epidemiology, despite the low prevalence rates in animals. Proper cooking or freezing of meat, including bovine meat, is suggested to mitigate the risk of toxoplasmosis, particularly for at-risk categories of people, such as pregnant women and immunocompromised individuals.

4. Future perspective

Toxoplasma gondii is a fascinanting parasite with a very complex life cycle allowing a variety of transmission routes. The infection of humans with *T. gondii* can generate important symptomatological patterns particularly in some categories such as pregnant women and immunocompromised person where abortus, ocular lesions and neurological diseases are possible consequences. However, pathogenic strains also showed the ability to cause symptomatic disease in immunocompetent individuals, and the link between seropositivity and various neurologial disorders has been hypothesized and demonstrated, in the light of the parasite's ability to manipulate host behaviour. Further research adressing this topic is warranted to disclose the real burden of this disease and to give adequate priority to prevention strategies addressing this neglected parasite.

In the meantime, it is important to understand to which extent each transmission route contributes to *T. gondii* epidemiology. This research topic should take advantage of the recent development of genomics and the ability to implement source attribution studies. The life cycle of *T. gondii* greatly relies on its definitive hosts (felids) and research into potential vaccination strategies is warranted, as a reduced delivery of oocysts into the enviroment would reduce prevalence in humans, in particular in the urban context. However, the parasite can succesfully rely on the horizontal transmission route among intermediate hosts, reducing the potential effect of a prevention strategy based exclusively on the definitive host. For this reason, mitigation strategies based on the food transmission route should not be neglected, as the potential presence of parasites is recognized in foods. Among foods, meat and vegetables are recognized as potential vehicles of *T. gondii*. The role of vegetables is largely unknown and should be targeted by future research to understand their real impact on cases of infection. Along the meat chain, prevention could be partially based on primary production, warranting the control of recognized risk factors linked to higher prevalences in animal populations, such as the presence of cats or wild animals on farms. As an additional, necessary measure, it would be important to address processing stages with reliable identification methods. Due to the inhability to easily detect live parasites in meat, historically, the *T. gondii* risk has never been managed through meat inspection strategies, as in the case of echinococcosis, or through analytical procedures, as in the case of *Trichinella spiralis* in the EU. The development of methods able to fill this gap has to be encouraged and suitable methods implemented to guarantee consumer safety.

Currently, the risk of acquiring *T. gondii* infection rely on an "unluckly" combination of events such as the presence of cysts in the meat portion, the lack of inactivation stages (freezing or cooking) and the immunological status of the consumer.

The education of consumers to properly manage food is the only sporadic mitigation strategy currently applied for avoidance of *T. gondii* infection. However, education is limited to at-risk individuals, and depends on health practicioners' knowledge. Information campaigns should be better planned and delivered, should go beyond education of at-risk individuals and should take into account food preparation habits.

In an ideal risk-based food safety system, meat harbouring live parasites should be identified and directed toward processing stages able to inactivate parasites, which would the exposure of consumers to *T. gondii*.

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Manuscript 1

Investigating the Determinants of Toxoplasma gondii Prevalence in Meat: A Systematic Review and Meta-Regression

Simone Belluco*[1,2], Marzia Mancin[1], Daniele Conficoni[2], Giulia Simonato[2], Mario Pietrobelli[2], Antonia Ricci[1]

[1]Food Safety Department, Istituto Zooprofilattico Sperimentale delle Venezie, viale dell'Università 10, 35020 – Legnaro (PD), Italy.
[2]Department of Animal Medicine, Production and Health, University of Padua, Viale dell'Università, 35020 – Legnaro (PD), Italy.

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RESEARCH ARTICLE

Investigating the Determinants of *Toxoplasma gondii* Prevalence in Meat: A Systematic Review and Meta-Regression

Simone Belluco[1,2]*, Marzia Mancin[1], Daniele Conficoni[2], Giulia Simonato[2], Mario Pietrobelli[2], Antonia Ricci[1]

1 Food Safety Department, Istituto Zooprofilattico Sperimentale delle Venezie, viale dell'Università 10, 35020, Legnaro, PD, Italy, **2** Department of Animal Medicine, Production and Health, University of Padua, Viale dell'Università, 35020, Legnaro, PD, Italy

* sbelluco@izsvenezie.it

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Abstract

Background

Toxoplasma gondii is one of the most widespread parasites in humans and can cause severe illness in immunocompromised individuals. However, its role in healthy people is probably under-appreciated. The complex epidemiology of this protozoan recognizes several infection routes but consumption of contaminated food is likely to be the predominant one. Among food, consumption of raw and undercooked meat is a relevant route of transmission, but the role of different meat producing animal species and meats thereof is controversial.

Objectives

The aim of the present work is to summarize and analyse literature data reporting prevalence estimates of *T. gondii* in meat animals/meats.

Data Sources

We searched Medline, Web of Science, Science Direct (last update 31/03/2015).

Eligibility Criteria

Relevant papers should report data from primary studies dealing with the prevalence of *T. gondii* in meat from livestock species as obtained through direct detection methods. Meta-analysis and meta-regression were performed.

Results

Of 1915 papers screened, 69 papers were included, dealing mainly with cattle, pigs and sheep. Pooled prevalences, based on random-effect models, were 2.6% (CI_{95} [0.5–5.8]) for cattle, 12.3% (CI_{95} [7.6–17.8]) for pigs and 14.7% (CI_{95} [8.9–21.5]) for sheep. Due to the high heterogeneity observed, univariable and multivariable meta-regression models were

fitted showing that the geographic area for cattle (p = 0.032), the farming type for pigs (p = 0.0004) and the sample composition for sheep (p = 0.03) had significant effects on the prevalences of *Toxoplasma* detected/estimated. Moreover, the role of different animal species was dependent on the geographic location of animals' origin.

Limitations

Limitations were due mainly to a possible publication bias.

Conclusions and Implications

The present work confirms the role of meat, including beef, as *T. gondii* sources, and highlights the need for a control system for this parasite to be implemented along the meat production chain. Moreover, consumer knowledge should be strengthened in order to reduce the impact of disease.

Introduction

Toxoplasmosis is a zoonotic disease caused by *Toxoplasma gondii*, one of the most widespread parasites among humans. The clinical importance of this disease is due largely to infection occurring during pregnancy or in immunocompromised individuals [1]. In contrast, its impact on healthy individuals is probably underestimated. Toxoplasmosis can cause serious health problems in immunocompetent people [1–4], and the parasite can reactivate in chronically infected individuals as a consequence of immunosupression due, for example, to organ transplant or HIV infection. In addition, there is a growing interest in the study of the potential relationship between *T. gondii* latent infections and neurological disorders [5]. *T. gondii*, both congenital and perinatal, has the greatest impact on public health in terms of Disability Adjusted Life Years (DALY) among all foodborne pathogens according to a study performed in the Netherlands [6], and the burden is suggested to be even higher in other countries [7].

The complex life cycle of *T. gondii* recognizes felids as definitive hosts, in which the parasite can complete its sexual cycle and from there spread millions of oocysts into the environment. Although the number of oocysts produced is a key element in environmental contamination and consequently in parasite transmission, *T. gondii* is able to rely also on its asexual cycle in almost all warm blooded animals. This is a key adaptation of life cycle [8], and enables the parasite to be transmitted through the ingestion of infected meat, as observed several decades ago [9]. Consumption of raw or undercooked meat is likely to be the major transmission route for humans [10].

T. gondii infection in food producing animals is a critical issue and, despite the high number of studies estimating prevalence through serology and/or direct detection of the parasite in animal samples, there is disagreement about the relative importance of different food animal species. The most controversial role concerns cattle. Their importance in *T. gondii* transmission was judged to be unresolved several years ago as the parasite was never isolated from beef tissue [11]. Moreover, a large study performed in the US recently failed to detect *T. gondii* in more than 2000 samples, supporting the theory that cattle are a poor host for the parasite [12]. In contrast, other authors support different theories. For example, Opsteegh and colleagues, despite agreeing on the low prevalence in cattle, argued that the risk posed to consumers by ingestion of contaminated beef is likely to be high due to consumption habits [13]. Efforts to

collect data on *T. gondii* prevalence have been made [14,15] but without recourse to meta-analysis, which, together with meta-regression, is a helpful technique to obtain insight into the reasons for such differences and to depict the current knowledge in an evidence-based way.

The aims of the present study are to systematically review literature on the prevalence and determinants of *T. gondii* in meat of food producing animals and analyse the data through meta-analysis and meta-regression.

Methods

Data sources and searches

Relevant studies were identified by searching multiple literature databases including Medline (through PubMed), Web of Science Core Collection, SciELO citation index (through Web of Science) and Science Direct. No time limitation was imposed. The search was executed on 30/06/2014 and last updated on 31/03/2015.

The search string used was the following: (Toxoplasma OR Toxoplasmosis) AND ("Dairy Products" OR Meat OR poultry OR beef OR pork OR horse OR vegetables OR milk OR consumption OR food OR carcas*). Only papers in English, Italian, French, Spanish and Portuguese were considered. References were imported in EPPI-4 software [16] and duplicates were removed. Relevant papers were manually cross checked in order to identify further references.

Study selection and data extraction

Several criteria were used to select eligible studies: 1) the prevalence of *T. gondii* had to be detected by direct methods (bioassay, PCR, microscopy); 2) samples had to originate from food of animal origins (except milk and dairy products) belonging to the main livestock species (cattle, pigs, sheep, goat and horses); 3) samples had to be collected from animals which had not been experimentally infected; 4) sampling strategy had to be directed toward a random population.

The selection process is detailed in Fig 1. Briefly, the screening process, both Title/Abstract and Full text, was performed by two reviewers (SB, DC) independently (parallel method). Disagreements were resolved through consensus. Data were extracted by one reviewer and checked by a second (sequential method). All studies were coded according to the previously chosen parameters and data were recorded on customized tables. The collective noun for each animal species (cattle, pigs, sheep, horses and goats) is used throughout the current paper to describe tissue (mostly edible) deriving from that meat-producing animal species.

Risk of bias in individual studies

Study-level risk of bias was likely to be high mainly because of differences in study design and sampling management. Studies describing a sampling campaign on farms already recognized as being at risk were excluded [17,18]. Additional efforts were made to collect data about randomization and sample selection, such as size of the population from which the animals originated, method of selection of individuals and geographic distribution, but these factors were poorly described in primary studies, impairing further analysis.

The minimum sample size was set to ten, and this choice caused the exclusion of two studies reporting data for cattle and pigs with four and nine samples respectively [19]. In addition, the impact of sample size on the pooled prevalence estimate was assessed, for each species, through a cumulative meta-analysis based on decreasing sample size. Moreover, potential sources of bias, such as sample composition, analytical technique and study design were assessed through

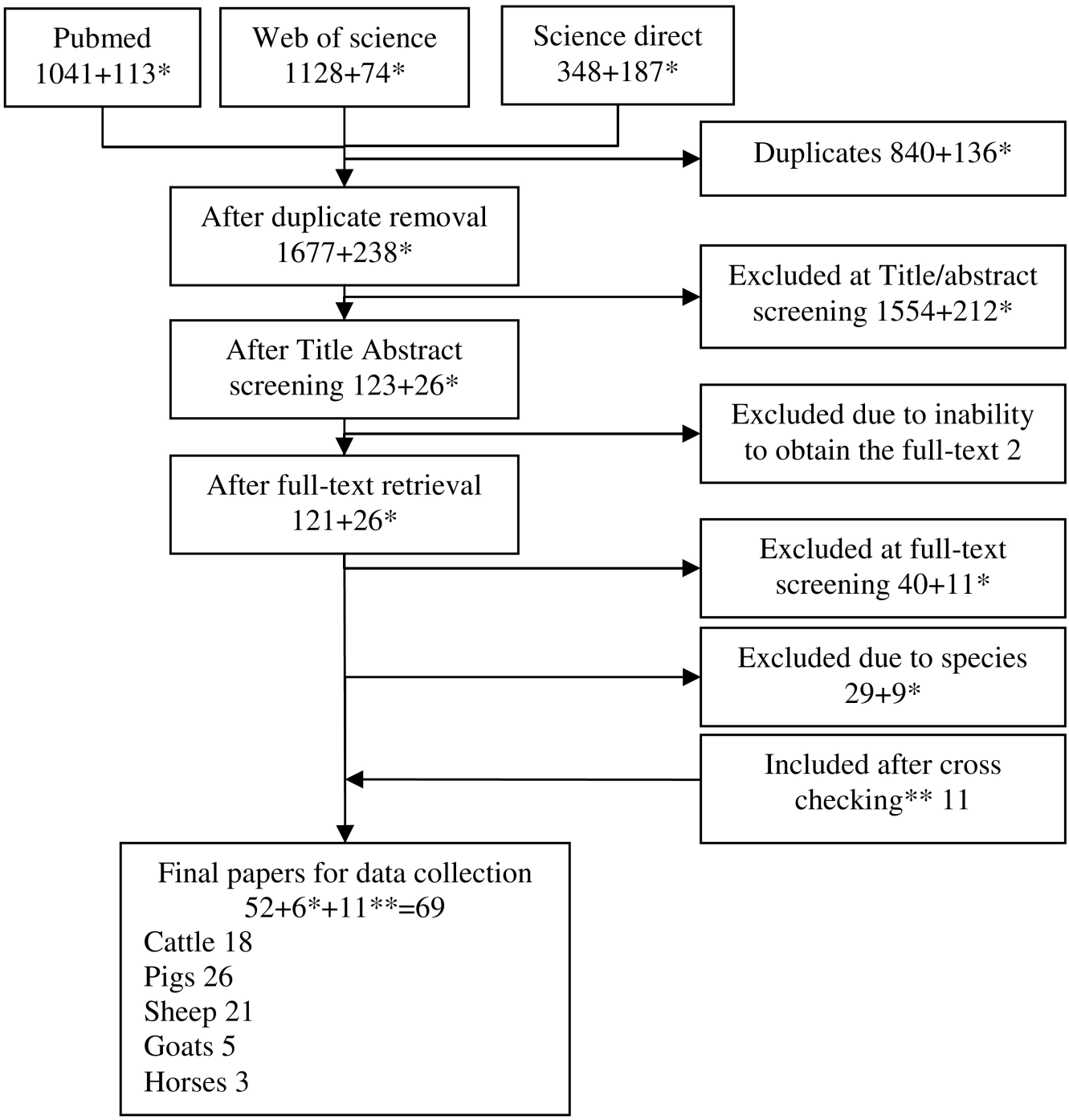

Fig 1. Flowchart describing the selection of relevant studies. *papers added during the last update ** papers added through cross checking.

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meta-regression. Outcome-level biases were not evaluated. However, an accurate sensitivity analysis was performed to detect influential studies.

The outcome selected for meta-analysis (event rate, defined as the number of events over the total sample size) was obtained from studies with the following rules. If studies reported different prevalence estimates obtained through different analytical methods or in different target

organs, the highest value was retained (prevalence at animal level), assuming that it represented the most sensitive estimate. When direct methods were applied only to seropositive samples, the proportion of positives was adjusted according to the size of the entire study population (i.e. 100 animals in the population, 50 seropositive, 10% of seropositive confirmed through direct method, prevalence in total population = 5%). Moreover, when direct methods were applied only to a fraction of seropositive animals, the proportion of positives was adjusted pro rata considering all seropositives and then by calculating according to the size of the original population (i.e. 100 animals in the population, 50 seropositive animals, 30 seropositive animals tested through direct method, 10 confirmed positive, prevalence in total population = ((30*10)/50)*100 = 16.7%).

Data analysis

Pooled prevalence. Meta-analyses were performed using the *metafor* package [20] of the statistical software R [21]. The proportion of positives among the total study population (event rate) was chosen as effect size. A study was designated as the unit of analysis, and was defined as an investigation performed on a group of animals which shared the same features (e.g. species, geographic location) in terms of variables used as moderators.

Meta-analysis is a statistical method that combines outcomes of primary studies with a weight assigned according to the inverse of the variance. For this reason, the variance is a critical parameter to be taken into account, and must also be calculated when studies reporting zero prevalences are included. The Freeman and TukeyDoubleArcsin transformation of the prevalence was used to obtain a variance stabilizing transformation without applying continuity corrections or removing studies from the meta-analysis, and to give an appropriate weight to those studies with zero prevalence and high numerousness [20,22]. Transformed prevalence estimates were combined in meta-analysis using a random-effect model and later back-transformed in the original metrics. The amount of heterogeneity was estimated using the Q, T^2 and I^2 [23] statistics obtained by Restricted Maximum Likelihood (REML), which is considered approximately unbiased and relatively efficient [24]. A separate meta-analysis was performed for each species (cattle, pigs and sheep). Data belonging to goats and horses were only described qualitatively.

Sensitivity analyses were performed in order to evaluate the presence of outliers or leverage studies and their potential influence on each model per species. Several parameters were examined: the externally studentized residuals, the DFFITS (DiFference in FIT, Standardized), the Cook's distance, the hat function and the covariance ratio. Influence was defined according to *metafor* package criteria (absolute DFFITS value $> 3\sqrt{[p/(k-p)]}$, where p is the number of model coefficients and k is the number of studies OR the lower tail area of a chi-square distribution with p degrees of freedom cut off by the Cook's distance being larger than 50% OR hat value $> 3(p/k)$). In addition, studies were excluded one by one from the model to evaluate relevant changes in heterogeneity (T^2 and Q) and pooled estimate. P-value<0.05 was considered significant in the statistical meta-analysis.

Heterogeneity. Heterogeneity was explored through uni-variable and multivariable meta-regression using the mixed-effects models [25]. Moderator significance for (nested) models was assessed through the Likelihood Ratio Test (LRT) by comparing the proportional reduction in the amount of heterogeneity (T^2 value) of the full and reduced models. Therefore, it was possible to evaluate the amount of (residual) heterogeneity accounted for by the moderator (R^2). Maximum Likelihood (ML) estimate instead of REML was use to evaluate the importance of the moderators [20].

Attempts were made to explain heterogeneity through epidemiological and methodological moderators: publication year (as a proxy variable for study year), geographic origin, animal age,

farming system, analytical technique, sampling location, serological screening presence and sample composition (details in Table 1). In addition, a multivariable meta-regression was performed, pooling all studies across species. This allowed us to use species as moderator and to evaluate the interaction between species and geographic area. In cases of moderator significance, determined according to the Likelihood Ratio Test, a pairwise comparison multitest was performed using the False Discovery Rate correction [26]. Publication bias was evaluated through the Trim and Fill method [27,28] and cumulative meta-analysis was based on sample size.

Results

Study selection and data extraction

The original literature search provided a total of 1677 records after duplicate removal, and 238 records were obtained during the last update (Details in Fig 1). After the first screening based on Title and Abstract, 149 papers remained and at the end of the selection procedure, 69 of which were considered as relevant according to the eligibility criteria. The final number included papers belonging to the original literature search, papers belonging to the last update and papers retrieved through cross checking the references in the included papers. Studies were identified within the included papers and coded according to review criteria. Details showing the result of study coding on the basis of relevant characteristics are presented in Table 1, whereas details for each eligible study are shown in Tables 2–4.

Cattle

The systematic review process identified 22 studies, presented in 18 papers, dealing with the direct identification of *T. gondii* in bovine meat [12,19,29–44]. However, one paper was not included in statistical analysis as it investigated only four samples [19]. General information about the 21 studies retrieved is presented in Table 2.

Meta-analysis, as summarized in S1 Fig, identified a pooled *T. gondii* prevalence of 2.6% (CI_{95} [0.5–5.8]). The 95% prediction interval ranged from 0% to 22%. Heterogeneity was high with significant Q test ($p<0.0001$), $T^2 = 0.0215$ and $I^2 = 92\%$ (details in Table 5).

Sensitivity analysis identified study n°1 [29] and n°6 [32] as outliers according to externally studentized residuals, and their removal one by one resulted in a noticeable reduction of combined estimate, with a final pooled prevalence that would reach 1.9% in both cases. However, the other sensitivity indexes applied (DFFITS, the Cook's distance, the hat function and the covariance ratio) did not identify these studies as influencing the final model according to *metafor* parameters. As regards publication bias, although the Trim and Fill test did not identify any asymmetry, a cumulative meta-analysis based on the number of samples (N) showed increasing prevalences as the number of samples in the studies decreased (Fig 2).

Because of the high level of heterogeneity observed, univariable meta-regressions were performed on publication year, geographic area, analytical technique, sample composition and sampling location. A significant effect was associated with geographic area, according to the Likelihood Ratio Test ($p = 0.032$), with a R^2 of 61.9%. The multitest for pairwise comparison identified only one statistically significant difference ($p = 0.0397$), between Central America (K = 3 in one paper) and North America (K = 3 in three papers) prevalence estimates. The other moderators tested through meta-regression did not show any relevant impact according to the Likelihood Ratio Test (Table 5), suggesting that neither the analytical technique used, nor sample type or sampling location influenced *T. gondii* prevalence in a statistically significant way. Details of model coefficients and prevalence estimates are presented in Table 6.

Table 1. Characteristics of 91 studies reporting prevalence for *T. gondii gondii* that were tested as sources of heterogeneity.

	Cattle		Pigs		Sheep	
	K	N	K	N	K	N
Total	21	3785	41	10894	29	4150
EPIDEMIOLOGICAL MODERATORS						
Geographic area						
-Africa	-	-	2	100	4	351
-Asia	3	170	2	439	8	376
-Central America	3	100	1	48	-	-
-Europe	4	651	14	3074	8	1061
-North America	3	2429	7	6318	2	469
-Oceania	1	80	1	30	2	64
-South America	7	355	14	885	5	1829
Animal age						
-<12 months	-	-	-	-	8	991
->12 months	-	-	-	-	9	543
-NS	-	-	-	-	12	2616
Farming system						
-Conventional	-	-	2	397	-	-
-Organic	-	-	4	86	-	-
-Small farms	-	-	2	433	-	-
-NS	-	-	33	9978	-	-
Publication Year						
METHODOLOGICAL MODERATORS						
Analytical technique						
-Bioassay in cats	2	2369	1	2094	-	-
-Bioassay in mice	12	600	28	5695	11	2137
-PCR	7	816	12	3105	16	1913
-Microscopy	-	-	-	-	2	100
Sample type						
-Single	14	3095	21	4670	17	2123
-Pooled within animal	7	690	12	5321	12	2027
-Meat products	-	-	5	336	-	-
-Cured meat products	-	-	3	567	-	-
Sampling location						
-Slaughterhouse	9	1315	21	7094	21	3686
-Retail	9	2332	18	3739	7	414
-NS	3	138	2	61	1	50
Serological screening						
-No	-	-	35	6808	22	1946
-Yes	-	-	6	4086	7	2204

K = number of studies (note that some individual published papers contained more than one study), N = number of samples, NS = non specified in the primary study.

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Pigs

The systematic review process identified 41 studies, presented in 36 papers, dealing with the direct identification of *T. gondii* in pigs and meat thereof (details in Table 3)

Table 2. General information about eligible studies reporting data for cattle.

ID	Reference	Country	Geographic area	Sampling location	Analytical technique	Technique specifications	Sampled organ
1	**Arias 1994**	Costa Rica	Central America	Retail	Bio mice	Feed	Liver
2	**Arias 1994**	Costa Rica	Central America	Retail	Bio mice	Feed	Heart
3	**Arias 1994**	Costa Rica	Central America	Retail	Bio mice	Feed	Muscle
4	**Azizi 2014**	Iran	Asia	NS	PCR	Nested	Brain/Liver/Muscle
5	**Berger Scoch 2011**	Switzerland	Europe	Slaughterhouse	PCR	Real-T PCR	Diaphragm
6	**Campo-Portacio 2014**	Colombia	South America	Retail	PCR	Nested	Muscle
7	**Catar 1969**	Czech Republic	Europe	NS	Bio mice	IP	Brain/Diaphragm
8	**Dubey 1976**	US	North America	Slaughterhouse	Bio cats	Feed	Heart/Diaphragm
9	**Dubey 2005**	US	North America	Retail	Bio cats	Feed	Muscle
10	**Ergin 2009**	Turkey	Asia	Slaughterhouse	PCR	Nested	Brain/Muscle
11	**Fortier 1990**	Portugal	Europe	Slaughterhouse	Bio mice	IP	Brain/Heart/ Diaphragm
12	**Jacobs 1960**	US	North America	Slaughterhouse	Bio mice	IP	Diaphragm
13	**Jacobs 1963**	New Zealand	Oceania	Slaughterhouse	Bio mice	IP	Diaphragm
14	**Jamra 1969**	Brazil	South America	Retail	Bio mice	IP	Muscle
15	**Jamra 1969**	Brazil	South America	Retail	Bio mice	IP	Liver
16	**Jamra 1969**	Brazil	South America	Retail	Bio mice	IP	Brain
17	**Martins 1989**	Brazil	South America	NS	Bio mice	IP	Muscle
18	**Opsteegh 2011**	The Netherlands	Europe	Slaughterhouse	PCR	MC-PCR	Heart
19	**Passos 1984**	Brazil	South America	NS	Bio mice	IP	Diaphragm
20	**Rahdar 2012**	Iran	Asia	Slaughterhouse/ Retail	PCR	PCR	Tongue/Heart/ Muscle
21	**Santos 2010**	Brazil	South America	Slaughterhouse	PCR	Nested	Brain/Heart

Bio mice = Bioassays in mice, IP = intra-peritoneal, MC-PCR = magnetic capture PCR, NS = not specified in the primary study.

doi:10.1371/journal.pone.0153856.t002

[12,31,33,36,37,39,40,45–69]. A univariable meta-regression was performed considering, as moderators, publication year, geographic area, analytical technique, farming system, sample type and sampling location.

The meta-analytical model (S2 Fig), without moderators, identified a *T. gondii* prevalence of 12.3% (CI_{95}[7.6–17.7]). The 95% prediction interval ranged from 0% to 55%. Heterogeneity was high with significant Q test ($p<0.0001$), $T^2 = 0.0534$ and $I^2 = 98\%$ (details in Table 7).

Sensitivity analysis identified one study [70] as an outlier according to externally studentized residuals, but it was judged non influential according to *metafor* parameters. Its removal from the analysis resulted in a reduction of estimated prevalence up to 11.2%.

A meta-regression based on publication year showed no significance. According to the Trim and Fill method, no asymmetry was identified. However, a cumulative meta-analysis based on the number of samples (N) showed increasing prevalences as the number of samples in the studies decreased (Fig 3).

Geographic area, analytical technique, sample type, sampling location and the presence of serological screening were not significant ($p = 0.172$, $p = 0.239$, $p = 0.476$, $p = 0.576$ and $p = 0.25$ respectively), and residual heterogeneity continued to be high according to T^2 and I^2 statistics (details in Table 7). Details of model coefficients and prevalence estimates are presented in

Table 3. General information about eligible studies reporting data for pigs.

ID	Reference	Country	Geographic area	Farm	Serological screening	Analytical technique		Sampled organ
1	**Aspinall 2002**	UK	Europe	NS	NA	PCR	PCR	Meat products (Mixed)
2	**Bacci 2015**	Italy	Europe	O	NA	PCR	Nested	Heart
3	**Bayarri 2012**	Spain	Europe	NS	NA	Bio mice	IP	Muscle
4	**Bayarri 2012**	Spain	Europe	NS	NA	Bio mice	IP	Cured Ham
5	**Belfort-Neto 2006**	Brazil	South America	NS	NA	PCR	PCR	Tongue/Diaphragm
6	**Berger Scoch 2011**	Switzerland	Europe	NS	NA	PCR	Real-T PCR	Diaphragm
7	**Bezerra 2012**	Brazil	South America	O	NA	Bio mice	SC	Brain/Tongue
8	**Cademartori 2014**	Brazil	South America	SF	Sero +	Bio mice	IP	Brain/heart
9	**Catar 1969**	Czech Republic	Europe	NS	NA	Bio mice	IP	Brain/Diaphragm
10	**Clementino andrade 2013**	Brazil	South America	NS	Sero +	Bio mice	IP	Heart
11	**Dias 2005**	Brazil	South America	NS	NA	Bio mice	Inoculation	Sausages
12	**Dubey 1995**	US	North America	NS	NA	Bio mice	SC	Heart
13	**Dubey 2005**	US	North America	NS	Bio cats	Bio cats	Feed	Muscle
14	**Dubey 2012**	US	North America	O	Na	Bio mice	SC	Heart
15	**Esteves 2014**	Portugal	Europe	NS	Sero +	PCR	Nested	Brain/Diaphragm
16	**Fortier 1990**	Portugal	Europe	C	NA	Bio mice	IP	Brain/Heart/Diaphragm
17	**Frazao-Texeira 2006**	Brazil	South America	O	NA	Bio mice	IP	Brain
18	**Frazao-Texeira 2011**	Brazil	South America	NS	NA	Bio mice	Inoculation	Heart
19	**Frazao-Texeira 2011**	Brazil	South America	NS	NA	Bio mice	Inoculation	Brain
20	**Feitosa 2014**	Brazil	South America	NS	Sero +	Bio mice	SC	Brain/Heart/Muscle
21	**Gajadhar 1998**	Canada	North America	NS	NA	Bio mice	SC	Heart/Diapraghm
22	**Galvan-Ramirez 2010**	Mexico	Central America	NS	NA	Bio mice	SC	Muscle
23	**Gomez-Samblas 2015**	Spain	Europe	NS	NA	PCR	MC-PCR	Serrano ham
24	**Halova 2012**	Ireland	Europe	NS	NA	PCR	Nested	Diaphragm
25	**Jacobs 1960**	US	North America	NS	NA	Bio mice	IP	Diaphragm
26	**Jamra 1969**	Brazil	South America	NS	NA	Bio mice	IP	Muscle
27	**Jamra 1969**	Brazil	South America	NS	NA	Bio mice	IP	Sausages
28	**Martins 1989**	Brazil	South America	NS	NA	Bio mice	IP	Muscle
29	**Medonca 2004**	Brazil	South America	NS	NA	PCR	SC	Sausages
30	**Navarro 1992**	Brazil	South America	NS	NA	Bio mice	IP	Muscle
31	**Navarro 1992**	Brazil	North America	NS	NA	Bio mice	IP	Brain
32	**Rothe 1985**	Australia	Oceania	NS	NA	Bio mice	IP	Muscle
33	**Samico Fernandes 2012**	Brazil	North America	NS	NA	PCR	Nested	Heart
34	**Siam 1979**	Egypt	Africa	NS	NA	Bio mice	IP	Diaphragm/Muscle
35	**Siam 1979**	Egypt	Africa	NS	NA	Bio mice	IP	Sausages and Mortadella
36	**Sousa 2006**	Portugal	Europe	SF	Sero +	Bio mice	SC	Brain/Heart
37	**Turcekova 2013**	Slovakia	Europe	NS	Sero +	PCR	Nested	Brain/Heart
38	**Vostalova 2000**	Czech Republic	Europe	C	NA	Bio mice	IP	Brain/Diaphragm
39	**Wang 2012**	China	Asia	NS	NA	PCR	Real-T PCR	Muscle
40	**Wang 2013**	China	Asia	NS	NA	Bio mice	IP	Brain
41	**Warnekulasuriya 1998**	UK	Europe	NS	NA	PCR	Nested	Sausages dried/ cured

Bio mice = Bioassays in mice, IP = intra-peritoneal, SC = subcutaneous, MC-PCR = magnetic capture PCR, NS = not specified in the primary study, NA = Not Appliable, Sero+ = seropositive.

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Table 8. The only significant moderator was farming system (p = 0.0004) with R^2 of 37.31, as organically farmed pigs had significantly higher *T. gondii* prevalences than pigs from conventional farms, small farms and from farms where this data was not reported (see Table 7).

Sheep

The systematic review process identified 29 studies, presented in 24 papers, dealing with the direct identification of *T. gondii* in sheep meat [31,35,37–39,43,59,62,71–84]. General information about the 29 studies retrieved is presented in Table 9. Geographic area, analytical technique and animal age (coded in two categories) were included in the univariable meta-analysis as moderators.

The meta-analytical model (S3 Fig), without moderators, identified a prevalence of 14.7% (CI_{95}[8.9–21.5]. The 95% prediction interval ranged from 0% to 57%. Heterogeneity was high with significant Q test (p<0.0001), T^2 = 0.0513 and I^2 = 97% (details in Table 9).

Table 4. General information about eligible studies reporting data for sheep.

ID	References	Country	Geographic area	Animal age	Analytical technique		Sampled organ
1	**Asgari 2011**	Iran	Asia	>12	PCR	Nested	Brain/Liver/Muscle
2	**Azizi 2014**	Iran	Asia	<12	PCR	Nested	Brain/Liver/Muscle
3	**Azizi 2014**	Iran	Asia	<12	PCR	Nested	Brain/Liver/Muscle
4	**Berger Scoch 2011(1)**	Switzerland	Europe	<12	PCR	Real Time PCR	Diaphragm
5	**Berger Scoch 2011(2)**	Switzerland	Europe	>12	PCR	Real Time PCR	Diaphragm
6	**Belbacha 2004**	Morocco	Africa	NS	Bio mice	Feed/IP	Brain
7	**Boughattas 2013***	Tunisia	Africa	>12	PCR	PCR	Heart
8	**da Silva 2009***	Brazil	South America	NS	Bio mice	NS	Heart/Diaphragm
9	**Dubey 2008***	US	North America	<12	Bio mice	Feed	Heart
10	**Dumetre 2006***	France	Europe	>12	Bio mice	IP	Heart
11	**Ergin 2009**	Turkey	Asia	NS	PCR	Nested	Brain/Muscle
12	**Ergin 2009**	Turkey	Asia	NS	PCR	Nested	Brain
13	**Gharbi 2013**	Tunisia	Africa	NS	PCR	Nested	Heart
14	**Glor 2013***	Switzerland	Europe	NS	PCR	Real Time PCR	Brain/Muscle
15	**Halos 2010**	France	Europe	<12	Bio mice	IP	Heart
16	**Halos 2010**	France	Europe	>12	Bio mice	IP	Heart
17	**Halova 2012**	Ireland	Europe	NS	PCR	Nested	Diaphragm
18	**Jacobs 1960**	US	North America	NS	Bio mice	IP	Diaphragm
19	**Jacobs 1963**	New Zealand	Oceania	>12	Bio mice	IP	Brain/Diaphragm/Muscle
20	**Jamra 1969**	Brazil	South America	>12	Bio mice	IP	Muscle
21	**Khayeche 2013**	Tunisia	Africa	>12	PCR	Nested	Heart
22	**Maciel 2014**	Brazil	South America	NS	PCR	Nested	Brain
23	**Opsteegh 2010**	The Netherlands	Europe	NS	PCR	MC-PCR	Heart
24	**Ragozo 2008***	Brazil	South America	NS	Bio mice	NS	Heart/Brain/Diaphragm
25	**Rahdar 2012**	Iran	Asia	<12	PCR	PCR	Tongue/Heart/Muscle
26	**Rothe 1985**	Australia	Oceania	<12	Bio mice	IP	Muscle
27	**Yildiz 2014**	Turkey	Asia	<12	Micro	NA	Brain/Diaphragm/Muscle
28	**Yildiz 2014**	Turkey	Asia	>12	Micro	NA	Brain/Diaphragm/Muscle
29	**Vieira 2001***	Brazil	South America	NS	PCR	PCR	Brain/Diaphragm

Bio mice = Bioassays in mice, IP = intra-peritoneal, SC = subcutaneous, MC-PCR = magnetic capture PCR, NS = not specified in the primary study, NA = Not Appliable,

*studies that performed a serological screening.

doi:10.1371/journal.pone.0153856.t004

Table 5. Summary of heterogeneity measures and Likelihood Ratio Test for each moderator tested in studies describing *T. gondii* prevalence in cattle.

	T^2 (95%CI)	I^2 (95%CI)	LRT p-value	R^2
No moderators	0.0215 (0.0113–0.0620)	91.6 (85.3–96.9)	-	-
Geographic area*	0.0150 (0.0066–0.0589)	84.8 (71–95.6)	0.032	61.86
Publication year	0.0206 (0.0106–0.0613)	90.76 (83.48–96.69)	0.16	10.86
Analytical technique	0.0166 (0.0084–0.0601)	85.56 (74.91–95.54)	0.063	38.37
Sample composition	0.0232 (0.0121–0.0674)	91.33 (84.58–96.84)	0.89	0
Sampling location	0.0213 (0.0107–0.0626)	88.52 (79.52–95.78)	0.22	13.74

LRT = Likelihood Ratio Test

*statistically significant results.

doi:10.1371/journal.pone.0153856.t005

Sensitivity analysis identified study n° 19 [38] as an outlier according to externally studentized residuals, but no influences in the model were highlighted according to other indexes investigated.

Cumulative meta-analysis based on publication year did not show any relevant trend. According to the Trim and Fill method, no asymmetry was identified. However, a cumulative meta-analysis based on the number of samples (N) showed increasing prevalences as the number of samples in the studies decreased (Fig 4).

None of the following moderators, studied using univariable meta-regression, were significant: geographic area (p = 0.0553), analytical technique (p = 0.1173), animal age (p = 0.1273), serological screening (p = 0.615), sampling location (p = 0.541), as summarized in Table 9. Sample composition was significant, with p = 0.031 and R^2 value of 14.12%. Details of meta-regression coefficients and prevalence estimates are presented in Table 10.

Multivariable meta-regression (cattle, pig, sheep)

Multivariable meta-regression was performed based on species, geographic origin of sampled animals and their interaction. Univariable analysis on the full dataset, using species as the

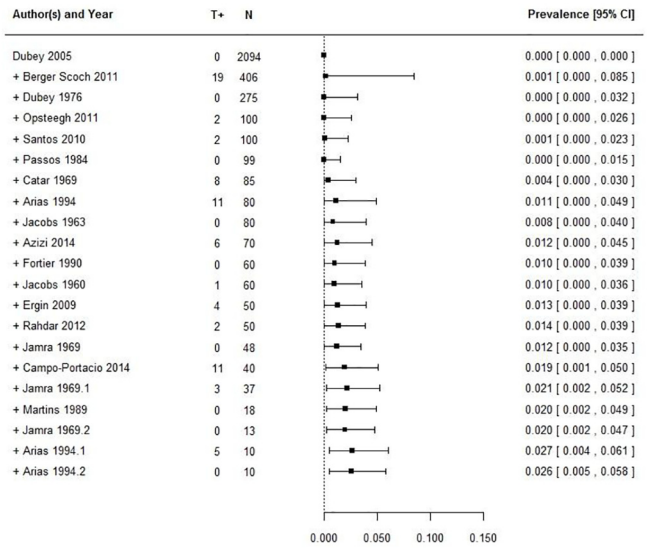

Fig 2. Cumulative meta-analysis on cattle studies based on decreasing sample size. T+ = positive samples, N = number of samples.

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Table 6. Summary of the output of univariable meta-regression in cattle or meat thereof for each category within moderators.

Moderator	K	N		B	SE	Prevalence (95%CI)
Epidemiological moderators						
*Geographic area**	3	170	Asia	0.2776	0.0806	0.060 (0.002–0.166)
	3	100	Central America	0.4263	0.0969	0.159 (0.040–0.328)
	4	655	Europe	0.1933	0.0661	0.022 (0.000–0.087)
	3	2429	North America	0.0592	0.0744	0.000 (0.000–0.026)
	1	80	Oceania	0.0557	0.1347	0.000 (0.000–0.085)
	7	355	South America	0.1988	0.0558	0.024 (0.000–0.078)
Publication year	21	3785		0.0027	0.0021	
Methodological moderators						
Analytical technique	2	2369	Bio cats	0.0203	0.0926	0.000 (0.000–0.025)
	12	600	Bio mice	0.19836	0.0447	0.024 (0.001–0.065)
	7	820	PCR	0.2672	0.0536	0.055 (0.012–0.0119)
Sample composition	14	3095	Single	0.2087	0.0460	0.032 (0.004–0.076)
	7	690	Pooled within	0.1973	0.0616	0.027 (0.000–0.087)
Sampling location	9	1315	Slaughterhouse	0.1499	0.0490	0.011 (0.000–0.048)
	9	2332	Retail	0.2792	0.0611	0.065 (0.014–0.142)
	3	138	NS	0.2256	0.0966	0.039 (0.000–0.154)

N = number of samples, K = number of studies, SE = Standard error, Bio = Bioassay,
*statistically significant results.

doi:10.1371/journal.pone.0153856.t006

moderator, resulted in a significant Likelihood Ratio Test (p = 0.0078). Moreover, the multitest pairwise comparison identified the estimate of *T. gondii* prevalence in cattle to be significantly lower than in sheep and pigs, whereas no differences were observed between these last two species. The addition of geographic area to this model gave no significant results, whereas the interaction between the two moderators was significant (p = 0.0212).

The multitest performed on the final model allowed the comparison of species prevalence within different geographic areas. In North America, Asia and Oceania, the *T. gondii* prevalences in sheep and pigs were significantly lower than the prevalence in cattle. In Europe and South America, *T. gondii* prevalences in sheep were significantly higher than in cattle but there was no difference in *T. gondii* prevalences in pigs and cattle.

Table 7. Summary of heterogeneity measures and Likelihood Ratio Test for each moderator tested in studies describing *T. gondii* prevalence in pigs.

	T^2 (95%CI)	I^2 (95%CI)	LRT p-value	R^2
No moderators	0.0534 (0.0346–0.0931)	98.1 (97.1–98.9)	-	-
Geographic area	0.0499 (0.0313–0.0930)	97.8 (96.6–98.8)	0.17	22.79
Farming system*	0.0365 (0.0223–0.0629)	97.2 (95.5–98.4)	0.0004	37.31
Publication year	0.0545 0.0351 0.0952	98 (97–98.9)	0.52	0.65
Analytical technique	0.0524 (0.0335–0.0930)	97.4 (96–98.5)	0.24	7.86
Sample composition	0.0547 (0.0348–0.0971)	97.8 (96.6–98.7)	0.48	6.32
Sampling location	0.0550 (0.0353 0.0970)	98 (96.8–98.8)	0.58	2.64
Serological screening	0.0529 (0.0341–0.0934)	97.8 (96.6–98.7)	0.25	3.9

LRT = Likelihood Ratio Test
*statistically significant results.

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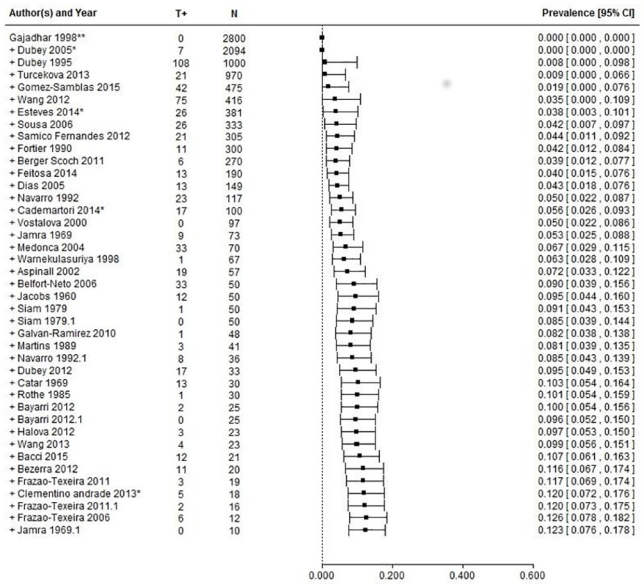

Fig 3. Cumulative meta-analysis on pig studies based on decreasing sample size. T+ = positive samples, N = number of samples.

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Goats and Horses

This systematic review process allowed the retrieval of the few studies available dealing with goats and horses, five and two studies, respectively.

Prevalence in goats is, according to literature, quiet heterogeneous. Samples of brain, tongue, liver, plus neck, intercostal, and femoral muscles from 22 goats from Shiraz abattoirs (Iran) were analysed in 2008. *T. gondii* was detected in five (23%) animals, and in at least one tissue, through nested PCR [85]. In East Brazil, mice bioassay demonstrated the presence of viable *T. gondii* in 1 out of 10 seropositive goats identified through ELISA anti IgG antibodies (DAT) among the total of 50 goats tested (prevalence 2.5%) [47]. A similar prevalence was described in the North East of Brazil after examination of tongues, brains and hearts from 102 goats at slaughter and positive results to nested PCR were found in 2.9%, 3.9% and 1% of these organs, respectively [86]. In North America, the hearts of 234 goats aged between 6 and 12 months and collected from local retail meat stores in Maryland were tested using Modified Agglutination Test (MAT) and 112 of them also using mice bioassay. *T. gondii* was isolated from 29 of 112 goats (26%) [87]. Finally, in China, liver, lung and lymph nodes from 403 Yunnan black goats were collected randomly from different administrative regions in Yunnan province, and B1 gene (a marker of *T. gondii*) was identified using PCR in 20 (5%) of the animals [88].

As regards horses, in a Brazilian study, Evers and colleagues in 2013 detected *T. gondii* in 14 out of 398 (3.5%) brain samples using bioassays in mice. The parasite was identified through PCR in two mice, but the others were found to be positive by IFAT (Indirect Fluorescent Antibody Test). All 398 horses were also tested by serology, and interestingly, 13 out of 14 horses positive by mouse bioassays tested negative by IFAT (<1:64). Moreover, only two bioassay positive horses tested positive by PCR [89]. In Egypt, meat and tissue samples from 150 horses were bioassayed in mice (pool of heart, liver, skeletal and diaphragmatic muscle) and 79 were positive (52.6%), with consequent isolation of the parasite from peritoneal fluid of inoculated animals [90].

Table 8. Summary of the output of univariable meta-regression analysis in pigs for each category within moderators.

Moderator	K	N		β	SE	Prevalence (95%CI)
Epidemiological moderators						
Geographic area	2	100	Africa	0.1201	0.1657	0.005 (0.000–0.178)
	2	493	Asia	0.4430	0.1660	0.176 (0.005–0.483)
	1	48	Central America	0.1734	0.2347	0.020 (0.000–0.347)
	14	3074	Europe	0.3159	0.0622	0.087 (0.027–0.172)
	7	6318	North America	0.3427	0.0868	0.104 (0.019–0.235)
	1	30	Oceania	0.2187	0.2411	0.037 (0.000–0.404)
	14	885	South America	0.4974	0.0643	0.221 (0.123–0.337)
*Farming system**	2	397	Conventional	0.1253	0.1383	0.006 (0.000–0.141)
	4	86	Organic	0.8189	0.1106	0.534 (0.316–0.746)
	2	433	Small Farms	0.3558	0.1381	0.112 (0.000–0.140)
	33	9978	NS	0.3386	0.0355	0.101 (0.061–0.149)
Publication year	41	10894		0.0020	0.0026	
Methodological moderators						
Analytical technique	1	2094	Bio cats	0.0598	0.2292	0.000 (0.000–0.231)
	28	5695	Bio mice	0.3563	0.0459	0.113 (0.059–0.179)
	12	3105	PCR	0.4314	0.0681	0.167 (0.076–0.281)
Sample composition	21	4670	Single	0.4080	0.0541	0.149 (0.079–0.236)
	12	5321	Pooled within	0.3475	0.0694	0.107 (0.034–0.209)
	5	336	Meat products	0.3935	0.1109	0.139 (0.020–0.325)
	3	567	Cured meat products	0.1881	0.1404	0.025 (0.000–0.0193)
Sampling location	21	7094	Slaughterhouse	0.3649	0.0531	0.119 (0.057–0.197)
	18	3739	Retail	0.3604	0.0585	0.116 (0.049–0.202)
	2	61	NS	0.5496	0.1789	0.267 (0.029–0.616)
Serological screening	35	6808	No	0.3911	0.0417	0.137 (0.083–0.200)
	6	4086	Yes	0.2805	0.0889	0.067 (0.003–0.186)

K = number of studies, N = number of samples, SE = Standard error,

*statistically significant results.

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Table 9. Summary of heterogeneity measures and Likelihood Ratio Test for each moderator tested in studies describing *Toxoplasma* prevalence in sheep.

	T^2 (95%CI)	I^2 (95%CI)	LRT p-value	R^2
No moderators	0.0513 (0.0309–0.0988)	96.6 (94.4–98.2)	-	-
Geographic area	0.046 (0.0241–0.0887)	95.6 (92.5–97.8)	0.055	33.8
Animal Age	0.0470 (0.0279–0.0956)	96.1 (93.6–98.1)	0.13	15.9
Publication year	0.0532 (0.0319–0.1040)	96.7 (94.6–98.3)	0.88	0.2
Analytical technique	0.0470 (0.0278–0.0947)	96.2 (93.7–98.1)	0.11	15.9
Sample composition*	0.0458 (0.0269–0.0868)	96 (93.3–97.8)	0.03	14.1
Sampling location	0.0536 (0.0316–0.1044)	96.8 (94.7–98.3)	0.54	3.48
Serological screening	0.0529 (0.0316–0.1032)	96.6 (94.3–98.2)	0.61	1

LRT = Likelihood Ratio Test

*statistically significant results.

doi:10.1371/journal.pone.0153856.t009

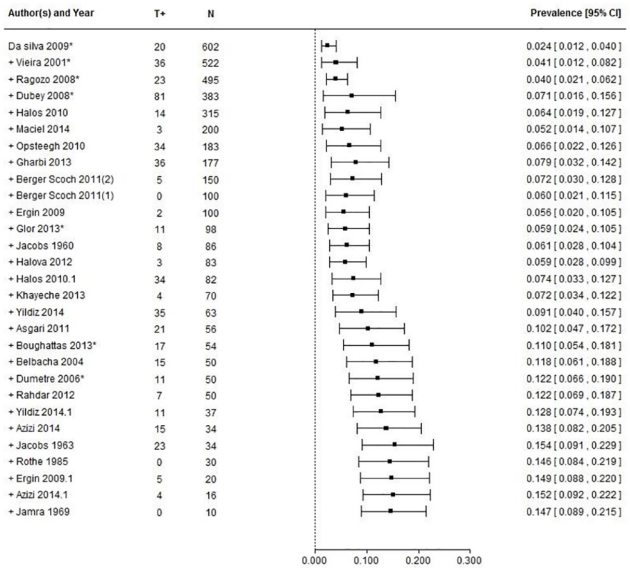

Fig 4. Cumulative meta-analysis on sheep studies based on decreasing sample size. T+ = positive samples, N = number of samples.

doi:10.1371/journal.pone.0153856.g004

Table 10. Summary of the output of univariable meta-regression analysis in sheep for each category within moderators.

Moderator	K	N		β	SE	Prevalence (95%CI)
Epidemiological moderators						
Geographic area	4	351	Africa	0.4752	0.1074	0.203 (0.059–0.399)
	8	376	Asia	0.5525	0.0786	0.271 (0.143–0.420)
	8	1061	Europe	0.3297	0.0750	0.096 (0.023–0.204)
	2	469	North America	0.4003	0.1489	0.144 (0.003–0.405)
	2	64	Oceania	0.5295	0.1586	0.250 (0.038–0.556)
	5	1829	South America	0.1946	0.0964	0.028 (0.000–0.132)
Age	8	991	<12 months	0.4125	0.0807	0.153 (0.054–0.287)
	9	543	>12 months	0.5171	0.0768	0.239 (0.120–0.381)
	12	2616	NS	0.3177	0.0643	0.089 (0.027–0.177)
Publication year	30	4150		0.0004	0.0031	
Methodological moderators						
Analytical technique	11	2137	Bio mice	0.4020	0.0685	0.146 (0.061–0.256)
	16	1913	PCR	0.3662	0.565	0.120 (0.055–0.204)
	2	100	Micro	0.7147	0.1616	0.428 (0.143–0.741)
*Sample composition**	17	2123	Single	0.3287	0.0540	0.096 (0.039–0.170)
	12	2027	Pooled within	0.5116	0.0651	0.234 (0.133–0.353)
Sampling location	21	3686	Slaughterhouse	0.4289	0.0510	0.166 (0.096–0.249)
	7	414	Retail	0.3084	0.1004	0.083 (0.004–0.228)
	1	50	Slaughter/retail	0.2419	0.3934	0.139 (0.000–0.584)
Serological screening	22	1946	No	0.4163	0.0514	0.156 (0.088–0.239)
	7	2155	Yes	0.3666	0.0886	0.120 (0.027–0.259)

K = number of studies, N = number of samples, SE = Standard Error, Bio = Bioassays,
*statistically significant results.

doi:10.1371/journal.pone.0153856.t010

Discussion

This review describes the current knowledge about *T. gondii* prevalence in meat-producing animals in a systematic way. The results showed a pooled prevalence for cattle, pigs and sheep of, respectively, 2.6% (CI_{95}[0.5–5.8]), 12.3% (CI_{95}[6–17.7]), and 14.7% (CI_{95}[8.9–21.5]). For goats and horses, the retrieved results can only provide some partial indications, but show that *T. gondii* infection is relevant in both species, and deserving of further attention.

Although pooling prevalence estimates originating from different animal species could be considered of limited value, it enabled us to statistically define *T. gondii* prevalence differences among them. Cattle are generally described as poor hosts for *T. gondii* and the role of this species is generally judged of limited importance in toxoplasmosis epidemiology [11]. Our results showed that prevalence estimates in cattle were usually lower than those of pigs and sheep. Interestingly, this lower prevalence was not observed in Europe and South America, highlighting the importance of geographic area in *T. gondii* prevalence estimation.

It should be noted that the pooled estimate of prevalence in cattle may suffer from some limitations that are likely to have caused an overestimation of mean prevalence. The first limitation is due to the problem of calculating variance for 0 prevalence estimates. The present work, in order to account for such results, applied the double arcsin transformation on event rates. This has the advantage of allowing this calculation without the use of continuity corrections that cause an underestimation of study weight [22]. The second limitation is due to a potential publication bias that was not detected by the Trim and Fill method but is suggested by the cumulative meta-analysis based on sample size. As an example, if we had considered only studies with sample sizes greater than 50, the pooled prevalence would have been 1% (CI_{95} [0.00–3.6]). This potential bias was not detected by the Trim and Fill method. This was probably because of the wide Confidence Interval in the final estimate that, starting from 0% in the case of cattle, also included the CI obtained after the exclusion of studies with $n<50$. However, this decreasing trend cannot be ignored. It can be supposed that an unknown number of small studies with results showing *T. gondii* prevalences of 0 would have failed to be published, or indeed, never started the publication procedure, thus supporting the theory of "winner's curse" [91]. The inclusion of grey literature in the search strategies probably would have corrected this overestimation. However, the present review focused solely on papers published in peer-reviewed journals to enhance the methodological rigor of the current study and the conclusions drawn regarding prevalence. The low prevalence in cattle, confirmed by our estimates, together with the short persistence of viable *T. gondii* in bovine tissues [11] can be used to support the theory of limited bovine role in *T. gondii* epidemiology. However, despite these considerations, the role of beef in *T. gondii* human epidemiology cannot be easily ruled out, as this meat is eaten raw or undercooked in several countries with a consequent high probability of infection if *T. gondii* is present, as demonstrated elsewhere [13].

As regards the other meat-producing animal species, a lack of difference was observed between sheep and pigs in each investigated area. This lack of difference was partly due to the inclusion, within pigs, of studies reporting *T. gondii* prevalence in organically farmed pigs. Pooled estimates from pigs and sheep suffered problems, in term of publication bias, similar to that already observed for cattle. Therefore, in these cases too, the *T. gondii* prevalence could be overestimated.

The pooled prevalences obtained in the present work should be interpreted cautiously, due to the high level of heterogeneity observed. Nonetheless, they provide important clues regarding the ranking of different meat-producing animal species that is of critical importance in the context of food safety. This is because, currently, there are no measures in place at the slaughter level able to identify animals carrying *T. gondii* [92–94] and prevention is left to consumer behaviour.

Univariable meta-regression models were fitted to account for variables explaining the high level of heterogeneity observed. A summary of moderators included is available in Table 1. Geographic area was an important variable affecting *T. gondii* prevalence in cattle, as it accounted for 61.87% of observed heterogeneity. Its significance was probably due to different farming systems in countries from which the different studies originated. However, this significance was not seen in studies dealing with sheep and pigs. In the case of sheep, it could be supposed that the lack of a widespread intensive farming system determines a common level of exposure in different countries. In the case of pigs, the lack of significance of geographic area is difficult to explain, although it could simply be due to a high level of heterogeneity within the geographic areas examined.

Analytical technique was expected to be a relevant moderator. Bioassay in cats is considered to be the gold standard because of the high sensitivity of these definitive host animals to *T. gondii* infection, and because samples of high quantity can be fed to cats, maximizing the probability of parasite ingestion [51]. Moreover, diagnosis of infection is performed through oocyst recovery from faeces, with widely accepted techniques optimized due to their routine use in small animal clinics. Only three studies were found reporting the use of bioassays in cats to assess prevalence, two performed with cattle samples and one with pig samples. Moreover, these studies were performed by the same research group in the same geographic area, and thus, it is difficult to evaluate the significance of these different factors. An alternative, more frequently used, bioassay technique is performed using mice, an animal more familiar to researchers and research centres. However mice are not the definitive hosts of *T. gondii* and their sensitivity is considered to be lower [12]. This disadvantage is commonly addressed through the intra peritoneal or subcutaneous injection of the parasite to maximize the likelihood of infection. However, a major drawback is the consistent low quantity of sample analysed compared to the amount used in cat bioassay. PCR is often applied as an alternative solution but in this case too, the low quantity of sample analysed is a cause for concern. Moreover, PCR is unable to assess parasite viability, so consequently, overestimation of prevalence can occur. This weakness could be balanced out by the presence of false negatives due to the low amount of tissue from which DNA is extracted. The meta-regression applied in the current study failed to detect such differences in the meat-producing animal species investigated, due to the wide confidence interval produced, within different species, by each technique. Despite the Likelihood Ratio Test never being significant, pairwise comparison with $p = 0.063$ was found in the comparison between PCR and bioassays in cats within studies dealing with cattle. It is worth mentioning, in this case, that studies using cat bioassay always reported a 0 prevalence. However, due to the low number of such studies, statistical analysis was unable to define the estimate as significantly different from other techniques. PCR was not shown to overestimate prevalence in a statistically significant way, but was shown to result in a large confidence interval among different studies. PCR can be considered as a useful and more ethical method than bioassays to assess *T. gondii* prevalence in meat, but needs to be improved. In this context, methods able to concentrate DNA from larger quantities of samples through innovative techniques should be preferred [81].

Animal age is considered an important factor, as higher seropositivity is usually found in older animals [95], because the probability of an animal having had contact with the parasite increases with age. Papers retrieved in the current study rarely reported the age of tested animals and the use of this variable was possible only in the case of sheep, where differences between young (<12 months) and old (>12 months) animals were not observed.

Another important factor is farming system, as increased biosecurity level is able to minimize the contact of farmed animals with wildlife, cats and other potential sources of *T. gondii*. This information would be very interesting to rank as a relevant risk factor for *T. gondii* in animals/meat. Unfortunately, farming systems were rarely reported in the analysed studies,

whereas these data are more common in studies dealing with seroprevalence as summarized elsewhere [15]. In the present work, only the organic farming system for pigs was able to be examined, and this system resulted in a significantly higher *T. gondii* prevalence rate in pigs/pork compared with pigs/pork from conventional pig farming. This result confirms published evidence, obtained through serological studies [96], and extends it, confirming *T. gondii* was significantly more prevalent in pork from organic farms than from conventional meat.

Other moderators classified as methodological were tested. Studies included in the present review considered different types of matrix and sample composition. We feel that examination of analysed organ as a moderator would have been very interesting. However, this was not possible because, often, studies reported that tissues from different organs were pooled within animals. Where analyses of individual organs were reported, results could not be defined as independent, impairing both subgroup analysis and moderator analysis.

Sample type was used as variable to differentiate samples composed of a single organ, samples composed of different organs from the same animal, meat products, assumed to be composed of meat from different animals, and cured meat products, assumed to be less contaminated. It is arguable that, following the uneven distribution of *T. gondii* within an animal [97], the pooling of different organs would increase the risk of positive findings. Moreover, meat products are considered to be of increased risk since they are, in effect, similar to a pooled sample [19]. Our analysis was not able to identify such differences according to a univariable meta-regression, in cattle and pigs. However, in sheep, samples pooled within the animal resulted in a significantly higher prevalence compared to single organ samples.

As expected, sampling location was found to be unrelated to prevalence because *T. gondii* exists along the meat chain, from freshly-slaughtered animals to meat and meat products. Finally the use of serological screening to detect seropositive samples, which were then further analysed through direct methods were applied in some studies within the pigs and sheep categories. There were no significant differences in *T. gondii* prevalences between studies where serological screening was used and studies where it was not used.

Goats are commonly considered as a species at high risk of *T. gondii* contamination and this has been confirmed by the collected data. However, the evidence is not strong and further analyses are needed. This is true in the case of horses too, as only two studies were retrieved, due, probably, to the fact that horse consumption is a local phenomenon. However the first isolation of *T. gondii* in this species dates back to 1979 [98] and horse meat is sometimes consumed raw or undercooked. Therefore, the role of this meat-producing animal species in the spread of *T. gondii* should no longer be overlooked.

Conclusions

The results of this systematic review show that *T. gondii* prevalence in meat animals worldwide is not negligible and that direct detection of this parasite in meat presents a heterogeneous situation. The relative prevalence of *T. gondii* in different meat-producing animal species varies worldwide, and no generalized assumption can be made regarding the role of these animals and meat thereof in the dissemination of the parasite to humans. This observation, together with differences in food habits suggests a high variability of human *T. gondii* infection worldwide. Further research should better evaluate and report the risk factors of the animal population in each study (and in each published paper), which would allow their proper evaluation. Furthermore, methodological and epidemiological sources of heterogeneity need to be clarified. In general, raw or undercooked meat from cattle, pigs, sheep, horses and goats is a potential source of *T. gondii* and should not be consumed by at-risk groups in the population. Control options should be studied to lower *T. gondii* impact on the human population.

Supporting Information

S1 Fig. Forest plot showing the estimated prevalence (with 95% CI) of *Toxoplasma* in cattle for each study. In addition, results for each category (geographic origins) identified through univariable meta-regression are shown. T+ = positive samples, N = number of samples, RE = Random Effects.
(PDF)

S2 Fig. Forest plot showing the estimated prevalence (with 95% CI) of *Toxoplasma* in pigs for each study. In addition, results for each category (farming system) identified through univariable meta-regression are shown. T+ = positive samples, N = number of samples, RE = Random Effects.
N = number of samples, C = Conventional farms, NS = studies not reporting farm features, O = Organic farms, SF = Small Farms, *studies applying serological screening before direction.
(PDF)

S3 Fig. Forest plot showing the estimated prevalence (with 95% CI) of *Toxoplasma* in sheep for each study. In addition results for each category (Sample type) identified through univariable meta-regression are shown. T+ = positive samples, N = number of samples, RE = Random Effects.*studies applying serological screening before direction.
(PDF)

S1 Table. PRISMA 2009 Checklist.
(DOC)

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Author Contributions

Conceived and designed the experiments: SB AR. Performed the experiments: SB DC GS. Analyzed the data: SB MM. Contributed reagents/materials/analysis tools: SB MM AR. Wrote the paper: SB MP AR.

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Manuscript 2

Toxoplasma gondii prevalence in bovine and swine meat from the Veneto region (Italy)

Simone Belluco[1,2,*], Elena Ramon[1], Annalisa Beccaro[2], Davide Giugno[2], Ilaria Patuzzi[1,3], Antonia Anna Lettini[1], Federica Marcer[2], Antonia Ricci[1]

[1]*Food Safety Department, Istituto Zooprofilattico Sperimentale delle Venezie, viale dell'Università 10, 35020 – Legnaro (PD), Italy.*
[2]*Department of Animal Medicine, Production and Health, University of Padua, Viale dell'Università 16, 35020 – Legnaro (PD), Italy.*

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- Davide Giugno
Prevalence of T. gondii in beef from emergency slaughtering carried out in the Veneto region.
Course: Food science and technologies, University of Padua
Supervisor: Prof. Paolo Catellani.
Co-supervisor: Simone Belluco

- Annalisa Beccaro.
Biomolecular investigation on the presence of T. gondii in pork products sold in the Veneto region.
Course: Food biotechnology, University of Padua.
Supervisor: Prof. Federica Marcer.
Co-supervisor: Simone Belluco

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[1]*Food Safety Department, Istituto Zooprofilattico Sperimentale delle Venezie, viale dell'Università 10, 35020 – Legnaro (PD), Italy.*
[2]*Department of Animal Medicine, Production and Health, University of Padua, Viale dell'Università 16, 35020 – Legnaro (PD), Italy.*
[3]*Department of Information Engineering, University of Padua, Padova, Italy.*

*corresponding author
Simone Belluco (sbelluco@izsvenezie.it)

Abstract

Toxoplasma gondii is a protozoan parasite with striking epidemiological success due to its ability to rely on several transmission routes. Food, particularly meat, is one of the most common infection sources, but the relative importance of commonly consumed meat from different animal species is debated and varies across years and countries. In Italy, the most widely consumed meats are pork and beef and both are able to harbour *T. gondii* cysts and transmit the parasite. This study estimated the parasite prevalence in cattle and pig meat through a nested PCR protocol. Investigated beef originated from emergency slaughter outside the slaughterhouse, whereas pork was obtained from a random sampling plan implemented at retail level. Results showed the *T. gondii* prevalence in beef was 20%, very high if compared with data from other studies, and a prevalence of 8.7% in pork, in agreement with literature data. Results confirm the potential for beef and pork to transmit *T. gondii* in Italy and the importance of properly cooking beef and pork before consumption, especially for at risk individuals.

Keywords

Pork, beef, PCR, *Toxoplasma gondii*, meat

Introduction

Toxoplasma gondii is one of the world's most successful parasites, with an estimated worldwide prevalence up to about 30% among the human population (Flegr, 2013). The clinical importance of this disease is largely due to infection occurring during pregnancy or in immunocompromised individuals (Montoya and Liesenfeld, 2004). Commonly used disease burden indicators, such as DALY (Disability Adjusted Life Years), rank *T. gondii* at the top of foodborne pathogens in the Netherlands (Havelaar et al., 2012) and worldwide (Torgerson and Mastroiacovo, 2013).

The complex life cycle of *T. gondii* recognizes felids as definitive hosts, in which the parasite can complete its sexual cycle and from there spread millions of oocysts into the environment. Although the number of oocysts produced is a key element in environmental contamination and consequently in parasite transmission, *T. gondii* is also able to rely on its asexual cycle in almost all warm blooded animals.

Food, particularly meat, has been shown to be a major route of transmission for human infection (Cook et al., 2000; Desmonts et al., 1965). The prevalence of *T. gondii* in meat-producing animals varies according to species (Belluco et al., 2016) and farming practices (Guo et al., 2015).

It is well established that sheep have the highest prevalence among common livestock, followed by pigs and bovines, the latter showing a low to null prevalence rate (Belluco et al., 2016; Dubey et al., 2005; Guo et al., 2015). Some authors argued that pork could be an important source due to high prevalence and huge consumption (Dubey et al., 2005). Others support the importance of bovine meat due to a high risk linked to the frequent consumption of raw or undercooked beef (Opsteegh et al., 2011). Epidemiological studies and risk assessment models are needed to solve this issue. Prevalence data from retail meats are useful to feed into risk assessment models and have special value for the geographical area from which they originate.

The main difficulty in obtaining reliable prevalence data is the laboratory method used. Published studies range from cat (gold standard) and mouse bioassays to PCR methods and, within PCR, from end-point procedure to the Magnetic Capture Real time technique. The Magnetic Capture protocol has very high sensitivity (Opsteegh et al., 2010), whereas other techniques, such as the Nested PCR protocol applied in this work, have the advantage of being easier to apply, an advantage in the light of their potential use in the food chain for diagnostic purposes.

The aim of this study was to estimate the prevalence of *T. gondii* in beef and pork produced or marketed in the Veneto region through a Nested PCR protocol and to discuss it in the context of epidemiological information.

2. Materials and methods

2.1 Sampling strategy

2.1.1 Beef sampling plan

The sampling plan for beef was defined to estimate the prevalence of *T. gondii* in meat from animals undergoing emergency slaughter in the Veneto region. *T. gondii* prevalence of 2.2% in European bovines was use to estimate the number of samples needed (Belluco et al., 2016). The number of samples (n) was calculated in order to demonstrate a prevalence lower than 2% with a confidence level of 95% (a) if no positive meat samples were obtained according to the following formula, where D is the assumed number of positive animals, P the assumed prevalence and *a* the value due to the selected confidence level (Thrusfield, 2005).

$$n = \left[1 - (1 - a)^{\frac{1}{D}}\right]\left(N - \frac{D-1}{2}\right)$$

Where N, in the case of an infinite population, is estimated as:

$$N = \frac{\ln(1-a)}{\ln(1-p)}$$

Following these considerations 149 samples were needed to confirm the prevalence obtained from scientific literature. The investigated meat samples originated from emergency slaughter outside the slaughterhouse as defined by Chapter VI of Reg. (EC) 853/2004. This emergency slaughter is practised when animals can't be moved to the slaughterhouses without causing pain to the animals. This sample origin was chosen for convenience, as this kind of samples routinely reached the food microbiology laboratory of Istituto Zooprofilattico Sperimentale delle Venezie. Moreover, following the amendment of Reg. 854/2004 by Reg. 218/2014, this meat, after appropriate laboratory analyses, can be marketed freely in the EU thus is not expected to have any different food safety risk than meat originating from conventional slaughter.

2.1.2 Pork sampling plan

The sampling plan for pork was defined to estimate the prevalence of *T. gondii* in meat at consumer level in the Veneto region. To provide wide coverage of the pork market, the sampling plan focused on food stores belonging to large-scale retailers active in the Veneto region. The total number of samples was divided proportionally among large-scale retail firms with more than 15 stores, according to the number of stores (data obtained from the Veneto region, 2014).

Given the prevalence of *T. gondii* in European pork is 8.7% (Belluco et al., 2016), a total of 140 samples was considered appropriate to estimate the prevalence with a confidence level of 95%, according to the following formula where P is the supposed prevalence and D the absolute precision (Thrusfield, 2005):

$$n = \frac{1.96^2 P(1-P)}{D^2}$$

Each store was visited one or more times, from March to June 2015, paying attention to avoid collecting duplicate samples by checking the traceability information as reported in the meat labels.

2.2 Biomolecular protocol

2.2.1 Extraction procedure

Briefly, 50g of muscular tissue were thoroughly homogenized using T25 digital Ultra Turrax® (IKA®) and kept refrigerated until DNA extraction. To obtain genomic DNA, 25mg of homogenized tissue was added to 180 µl of buffer ATL and 20 µl of proteinase k and incubated at 56°C until the complete lysis of muscle fibres. After that, extraction was carried out using a commercial kit (QIAamp DNA Mini Kit, Qiagen) according to the manufacturer's instructions.

2.2.2 Amplification technique

Nested PCR reactions were carried out in duplicate as described earlier (Burg et al., 1989) with the following primers: outer (5'-GGAACTGCATCCGTTCATGAG-3' and 5'-TCTTTAAAGCGTTCGTGGTC-3') inner (5'-TGCATAGGTTGCAGTCACTG-3' and 5'-GGCGACCAATCTGCGAATACACC-3'). First round amplification was carried out as previously reported (Burg et al., 1989), whereas the protocol for the nested reaction was slightly modified as follows. The PCR reaction contained: 4mM of $MgCl_{2,}$ 0.2 mM of each dNTP, 0.2 mM of each primer. The number of cycles was reduced to 30. A 2% agarose gel was used for fragment separation.

2.2.3 Sensitivity of the B1 Nested PCR assays for T. gondii DNA *detection*

To assess the analytical sensitivity of the Nested-PCR assay, 10-fold serial dilutions of *T. gondii* DNA (RH strain, 50174D™, ATCC – American Type Culture Collection) were prepared, ranging from 4 ng to 0.04 fg per 25 µl PCR reaction. Assuming a content of 112 fg genomic parasite DNA (Opsteegh et al., 2010) the number of parasites per dilution would be 3.6×10^{4} down to 3.6×10^{-5}. The dilution series were tested in duplicate.

2.2.4 Sequencing

Positive samples were processed using a Sanger sequencer ABI 3130 (Applied Biosystems) and sequence reader software (Data Collection Software v3.1. Applied Biosystems). The sequences obtained were checked against GenBank® sequences and then aligned through MultAlin (Multiple sequence alignment with hierarchical clustering) (Corpet, 1988).

2.3 Statistical analysis

Confidence interval and statistical analyses were carried out using R software facilities (R Core Team, 2012). Differences between categories were assessed using the Z-test test for the equality of proportions.

3. Results

3.1 Sample distribution and prevalence of T. gondii in beef

The 150 beef samples examined had an overall *T. gondii* prevalence of 0.2 (CI 95%; [0.14-0.27]).

The age distribution of animals in the investigated population was very different from the distribution of slaughtered animals as shown in figure 1.

The prevalence rate was significantly higher for female than for male (p=0.046) animals, for cattle belonging to breeding farms than to fattening farms (p=0.057), and for cattle of Italian origin (0.027) if compared with animals of French origin. No linear association was found with increasing bovine age.

3.2 Sample distribution and prevalence of T. gondii in pork

One hundred and forty-one samples of pork meat were obtained, 81 fresh meat and 60 meat preparations. The estimated prevalence of *T. gondii* in pork was 0.085% (CI 95%; [0.045-0.144]). No significant differences were observed between fresh meat (7/81) and meat preparations (5/60). No analysis of animal origins was possible because this was not reported in the label for the majority of the meat preparations and because when it was reported, the country of origin was commonly Italy.

3.3 Sensitivity analysis

When the B1-Nested PCR protocol was applied to the 10-fold dilution series of *T. gondii* genomic DNA, a detection limit of 40 fg of DNA was determined. This corresponded to the DNA content of 0.36 of a parasite, calculated according to a total genomic DNA content of 112 fg per parasite (Opsteegh et al., 2011). This level of analytical sensitivity agreed with results from the original method (Burg et al., 1989) and was better than the sensitivities obtained by some Real time PCR techniques (Reischl et al., 2003).

3.4 Sequencing results

Positive samples were confirmed through sequencing, but a consensus sequence was not obtained in all cases due to the short amplification product.

4. Discussion

The role of bovine meat in *T. gondii* transmission is debatable. The association between undercooked beef consumption and *T. gondii* has been demonstrated in epidemiological case-control studies (Cook et al., 2000). Worldwide, a mean *T. gondii* prevalence of 2.6% (CI 95% [0.5–5.8]) was recently calculated, ranging from values very close to 0% in North America to up to 16% in Central America, depending also on the diagnostic test used (Belluco et al., 2016). In particular, an important study carried out in the US resulted in 0 positives to cat bioassay out of 2,094 tested beef samples originating from the whole country (Dubey et al., 2005), and concluded that beef consumption is not an important risk factor for human toxoplasmosis in the US.

In contrast, we determined a high *T. gondii* prevalence in bovine meat (20%), which is very different from prevalences given in the literature and comparable only to South American prevalences. To our knowledge, no other studies assessed the prevalence of *T. gondii* in

Italian bovine meat through direct methods. However, according to seroprevalence data, the prevalence of positive bovines in Italy varies between 11.3% in Sicily to 92% in the North (Rinaldi and Scala, 2008). These rates are much higher than rates from all the other countries considered in a review with data from several countries worldwide (Tenter et al., 2000). Seroprevalence alone cannot conclusively explain the high prevalence (20%) found in the present investigation. However, the applied sampling plan, which investigated bovine meat from emergency slaughter outside the slaughterhouse, could partially explain this result. This slaughter practice is mainly applied to aged animals often belonging to the dairy sector. Only 7% of slaughtered bovines in Veneto in 2015 were more than 24 months old, whereas 43% of the bovines in the current study belonged to this age category. Despite no linear association being found with age, a higher prevalence was observed among bovines more than 24 months old. Moreover, significantly more bovines of Italian origin were *T. gondii*-positive than were bovines from France. Moreover, female bovines had a higher *T. gondii* prevalence than male bovines. This result can be explained by the fact that, in our sampled population, bovines of Italian origin and female bovines were more likely to belong to dairy farms. The management of dairy farms, at least in some temporal periods in the farming process, allows for outdoor farming, increasing the likelihood of bovine contact with potential environmental *T. gondii* sources.

A recent risk assessment indicated beef was likely the major food source of *T. gondii* in the Netherlands (Opsteegh et al., 2011), not because of high prevalence rates, but because of the frequent consumption of this meat without any treatment able to inactivate parasites. According to this model, beef meat was responsible for 68% of the meatborne infections, followed by sheep (14%) and pork (11%). Beef was also the major transmission vehicle even when a 0.5% prevalence rate was considered (Opsteegh et al., 2011). Interestingly, meat from animals older than 24 months, due to its lower cost, is more likely to be used in the preparation of some specific meat products (i.e. hamburger or typical regional food) which would be potentially higher risk for consumers.

However, it is important to bear in mind that PCR is unable to discriminate between viable and non-viable parasites, and thus, not all positive samples are necessarily able to cause infection. This is why bioassay is recognized as a more reliable technique to predict the infectivity of *T. gondii*-positive meat. However, bioassays are costly, time consuming and rely on animal use, this last raising ethical concerns. For these reasons, despite some limitations, great interest has been generated for molecular techniques which have significant advantages in terms of costs, speed and feasibility.

The prevalence rate for *T. gondii* in pork in our study was 0.085%, comparable with a previous estimation (Belluco et al., 2016). In the current study, the sampling plan was designed to provide a reliable estimate of *T. gondii* in pork meat available for consumers from conventional retail channels and confirmed the potential role for this meat if consumed raw or undercooked. This result, on one hand, could be an overestimation of the real prevalence due to the inability of PCR technique to discriminate between non-viable and viable parasites, but on the other hand, it could be an under-estimation due to detection

limits of the protocol applied. It is also noteworthy that the risk of *T. gondii* infection for consumers depends strongly on their cooking habits (Cook et al., 2000) and on the amount of cysts in the edible tissues. The number of parasites in animal tissues varies widely, starting from <0.2 parasites per gram (in hind limb muscles, liver and kidney) as calculated under experimental conditions in inoculated pigs (Juránková et al., 2014). However, our method was unable to detect such low numbers of parasites.

To conclude, our results confirm the *T. gondii* prevalence rate in pork from the Veneto region at retail level is not negligible. This supports the importance of consuming this meat well-cooked, and suggests the need for studies directed towards local cured pork products to evaluate their epidemiological potential.

As regards beef, our study highlights a very high prevalence of *T. gondii*, suggesting that the consumption of uncooked or rare meat from old bovines could have great potential to cause human toxoplasmosis.

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Manuscript 3

Toxoplasmosis and food consumption: a systematic review and meta-analysis of case-controlled studies.

Simone Belluco[1,2,*], Giulia Simonato[2], Marzia Mancin[1], Mario Pietrobelli[2], Antonia Ricci[1].

[1]*Food Safety Department, Istituto Zooprofilattico Sperimentale delle Venezie, viale dell'Università 10, 35020 – Legnaro (PD), Italy.*
[2]*Department of Animal Medicine, Production and Health, University of Padua, Viale dell'Università, 35020 – Legnaro (PD), Italy.*

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Simone Belluco[1,2,*], Giulia Simonato[2], Marzia Mancin[1], Mario Pietrobelli[2], Antonia Ricci[1].

[1]*Food Safety Department, Istituto Zooprofilattico Sperimentale delle Venezie, viale dell'Università 10, 35020 – Legnaro (PD), Italy.*
[2]*Department of Animal Medicine, Production and Health, University of Padua, Viale dell'Università, 35020 – Legnaro (PD), Italy.*
*corresponding author
Simone Belluco
Viale dell'Università 35020, Legnaro (PD)
+39 049 8084169

Abstract

Background Toxoplasmosis is a zoonotic disease causing severe diseases in pregnant women and immunocompromised individuals. On average, worldwide, around 30% of people are seropositive. The oral transmission route is of great significance and food, particularly meat, is an important transmission vehicle for *T. gondii*. However, the role of different food matrices is debated.

Objectives The aim of this review was to assess the risk of humans developing acute *T. gondii* infection via the foodborne route.

Study eligibility criteria Case-control studies including acute cases of toxoplasmosis were included after literature searches, without time limits, in several databases. All studies estimating the risk of acquiring toxoplasmosis after consumption of specific food categories were included.

Results Three risk factors proved to be significantly associated with acute *T. gondii* infection in humans: consumption of raw/undercooked meat OR 3.44 (1.29-9.16), consumption of raw/undercooked beef OR 2.22 (1.57–3.12) and consumption of raw/undercooked sheep meat OR 3.85 (1.85–8.00). Consumption of raw/undercooked pork, raw eggs and unpasteurised milk proved to be non-significant risk factors.

Limitations Limitations in the present review and meta-analysis are due to the low number of case-control studies available for analysis and by the lack of a search strategy targeting grey literature.

Conclusion Consumption of raw/undercooked beef and sheep meat are important risk factors for toxoplasmosis. Their consumption should be avoided in order to prevent toxoplasmosis, particularly by those in at-risk categories, including pregnant women.

The review protocol is registered in PROSPERO database (CRD42016043295).

Keywords

Toxoplasma gondii, meat, beef, risk.

Introduction

In 1965, when the of toxoplasma research (Desmonts et al., 1965) met zoomotherapy (Lowy, 2010), the scientific community attained the first solid proof of meat's role in *T. gondii* transmission to humans. However, some aspects of the role of meat in human toxoplasmosis remain unclear to this day.

Worldwide, *T. gondii* prevalences range up about to 30% (Flegr, 2013) and, according to disease burden indicators such as DALY, this parasite ranks at the top of foodborne disease-causing agents (Havelaar et al., 2012; Torgerson and Mastroiacovo, 2013). *T. gondii* can cause abortions or severe foetal malformations when women acquire infection during pregnancy, but toxoplasmosis is also an important disease for immunocompromised individuals (Montoya and Liesenfeld, 2004). The impact of *T. gondii* in healthy individuals is still unclear but the parasite can encyst in human tissues with a potential for reactivation during strong immunosuppression events.

Felids hold a leading role in *T. gondii* epidemiology, as they are the only definitive host able to facilitate the sexual propagation of the parasite. Felids can spread millions of oocysts during infection, causing huge environmental contamination as, after sporulation, oocysts become infective for hosts accidentally ingesting contaminated products. Risk factors for oocyst ingestion are poor hygiene and consumption of contaminated water or vegetables.

However, the epidemiological importance of bradyzoites from tissue cysts is well known, and food is estimated to contribute to a proportion (between 42-61%) of all cases, depending on the geographical area (WHO, 2014). Acquisition of infection via the oral route is a fundamental event for *T. gondii* biology. The route is independent from the parasite's sexual reproduction, differentiating *T.gondii* from other closely related parasites such as *Neospora*, *Sarcocystis*, and *Hammondia*, and expediting the infection of almost all warm blooded animals (Su, 2003). The acquistion of this alternative transmission route probably explains the evolutionary success of *T. gondii.*

A third infective parasite stage is the tachyzoite, which can be transmitted by animal fluids. Milk is considered as a potential source of infection as, during acute animal infection, circulating tachyzoites can be transferred from blood to milk (Tenter et al., 2000), but debate on this is still ongoing in the scientific community (Boughattas, 2015a; Dehkordi et al., 2013; Dubey and Jones, 2014). According to this premise, food plays a critical role in *T. gondii* transmission but the differential role of food sources, particularly within the meat category, has not been disclosed.

Oral exposure to *T. gondii* through food mainly depends on parasite prevalence and people's food consumption habits. *T. gondii* has been detected in different kinds of meats (Belluco et al., 2016; Guo et al., 2015), vegetables (Lass et al., 2012) and milk (Dehkordi et al., 2013). Whatever the food source is, consumption habits play a critical role, as *T. gondii* can be inactivated through cooking, freezing and salting.

Epidemiological studies provide the opportunity to assess the risk associated with a particular food in the absence of Randomized Control Trials. Among observational studies, cohort and case-control study designs offer the opportunity to evaluate the causality associations.

In this systematic review, we mapped all relevant literature reporting the result of primary observational studies on food-related risk factors associated with *T. gondii* infection in the human population. We then narrowed the review question to case-control studies and, after appraising the quality of individual studies, we summed up the evidence using meta-analysis to estimate the risk of *T. gondii* infection associated with consumption of different foods.

Materials and methods

Selection of relevant studies

The review question was aimed at estimating the role of different foods (E) on *T. gondii* infection (O) in human population (P). We considered all studies published in peer reviewed journals in English, French, Italian, Spanish and Portuguese. No time limits were imposed.
We searched multiple literature databases: PUBMED, Web of Science core collection - KCI-Korean Journal Database - Russian Science Citation Index - SCIELO Citation Index, and CAB Abstracts with the following search terms: (Toxoplasma OR Toxoplasmosis) AND risk factor.
The last date searched was 7 April 2016. To implement the search process, we used the final list of studies to carry out both a backward and a forward reference search using Google scholar as a search engine.
The review design is presented in Figure 1. Due to our inability to estimate the available number of case-control and cohort studies, we decided not to use study design as an eligibility criterion in the first screening but to code this item and to use it to narrow the review question in the second stage. The review protocol is registered in PROSPERO database (CRD42016043295).

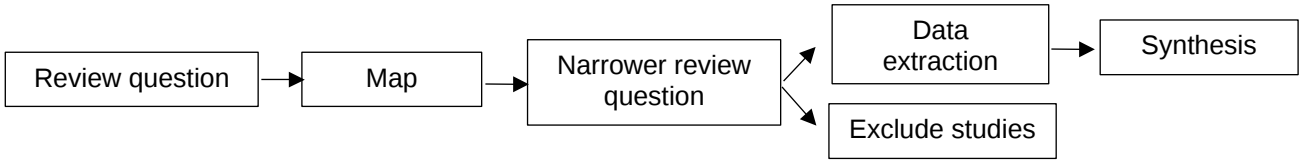

Figure 1 review design

Several criteria were used to select eligible studies beyond language restriction: 1) reported data had to belong to primary research; 2) cases had to belong to the human population; 3) cases had to be diagnosed with *T. gondii* infection; 4) food-related risk factors had to be considered. In the case of a poorly explicative abstract or in the case of doubt about the available data, the study was included.
Thereafter, two reviewers (SB, GS) screened all studies obtained via the initial literature search according to Title/Abstract and Full text, independently (parallel method). Disagreements were resolved through consensus. One reviewer (SB) collected data from relevant articles and a second reviewer (GS) checked the collected data against the original studies (sequential method). All studies were coded according to the previously chosen parameters and data were recorded. In the case of reviewer doubt about the reported data or in the absence of useful effect size, the study authors were contacted via e-mail.

Data were sought for the following variables: country, study year, population studied, case definition, case selection, selection of controls, exposure window, investigated risk factor and outcome. Due to our study design, we used the Odds Ratio (OR) as the outcome.
The review process was carried out using EPPI-4 Reviewer software (Thomas et al., 2010).

Risk of bias in individual studies

Individual study quality was assessed using a score modified from the Newcastle-Ottawa Quality Assessment Scale for Case-Control Studies (GA Wells, B Shea, D O'Connell, J Peterson, V Welch, M Losos, 1993). Relevant confounders eligible for scoring were age and residency. Both statistical adjustment and matching by design were considered.
In addition to the Newcastle-Ottawa scale, we added two other relevant items; the inclusion of an exposure window (a time limitation) for exposure assessment purposes and the inclusion of only recent cases of infection. A recent case was defined as a patient with either: 1) a positive test result following a negative test result; 2) IgM and IgG positivity, or; 3) with serological positivity and low-avidity to IgG.

Synthesis of results

Meta-analyses were performed using the metafor package (Viechtbauer, 2010) of the statistical software R (R Core Team, 2012) through the interface developed in EPPI-4 Reviewer, and also directly without the interface.
A meta-analysis was run for each food-related risk factor considered, where the number of available studies was equal or higher than three and the risk factors investigated were suitable for aggregation.
Some included studies considered more than one risk factor, and thus, outcomes within the same studies are not independent. This has been considered where relevant.
All the other results were collected and discussed. The OR was selected as a relevant outcome and collected from primary studies according to adjustment for relevant confounders, if present. If an OR was calculated in the original study from a subpopulation (e.g. consumption of raw beef among people consuming raw meat), the OR was used to extrapolate the result to the entire population of respondents.
ORs from different studies were aggregated through a Random-Effects Model, using Restricted Maximum Likelihood (REML) as an estimator, which is considered approximately unbiased and relatively efficient. Knapp-Hartung adjustment was applied (Knapp and Hartung, 2003).
Heterogeneity was assessed using the Q, T^2 and I^2 (Higgins and Thompson, 2002) parameters.

Risk of bias across studies

The potential for publication bias was assessed through the Trim and Fill method (Duval and Tweedie, 2000). Sensitivity analyses were performed for each meta-analysis, to evaluate the potential for studies exerting high influence on the model. Briefly, several parameters were examined: the externally studentized residuals, the DFFITS (DiFference in FIT, Standardized), the Cook's distance, the hat function and the covariance ratio. Influence was defined according to metafor package criteria (absolute DFFITS value>3p[p/(k-p)], where p is the number of model coefficients and k is the number of studies *or* the lower tail area of a chi-square distribution with p degrees of freedom cut off by the Cook's distance being larger than 50% *or*

hat value>3(p/k)). In addition, studies were excluded one by one from the model to evaluate relevant changes in heterogeneity (T^2 and Q) and pooled estimate. A P-value<0.05 was considered significant in the statistical meta-analysis (Viechtbauer, 2010).

Results

Study selection

A total of 2215 articles were retrieved according to the search criteria. In total, 285 full text articles were considered eligible for the initial review question and mapped particularly against study design. After narrowing the review question to include only case-control designs, eleven studies were retained and considered eligible for data extraction (Details are shown in Figure 2).

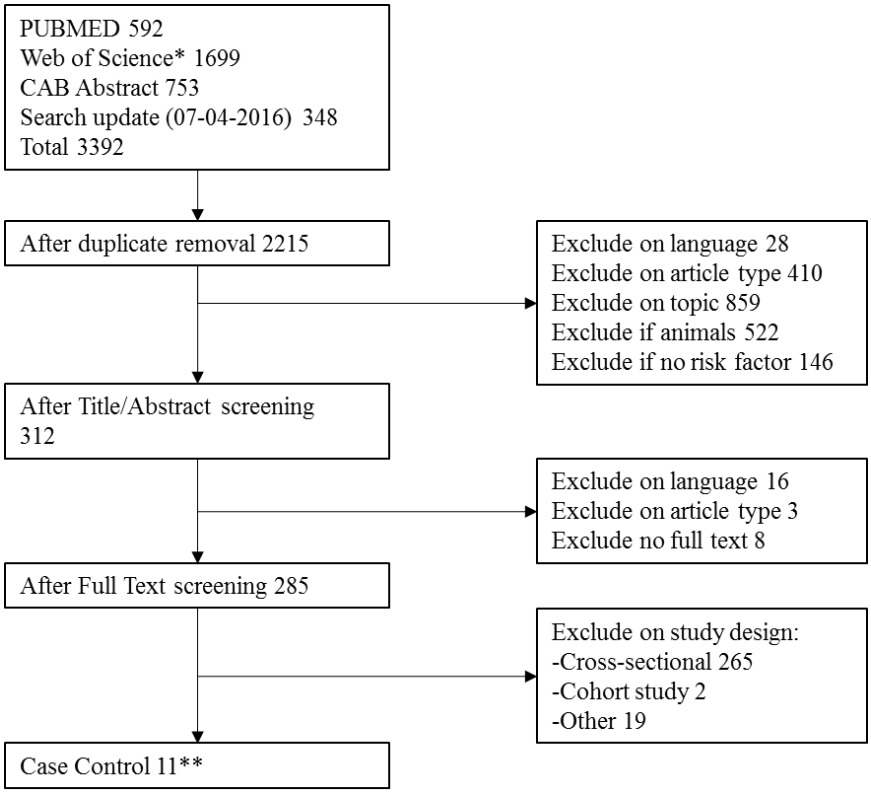

Fig 2 PRISMA diagram showing the detailed results of the study selection process
*Web of Science allowed the search to be conducted in multiple databases as specified in the Materials and Methods section.
**Two studies were considered eligible but did not report results which could be included.

Study characteristics

All studies included after full-text screening were mapped against relevant characteristics to select criteria for narrowing the review question for meta-analytical purposes. The vast majority of studies mapped in our systematic review were carried out according to the cross-sectional design, and targeted pregnant women. Details are reported in Figure 3.
Study characteristics of the 11 case-control studies were collected in detail and are reported in Table 1. Briefly, publication year ranged from 1980 to 2014, and studies were carried out in

Europe (6), South America (2), Asia (1) or in North America (1). The investigated populations comprised pregnant women (5) or the general population (4), whereas in one study only, mothers of newborns diagnosed with *T. gondii* were selected as the target population. Cases were always confirmed through serology and in general, all positive subjects were recruited. Several food-related risk factors were included in the exposure assessment design of the selected studies beyond the six food categories included in meta-analyses. A full list is reported in Table S1.

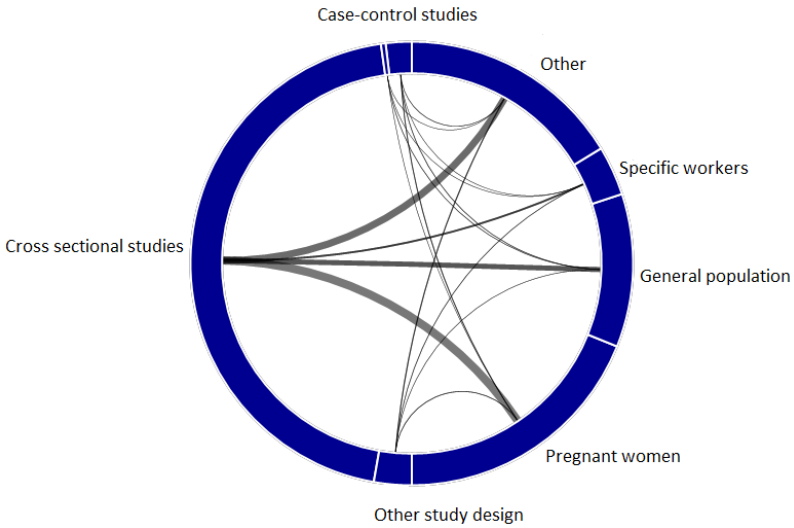

Fig 3 circular relation diagram showing the results of mapping as regards study design and population. The thickness of the connection line is proportional to the number of studies

Risk of bias within studies

The Newcastle-Ottawa scale was used to evaluate the potential for bias within each included study. The case definition was of no concern, as all studies were based on serological findings. As regards case selection in general and due to the low number of acute cases identified, all positive subjects giving consensus were included. However, in one study (Kapperud et al., 1996) the number of cases was increased by 22 individuals from sporadic testing. Controls were selected within the same community as the cases, with two exceptions (Carellos et al., 2014; Chiang et al., 2014), where controls were selected from previous programs or from the general population. In particular, the study of Carellos et al. (2014) has a major limitation, as controls were not serologically tested to exclude *T. gondii* antibodies, whereas they were in all the other studies. Different matching strategies were applied and most studies matched cases and controls according to age and/or residency. Details are reported in Table 2.
Assessments of exposure were always carried out through questionnaire or interview but blinding was never reported. In one case, there is no detail about how exposure was assessed (Bobić et al., 2007). As regards the non-respondent rate, it was generally reported in the included studies.

Synthesis of results

Eleven studies were eligible for meta-analysis, although of these, two studies did not have eligible data for inclusion. One study reported data from multivariate analysis accounting for

different risk factors (Jones et al., 2009), but our e-mail request to be supplied with univariate data garnered no response. The other study reported only one risk factor relevant for inclusion, but due to the low number of exposed people did not estimate the OR for such risk (Lopez-Castillo et al., 2005). Additional data and explanations were obtained by e-mail for two studies (Bobić et al., 2007, 2010).

Three food consumption risk factors were significantly associated with *T. gondii* infection: raw/undercooked meat, OR 3.44 (1.29-9.16) (Fig 4), raw/undercooked beef, OR 2.22 (1.57–3.12) (Fig 5) and raw/undercooked sheep meat, OR 3.85 (1.85–8.00) (Fig 6). No heterogeneity was observed according to I^2 statistics in the cases of beef and sheep meat consumption, confirming the agreement among the results of individual studies. A high heterogeneity of 73% was observed within the category raw meat consumption, producing a more uncertain result as the prediction interval (0.47-25.20) had the potential to generate non-significant results. Consumption of raw/undercooked pork (Fig 7), raw milk (Fig 8) and raw eggs (Fig 9) were not significantly associated with toxoplasmosis. In the case of raw eggs, slightly different risk factors were considered in the studies included in our meta-analysis: eating raw eggs (Baril et al., 1999; Kapperud et al., 1996), eating eggs with soft yolk (Carellos et al., 2014), and frequent consumption of soft-boiled eggs (Stray-Pedersen and Lorentzen-Styr, 1980). Details of individual study results are shown in Figure 4, while details of our meta-analysis results are reported in Table 3.

Publication bias analysis was carried out to account for potentially missing studies. Results should be interpreted cautiously due to the low k of different meta-analyses. Publication bias was observed in the raw meat analysis, with a potential loss of statistical significance, in the raw beef analysis, suggesting underestimation of the real OR, and more interestingly in the pork analysis, where, according to the Trim and Fill test, missing studies could lead to the acquisition of statistical significance.

Sensitivity analyses were performed for meta-analyses involving raw meat and raw beef as other meta-analyses included only a limited number of studies. No individual study influenced the model according to the statistical parameter evaluated. The multicentric study of Cook et al. (2000) contributed heavily to the result of our meta-analysis when it was included, but the high appraised methodological quality of this study makes the introduction of potential biases unlikely. Meta-analysis on raw/undercooked beef was conducted both with and without the study by Carellos et al. (2014) due to methodological concerns we observed in that study. When the study was not included, the resultant OR 2.10 (1.21-3.64) did not significantly differ from the OR obtained when meta-analysis was conducted on all studies.

Author	Country	Study year	P	Case definition	Case number and selection	Control number and selection	EW
Baril 1999	*France*	*1995*	PW	Seroconversion Negative test to specific IgG and IgM followed by a positive test	80 All positives *Giving consensus*	80 Non random Matched	1
Bobić 2007	*Serbia*	*2001-05*	PW	IgG and IgM positivity	53 All positives	53, Non random Matched	NS
Bobić 2010	*Serbia*	*2004-08*	GP	IgG and IgM positivity IgG avidity low	35 All positives	35, Non random Matched	NS
Carellos 2014	*Brazil*	*2006-07 cases, 2011 controls*	M	Clinical and serologiocal o*n newborns*	175 All positives	278 Random (*stratified per municipalities).*	9
Chiang 2014	*Taiwan*	*2008-2013*	GP	IgG and IgM positivity IgG avidity low or PCR positive in blood or body fluids	30 All positives (from surveillance)	224 Random (from blood donors)	NS
Cook 2000	*Italy, Switzerland, Denmark, Norway, Belgium*	*1994-1995*	PW	Negative test to specific IgG and IgM followed by a positive test or IgG and IgM positivity, IgG avidity low, or IgA positive	252 All positives	852 Random (*Next 4 women negative for Toxoplasma)*	4
Jones 2006	*Brazil*	*2003-04*	GP	IgG and IgM positivity	All positives *Patients from an opthalmology clinic*	Non random (*Next patient which was seronegative)*	12
Jones 2009	*USA*	*2002-07*	GP	IgG and IgM positivity, IgG avidity low, or IgA positive	All positives *>18 years, infection within 6 months*	Random (*Among T.gondii seronegative tested in the laboratory)*	12
Kapperud 1996	*Norway*	*1992-94*	PW	Seroconversion OR dye test > 300IU/ml and specific IgM	*All positive to specific program + positives from sporadic testing*	Non Random Matched	4
Lopez-Castillo 2005	*Colombia*	*2004-2005*	PW	IgG and IgM or IgA positivity or newborn with *T.gondii*	*Not described*	Non Random Matched	9
Stray-Pedersen 1980	*Norway*	NS	PW	Negative test to specific IgG and IgM followed by a positive test or IgM detection	Random *(specified)*	Randomisation non described	NS

Table 1 relevant information collected from relevant papers. PW= Pregnant women, GP=General Population, M=mothers of positive newborns; NS=not specified, EW= Exposure Window (months)

Other results

Results not included in our meta-analyses comprised the multivariate model of Jones et al. (2009) and individual ORs from relevant but sporadic food-related risk factors investigated in the studies.

The results from the multivariate model identified several risk factors which increased the risk of *T. gondii* infections. These were: eating raw ground beef, eating rare lamb, eating locally

produced cured, dried or smoked meat and drinking unpasteurized goat milk (Jones et al., 2009).

All other relevant but sporadic risk factors, which were not included in our meta-analysis, are available in Table S2. In some cases, different risk factors from different studies looked to be classifiable in a common category, but due to heterogeneity in the definitions of these factors, we finally judged them as not suitable for aggregation.

Cook et al. (2000) found a significant association between toxoplasmosis and consumption of dry or cured meat and salami more than once a week, whereas this association was not found in the other study (Stray-Pedersen and Lorentzen-Styr, 1980). Several eating habits were investigated by Cook et al. (2009), and participant's preference for raw/rare beef was significantly associated with an increased risk of toxoplasmosis, similarly to actual consumption of this kind of meat. Among hygienic habits, only infrequent knife washing was significantly associated with infection (Kapperud et al., 1996) whereas none of the other eight risk factors similarly associated with the potential for cross-contamination were significant. Meat consumption and meat consumption frequency were never associated with an increased risk of toxoplasmosis. As regards other raw/undercooked meat or fish, data for which were not included in our meta-analysis, there was a high variation in the definitions of risk. Therefore, details from these studies can be found in Table S2.

Interestingly, tasting meat while cooking was found to be a significant risk factor for toxoplasmosis in two studies (Cook et al., 2000; Kapperud et al., 1996), whereas the tasting of condiments in general was not significant (Carellos et al., 2014). Finally, as regards the consumption of vegetables, acquisition of toxoplasmosis was significantly associated with eating raw vegetables or unpeeled fruit (Kapperud et al., 1996) and with eating raw vegetables away from home (Baril et al., 1999). However, other similar risk factors failed to be significantly associated with *T.gondii* infection in four studies (Baril et al., 1999; Carellos et al., 2014; Chiang et al., 2014; Kapperud et al., 1996).

	Baril	Bobić		Carellos	Chiang	Cook	Jones		Kapprud	Lopez-Castillo	Stray-Pedersen
	1999	2007	2010	2014	2014	2000	2006	2009	1996	2005	1980
SELECTION											
1) Independent case definition	■	■	■	■	■	■	■	■	■	■	■
2) Representativeness of the cases	■	■	■	■	■	■	■	■		■	■
3) Selection of controls	■	■	■			■	■	■	■	■	■
4) Definition of controls (no history of disease)	■	■	■		■	■	■	■	■	■	■
COMPARABILITY											
1) Comparability of cases and controls											
a) study controls for age*		■	■		■	■			■	■	■
b) study controls for residency*	■				■	■		■	■	■	
EXPOSURE											
1)Blinded ascertainment of exposure											
2) Same method of ascertainment for cases and controls	■	■	■			■	■	■	■	■	■
3) Same non-Response rate											
ADDITIONAL ITEMS FOR QUALITY EVALUATION											
Is the exposure window specified?	■			■		■	■	■	■	■	
Are selected case acutes?	■		■	■	■	■		■	■	■	■
Quality SCORE	8	6	7	4	6	9	8	8	8	9	8

Table 2 Ottawa-Newcastle checklist details for each relevant study with items added for quality assessment purpose

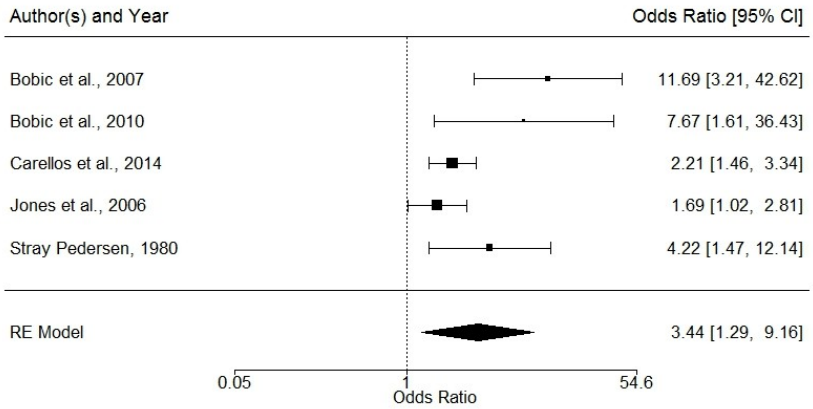

Fig 4 Forest plot of studies investigating the consumption of raw/undercooked meat as a risk factor for *T. gondii* infection in humans

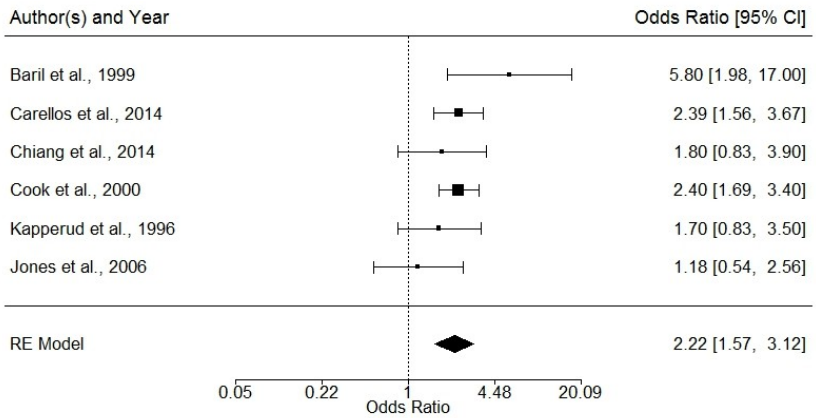

Fig 5 Forest plot of studies investigating the consumption of raw/undercooked beef as a risk factor for *T. gondii* infection in humans

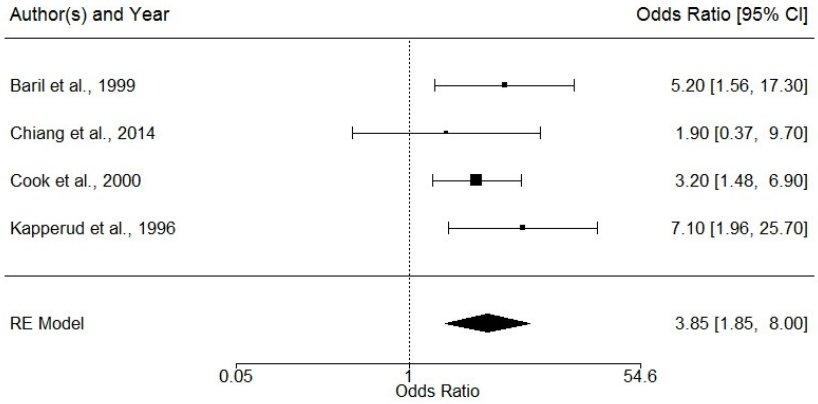

Fig 6 Forest plot of studies investigating the consumption of raw/undercooked sheep meat as a risk factor for *T. gondii* infection in humans

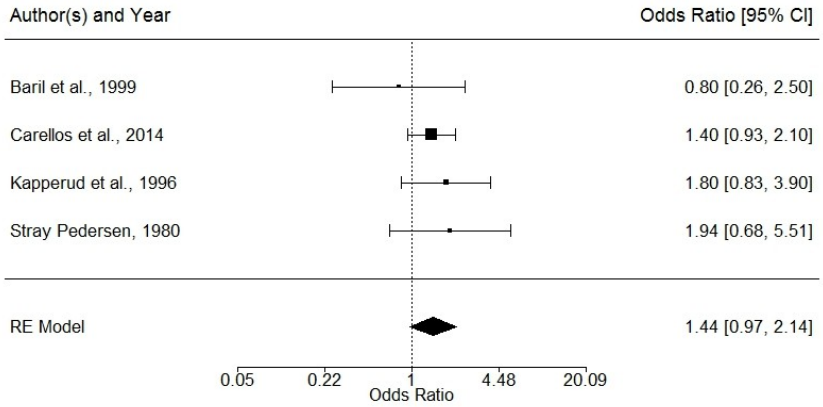

Fig 7 Forest plot of studies investigating the consumption of raw/undercooked pork as a risk factor for *T. gondii* infection in humans

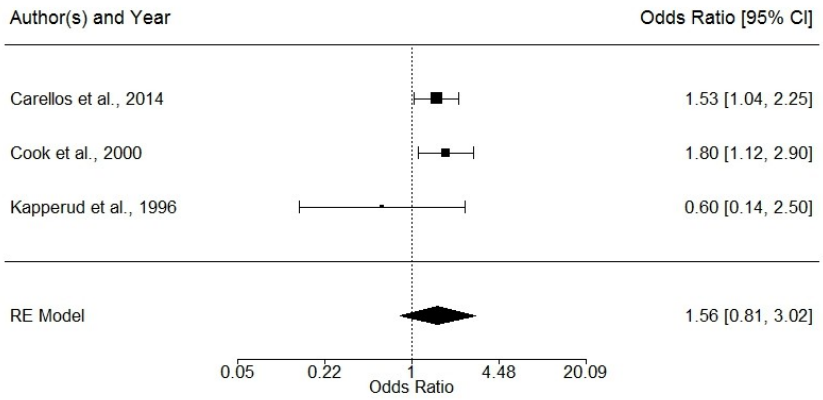

Fig 8 Forest plot of studies investigating the consumption of unpasteurized milk as a risk factor for *T. gondii* infection in humans

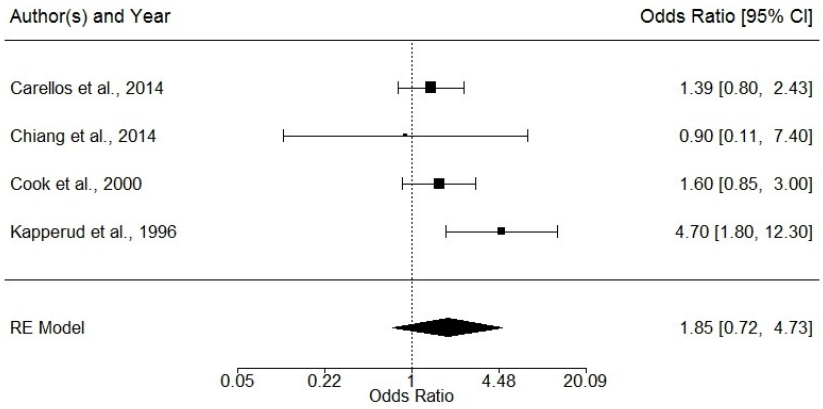

Fig 9 Forest plot of studies investigating the consumption of raw/undercooked eggs as a risk factor for *T. gondii* infection in humans

Food consumed	K	OR (95%CI)	OR Prediction interval (95%)	T^2 (95%CI)	I^2	PB
Raw meat	5	3.44 (1.29-9.16)	0.47-25.20	0.39 (0.00-5.20)	73%	Yes, 2.27 (0.98-5.24)
Raw beef	6	2.22 (1.57–3.12)	-	0.00 (0.00–1.50)	0%	Yes, 2.35 (1.89-2.91)
Raw pork	4	1.85 (0.72–4.73)	0.40–8.46	0.14 (0.00–6.36)	45%	Yes, 1.94 (1.13-3.33)
Raw sheep	4	3.85 (1.85–8.00)	-	0.00 (0.00–4.08)	0%	No
Raw milk	3	1.56 (0.81–3.02)	-	0.00 (0.00–13.52)	0%	NC
Raw eggs	4	1.44 (0.97 – 2.14)	-	0.00 (0.00 – 1.93)	0%	No

Table 3 results of meta-analyses carried out on different risk factors associated with food-related acquisition of acute *T. gondii* infection K= number of studies, PB=Publication Bias, NC=Not Calculated

Discussion

Summary of evidence

The consumption of raw/undercooked beef or sheep meat is significantly associated with acute *T.gondii* infection. The recognition of sheep meat as an important risk factor for toxoplasmosis is not surprising, since sheep meat has been shown to have, on average, the highest prevalence among the commonly eaten livestock species (Belluco et al., 2016). The association of raw/undercooked beef consumption with toxoplasmosis is more interesting and holds major implications. The role of beef in toxoplasma transmission has not always been acknowledged. Despite epidemiological evidence, now included in our meta-analysis, showing that the consumption of raw/undercooked beef is a risk factor for human toxoplasmosis (Baril et al., 1996; Cook et al., 2000), and that the seroprevalence in bovines can be as high as 92% (Tenter et al., 2000), scepticism exists about the role of this animal species. Experimental studies showed that cattle is a poor host for *T. gondii* (Dubey and Jones, 2008) and in the US, beef has been judged as an unlikely source of *T. gondii* infection for humans (Dubey et al., 2005). Some authors support the opinion that conclusive evidence able to correlate toxoplasmosis with the ingestion of naturally infected beef is lacking (Dubey, 1986; Kijlstra and Jongert, 2008). An outbreak has been reported following consumption of raw beef (Kean et al., 1969), but the evidence was judged to be uncertain (Dubey and Jones, 2008). Our result shows that not only is the overall OR significant, but also that heterogeneity is null and that the effect size of all the six individual studies included in the model lie in the same direction. The importance of applying the meta-analytical technique in this case is clear, as using the vote counting technique would have produced inconsistent evidence, whereas looking at individual effect sizes and at their aggregation gives a clear picture. This result is supported by a risk assessment model carried out in the Netherlands where beef, even though it had very low prevalence levels, proved to be the major source of human cases due to consumption habits (Opsteegh et al., 2011).

As regards the general category “raw/undercooked meat” our result is significant but with a high heterogeneity and a prediction interval which also included non-significant values. However, the high heterogeneity is a logical consequence of the width of this category, with the potential for inclusion of different kinds of meat and meat consumption patterns according to the population studied.

Another interesting result occurred for the consumption of raw/undercooked pork; this did not prove to be a significant risk factor in the current study. This result is in contrast with published literature, as swine are recognized as an important source of *T. gondii (Dubey and Jones, 2008; Kijlstra and Jongert, 2008; Tenter et al., 2000)*. However, the inability of the present meta-analysis to find a significant result could be due to several factors. Firstly, the final estimate of the OR had wide confidence intervals and noticeable heterogeneity (44%). Secondly, the low number of included studies (4) might not be enough to uncover small effects. This consideration is supported by the fact that the effect size of three out of four studies lie to the right of the plot, showing a positive but not significant association. Thirdly, the potential for missing studies, as disclosed by Trim and Fill, suggests that the real OR could be significant.

Finally, the categorization of pork meat in the primary studies is a concern, as it was not always clear if cured meat was included or excluded, nor how the meat was categorised. In our opinion, the consumption of undercooked pork could often be an accidental event due to improper cooking, but consumers may not be aware of the cooking status of pork they cook and/or eat.

As regards milk and eggs, no statistically significant evidence of association with toxoplasmosis was found. The literature contains only sporadic evidence of *T. gondii* isolation from eggs (Pande et al., 1961).

Milk could be theoretically a source for *T. gondii* tachyzoites, the infective *T. gondii* stage responsible for mother-to-foetus transmission. However, their low resistance to environmental stresses makes tachyzoites unlikely to survive the acidic conditions of the stomach during digestion. The available evidence about the potential for *T. gondii* transmission, highlighting the potential for goat milk to act as a transmission vehicle, has been discussed elsewhere (Dubey and Jones, 2008; Tenter et al., 2000). Significant prevalence rates were found in a study conducted in Iran (Dehkordi et al., 2013) but results have been debated (Boughattas, 2015a; Dubey and Jones, 2014), and a demand for analysis repetition has been invoked to produce more conclusive evidence. A more detailed discussion about milk and *T. gondii* transmission can be found elsewhere (Boughattas, 2015b).

The results from the studies we were unable to include in our meta-analysis (Jones et al., 2009; Lopez-Castillo et al., 2005) agree with what we observed and these data, if included, would have strengthened our outcomes. The only difference between the results of our meta-analysis and other studies concerns milk. However, in Jones et al. (2009), goat milk only was considered, whereas in our current systematic review, milk was included as a general category, and was not species-specific. Finally, we were unable to include some risk factors in the meta-analysis but which are worthy of more investigation. These are the habit of tasting raw meat while cooking and the consumption of vegetables, as these factors were found to be significant risks for toxoplasmosis in some studies.

Limitations

Our systematic review has some limitations that have been taken into account during the analysis and discussion stages. Firstly, a limitation could be linked to the search strategy, as it lacked complex search strings. Although we made every attempt to find additional evidence for case-control studies not included in the initial search strategy, all such attempts failed. This was despite our recourse to forward and backward reference searches of both the included studies and of relevant reviews. A second limitation is due to our not searching in the grey literature, and this could account for publication bias due to the file drawer effect. However, the studies we finally included were never based on the evaluation of a single exposure and thus, it is unlikely that a non-significant result for a relevant risk factor could have influenced the publication success of any of the 11 primary studies, despite the potential for publication bias which we observed in three meta-analyses. The included studies were of varied quality, and the main risk of bias at individual study level was linked to exposure assessment for two main reasons: the absence of blinding and the recall bias. As regards our meta-analysis, it is

noteworthy that the number of studies for each risk factor was limited. In spite of that, the major results, concerning the link of toxoplasmosis with consumption of raw/undercooked beef or sheep meat, were in agreement among individual studies, as discussed before. On the other hand, the low number of studies could have impaired the ability to disclose other significant relationships and further studies are warranted in this direction. Another strategy to increase the number of studies could be to include cross-sectional designs. In this case, the amount of evidence would greatly increase, as the results of our mapping exercise showed. This is an interesting solution that should, however, take into account the different effect sizes and also the limitation of cross-sectional studies in disclosing causality.

Conclusions

Consumption of raw or undercooked beef or sheep meat is an important source of *T. gondii* transmission to humans as shown by epidemiological studies. It is important to take this risk into account, particularly when counselling at-risk individuals, due to the severe effects of toxoplasmosis in particular circumstances. In general, proper cooking is needed for meat of all species. Moreover, even for healthy individuals, caution must be suggested for the consumption of undercooked meat due to the unknown effect of chronically encysted *T. gondii* bradyzoites. Furthermore the role of improper cooking, tasting while cooking and/or consumption of cured and/or dried products deserves to be elucidated in future studies on the epidemiology of *T. gondii* in humans.

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Appendix

	Baril	Bobić		Carellos	Chiang	Cook	Jones		Kapperud	Lopez-Castillo	Stray-Pedersen
	1999	2007	2010	2014	2014	2000	2006	2009	1996	2005	1980
SELECTION											
1) Is the case definition adequate?											
a) yes, with independent validation*	1	1	1	1	1	1	1	1	1	1	1
b) yes (record linkage or self reports)											
c) no description											
2) Representativeness of the cases											
a) consecutive/obviously series of cases*	1	1	1	1	1	1	1	1		1	1
b) potential for selection biases									1		
3) Selection of controls											
a) community controls *	1	1	1			1	1	1	1	1	1
b) hospital controls				1	1						
c) no description											
4) Definition of controls											
a) no history of disease (endpoint) *	1	1	1		1	1	1	1	1	1	1
b) no description of source				1							
COMPARABILITY											
1) Comparability of cases and controls											
a) control for age*		1	1		1	1			1	1	1
b) control for residency*	1				1	1		1	1	1	
c) control for sex			1		1						
d) control for gestational age	1								1	1	
e) control for period infection-diagnosis			1			1					
EXPOSURE											
1) Ascertainment of exposure											
a) secure record (eg surgical records)*											
b) interview blind to case/control status*											
c) interview not blind (case/control status)	1		1	1		1			1	1	1
d) written self report/medical record only					1		1	1			
e) no description		1									
2) Same method of ascertainment for cases and controls											
a) yes*	1	1	1			1	1	1	1	1	1
b) no				1	1						
3) Non-Response rate											
a) same rate for both groups *							1				
b) non respondents described	1		1			1			1		
c) rate different and no designation				1	1						
ADDITIONAL ITEMS FOR QUALITY EVALUATION											
Is the exposure window specified?											
a) Yes*	1			1		1	1	1	1	1	1
b) No		1	1		1						
Are selected case acutes?											
a) Yes*	1		1	1	1	1	1	1	1	1	1
b) No											
Quality SCORE	8	6	7	4	6	9	8	8	8	9	8

Table 4 Details of the Quality appraisal process for each included study *item that assign a score point according to Newcastle-Ottawa scale

Authors	Ca	Co	Category	Risk factor	OR	LL	UL
Baril et al. 1999	80	80	Dairy products	Eating unpasteurised cheese	0.6	0.3	1.2
Cook et al. 2000	252	852	Dry/cured product	Eating dry or cured meat < 1/week	1.2	0.8	1.7
Cook et al. 2000	252	852	Dry/cured product	Eating dry or cured meat > 1/week	**1.8**	**1.2**	**2.8**
Cook et al. 2000	252	852	Dry/cured product	Eating salami < 1/week	1	0.7	1.6
Cook et al. 2000	252	852	Dry/cured product	Eating salami > 1/week	**1.6**	**1.1**	**2.4**
Stray Pedersen 1980	27	85	Dry/cured product	Frequently eating cured meat	0.6		
Baril et al. 1999	80	80	Eating habits	Eating away from home	0.3	.003	1.3
Carellos et al. 2014	175	278	Eating habits	Eating away from home pregnancy	0.68	0.38	1.24
Cook et al. 2000	252	852	Eating habits	Eating food prepared in microwave	1.3	0.8	2.2
Kapperud et al. 1996	63	128	Eating habits	Preferring beef raw or rare	**3.5**	**1.1**	**10.7**
Kapperud et al. 1996	63	128	Eating habits	Eating meat at a barbecue	1.1	0.4	2.6
Kapperud et al. 1996	63	128	Eating habits	Eating meat prepared in microvawe	2	0.6	6.9
Lopez Castillo 2005	14	34	Eating habits	Eating at restaurant	2.16	0.4	11.6
Baril et al. 1999	80	80	Frozen meat	Never or rarely freezing meat	2	1	4.2
Carellos et al. 2014	175	278	Frozen meat	Eating fresh (not frozen) meat	**3.59**	**2.19**	**5.89**
Cook et al. 2000	252	852	Frozen meat	Eating frozen meat	0.8	0.5	1.2
Jones et al. 2006	131	110	Frozen meat	Eating frozen lamb	**1.89**	**1.01**	**3.52**
Baril et al. 1999	80	80	Hygienic habits	Not washing hands after prep food	3.4	0.75	16.7
Baril et al. 1999	80	80	Hygienic habits	Not washing hands before meals	ND		
Baril et al. 1999	80	80	Hygienic habits	Unwashed knives for vegetables	2.5	0.49	12.5
Carellos et al. 2014	175	278	Hygienic habits	Washing hands after cooking	0.94	0.58	1.54
Carellos et al. 2014	175	278	Hygienic habits	Washing hands before eating	0.63	0.4	0.99
Kapperud et al. 1996	63	128	Hygienic habits	Washing hands infrequently	3	0.9	10.4
Kapperud et al. 1996	63	128	Hygienic habits	Washing knives infrequently	**4.6**	**1.2**	**17.6**
Kapperud et al. 1996	63	128	Hygienic habits	Washing cutting boards infrequently	4	1	15.6
Kapperud et al. 1996	63	128	Hygienic habits	Washing countertops infrequently	3.7	0.9	14.6
Baril et al. 1999	80	80	Meat Consumption	Eating meat daily	1.3	0.7	2.7
Baril et al. 1999	80	80	Meat Consumption	Eating beef	ND		
Baril et al. 1999	80	80	Meat Consumption	Eating lamb	1.3	0.4	3.6
Baril et al. 1999	80	80	Meat Consumption	Eating pork	0.7	0.2	2.1
Baril et al. 1999	80	80	Meat Consumption	Eating horse meat	2	0.5	9.1
Baril et al. 1999	80	80	Meat Consumption	Eating chicken	ND		
Baril et al. 1999	80	80	Meat Consumption	Eating rabbit meat	0.7	0.3	1.6
Baril et al. 1999	80	80	Meat Consumption	Eating duck	0.6	0.2	1.6
Baril et al. 1999	80	80	Meat Consumption	Eating game	1.8	0.4	8.2
Cook et al. 2000	252	852	Meat Consumption	Eating cooked meat < 1/week	0.8	0.3	2.1
Cook et al. 2000	252	852	Meat Consumption	Eating cooked meat > 1/week	0.8	0.3	1.9
Kapperud et al. 1996	63	128	Meat Consumption	Frequency of meat consumption	1.5	0.7	3
Baril et al. 1999	80	80	Raw/UC meat	Eating UC meat outside home	**8.3**	**2.5**	**43.1**
Carellos et al. 2014	175	278	Raw/UC meat	Eating raw or UC meat (<1 a week)	1.31	0.58	2.97
Carellos et al. 2014	175	278	Raw/UC meat	Eating raw or undercooked chicken	ND		
Cook et al. 2000	252	852	Raw/UC meat	Eating raw sausage < 1/week	1.2	0.7	2
Cook et al. 2000	252	852	Raw/UC meat	Eating raw sausage > 1/week	**3.2**	**1.2**	**9**
Cook et al. 2000	252	852	Raw/UC meat	Eating other raw/undercooked meat	**3.9**	**1.6**	**9.5**
Kapperud et al. 1996	63	128	Raw/UC meat	Eating raw or UC minced meat	**3.2**	**1.5**	**6.6**
Kapperud et al. 1996	63	128	Raw/UC meat	Eating raw or undercooked poultry	**8.9**	**1.9**	**41.5**
Kapperud et al. 1996	63	128	Raw/UC meat	Eating tartare meat	**4.6**	**1.4**	**15.1**
Kapperud et al. 1996	63	128	Raw/UC meat	Eating roast beef	0.7	0.4	1.2
Kapperud et al. 1996	63	128	Raw/UC meat	Eating gravet meat	8	0.9	71.6
Jones et al. 2009	89	79	Raw/UC meat	Eat raw ground beef	0.78		
Jones et al. 2009	88	78	Raw/UC meat	Eat raw ground chicken	ND		
Lopez Castillo 2005	14	34	Raw/UC meat	Eating raw/undercooked meat	ND		
Carellos et al. 2014.	175	278	Raw/UC fish	Eating raw or undercooked fish	ND		
Chiang et al. 2014	30	224	Raw/UC fish	Eating raw fish	1.4	0.6	3.5
Chiang et al. 2014	30	224	Raw/UC fish	Eating raw oysters	1.5	0.6	3.4
Chiang et al. 2014	30	224	Raw/UC fish	Eating raw clams	**3.6**	**1.4**	**9.3**

Table 5 Odds Ratio or eligible risk factors not included in meta-analysis. Ca=case; Co= Controls; UC=Undercooked

Authors	Ca	Co	Category	Risk factor	OR	LL	UL
Carellos et al. 2014	175	278	Tasting	Tasting condiments while cooking	1.05	0.67	1.65
Cook et al. 2000	252	852	Tasting	Tasting meat when cooking < 1/week	**2.5**	**1.5**	**3.4**
Cook et al. 2000	252	852	Tasting	Tasting meat when cooking > 1/week	**4.7**	**2.1**	**10.9**
Kapperud et al. 1996	63	128	Tasting	Tasting raw meat while preparing food	**5.6**	**2.4**	**13.1**
Baril et al. 1999	80	80	Vegetables	Raw vegetables prepared at home	ND		
Baril et al. 1999	80	80	Vegetables	Frequently eating raw vegetables outside the home	2.8	1.4	5.9
Baril et al. 1999	80	80	Vegetables	Eating garden produce	2	0.5	9.1
Carellos et al. 2014	175	278	Vegetables	Eating raw vegetables outside the home	1.1	0.74	1.64
Chiang et al. 2014	30	224	Vegetables	Eating uncooked vegetables	1.5	0.5	3.9
Kapperud et al. 1996	63	128	Vegetables	Eating unwashed raw vegetables	5.7	2	15.7
Kapperud et al. 1996	63	128	Vegetables	Eating unwashed unpeeled fruit	2.4	1.2	4.8
Kapperud et al. 1996	63	128	Vegetables	Eating unwashed raw vegetables/unpeeled fruit	2.3	1.2	4.5
Kapperud et al. 1996	63	128	Vegetables	Eating unwashed berries	1.6	0.7	3.4

Table 5 (continue) Odds Ratio or eligible risk factors not included in meta-analysis. Ca=case; Co= Controls; UC=Undercooked

Survey

Survey on meat preparation habits (cooking and freezing) carried out to inform the Quantitative Microbial risk Assessment model.

Data on meat consumption habits of the Italian population were acquired through an *ad hoc* designed survey. Briefly, a questionnaire comprising 23 multiple-choice questions, nine about socio-demographic features and 14 about food manipulation habits, was created.

The questionnaire was first validated on a subsample of 25 individuals and modified according to feedback received. The survey then investigated the frequency of raw/undercooked meat consumption and the frequency of consumption of frozen meat for different meat categories (beef and veal, pork, pork sausages, horse meat, ovine meat). Raw/undercooked meat was defined with photographs showing meat which had undergone various degrees of cooking. A temperature of 60° was considered effective for parasite inactivation (Dubey, 1990) and thus, qualitative data was obtained to describe the impact of cooking on *T. gondii* inactivation. Freezing was considered as an effective strategy for parasite inactivation at temperature <8 °C for one hour.

The questionnaire was built through the online free tool Google Forms and disseminated through social networks and a mailing list of consumers, available at the Istituto Zooprofilattico Sperimentale delle Venezie, for survey purposes.

Questions

Gender

Male

Female

Date of birth

Italian region of residency

Which is your level of education?

Primary school

Junior High school

High school

Undergraduate degree

Post graduate degree

Do you live alone?

Yes/No

If not, who do you live with?

Do you have any sons?

If yes, how many are under 16 years of age?

What is your occupation?

Student

Employed

Unemployed

Retired

Other

Meat categories investigated in the present survey:

-Bovine meat: veal, beef.

-Swine meat: meat from pigs excluding sausages

-Sausages: fresh products with minced pork.

-Horse meat

-Sheep meat

Meat consumption after freezing

Management of meat at home: how many times do you eat frozen meat out of the total times you eat that kind of meat?

	I never eat this kind of meat	Never (0/5)	Rarely (1/5)	Seldom (2/5)	Often (3/5)	Very often (4/5)	Always (5/5)	Don’t know
Bovine meat								
Swine meat								
Sausages								
Horse meat								
Sheep meat								

Meat consumption after cooking

Management of meat at home: how many times do you eat well-done meat out of the total times you eat that kind of meat?
The picture helps to define a well-done piece of meat

WELL-DONE

	I never eat this kind of meat	Never (0/5)	Rarely (1/5)	Seldom (2/5)	Often (3/5)	Very often (4/5)	Always (5/5)	Don’t know
Bovine meat								
Swine meat								
Sausages								
Horse meat								
Sheep meat								

How many times do you eat raw bovine meat which has not been previously frozen?

(How many portions are raw out of a total of 10 portions you eat?)

I do not eat bovine meat

I do not eat raw bovine meat

Only in exceptional cases (1/10)

Rarely (1 or 2 times every 10)

Sometimes (3 or 4 times every 10)

Often (5, 6, 7 times every 10)

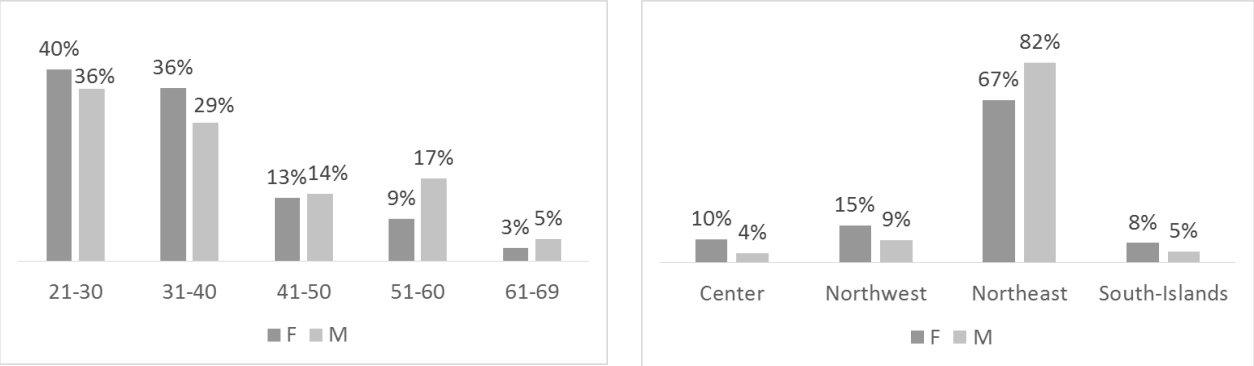
Very often (8 or 9 times every 10)

Always

Survey results

Number of respondents: 313

Description of the investigated population



Age (left panel) and geographical (right panel) sex based distribution of respondents to the survey on meat consumption habits. Northwest (Piemonte, Lombardia, Liguria), Northeast (Veneto, Friuli Venezia Giulia, Trentino-Alto Adige, Emilia Romagna), Center (Toscana, Marche, Lazio), South and Islands (Campania, Calabria, Puglia, Sicilia)

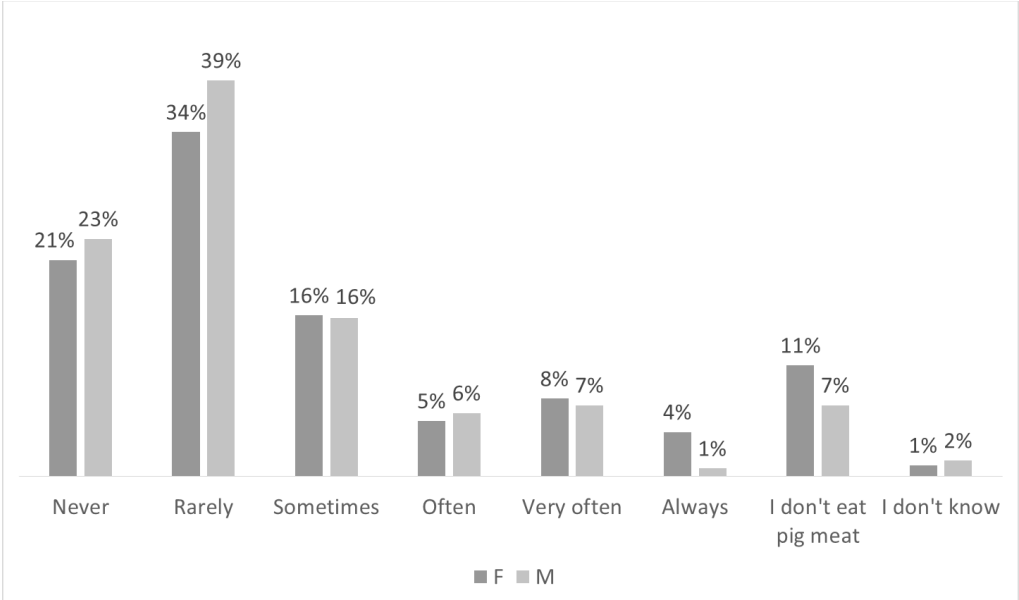
Results of meat preparation habits (cooking and freezing) for different species.

The bar charts describe the frequency of different behaviours (cooking and freezing) on the total number of times a specific meat is consumed, according to the answers obtained trough the web based survey.

Freezing habits

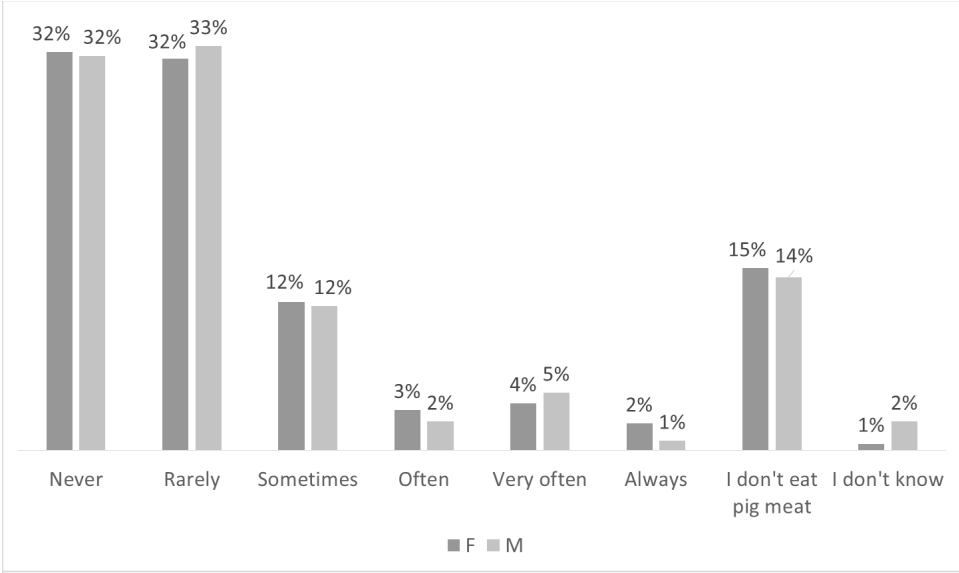
PIGS

Freezing habits



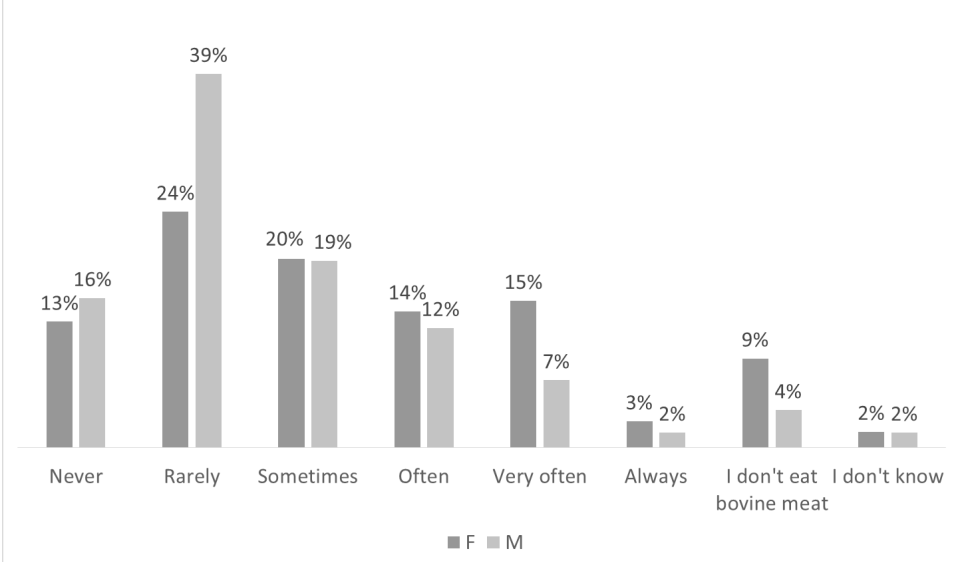
PORK SAUSAGES

Freezing habits



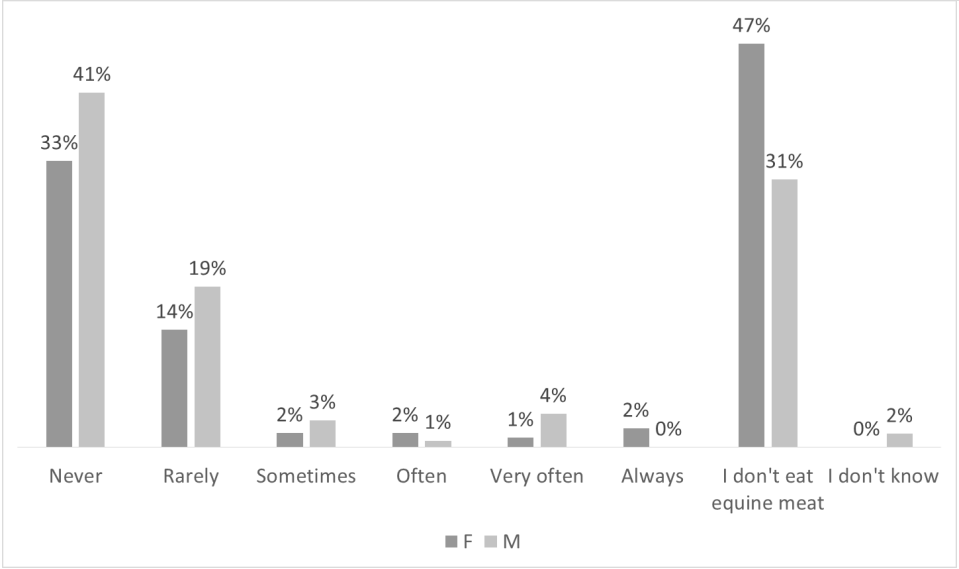
BOVINE MEAT

Freezing habits



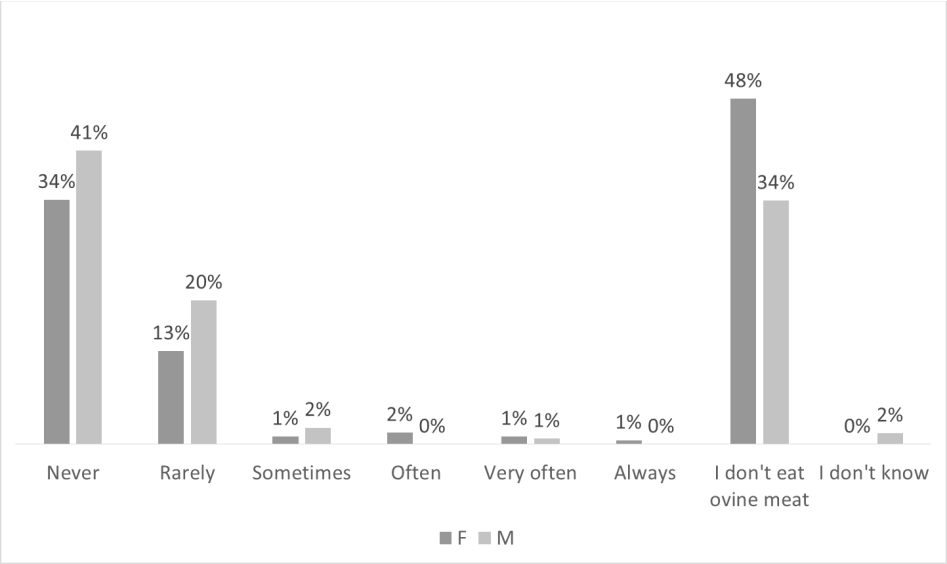
HORSE MEAT

Freezing habits



SHEEP MEAT

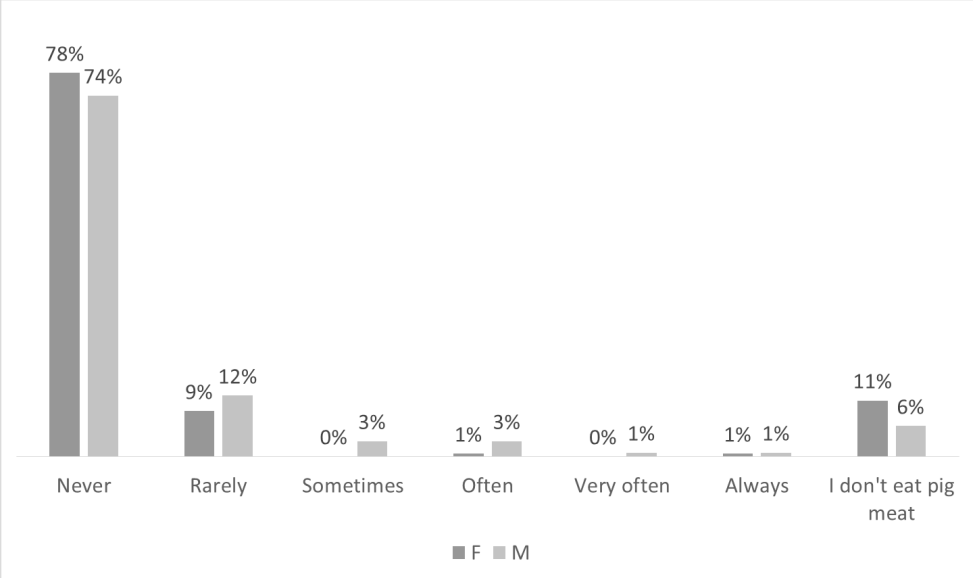
Freezing habits



Cooking habits

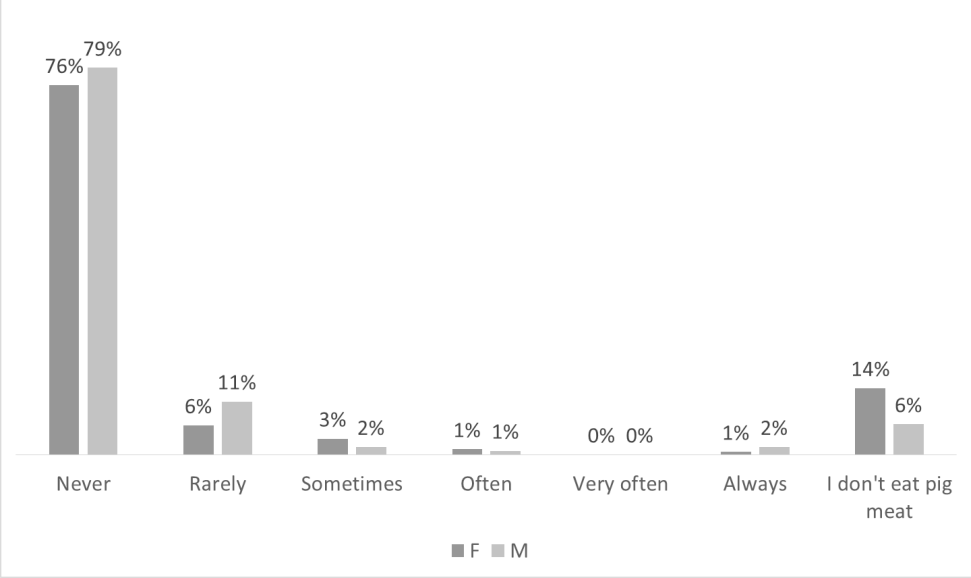
PIGS

Cooking habits



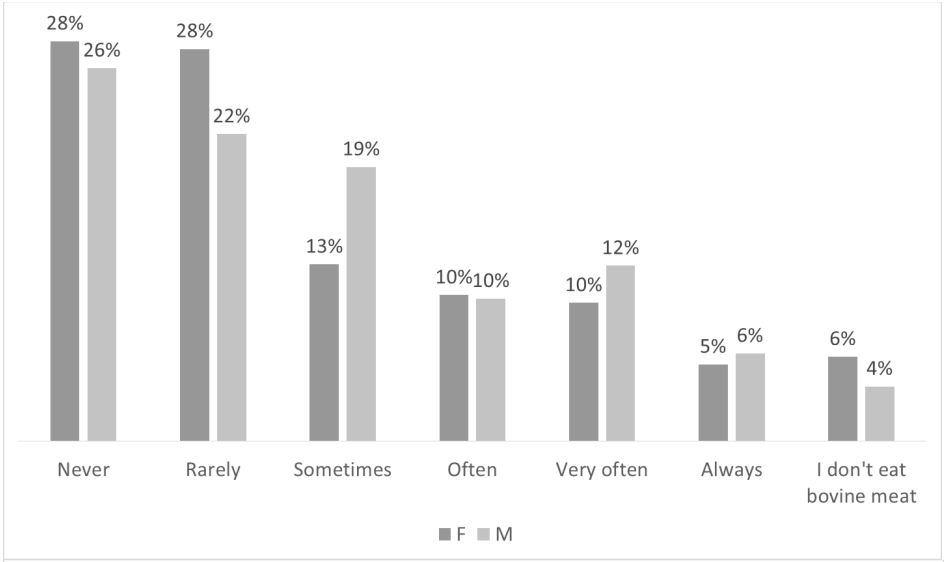
PORK SAUSAGES

Cooking habits



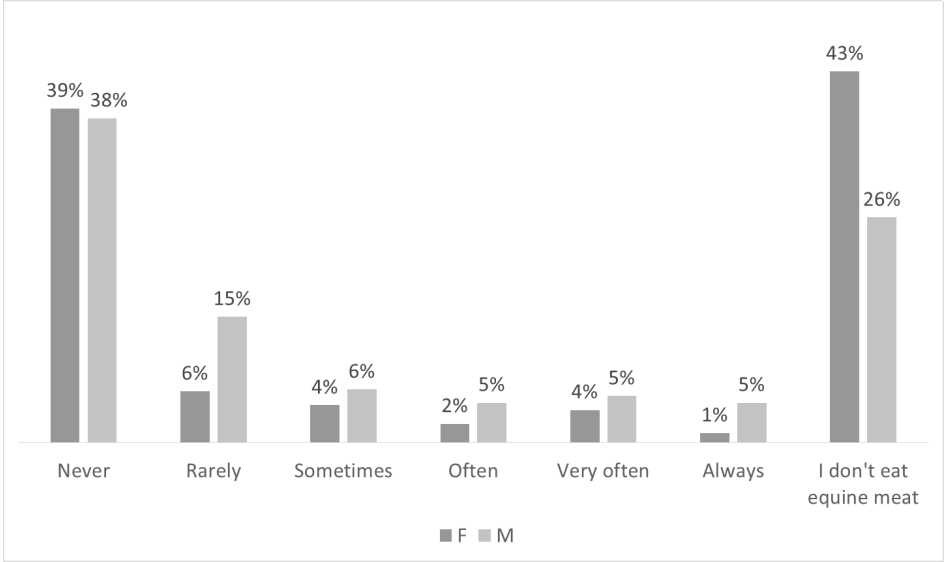
BOVINE MEAT

Cooking habits



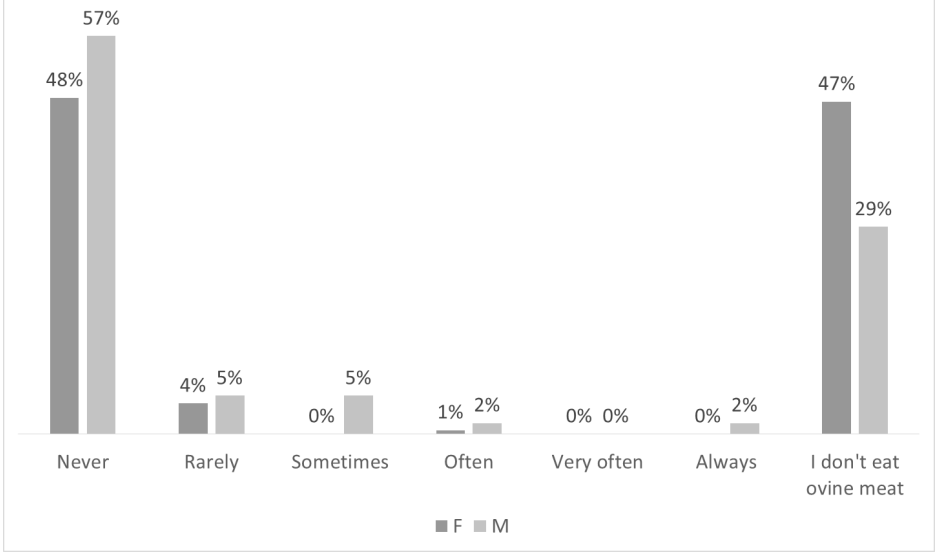
HORSE MEAT

Cooking habits

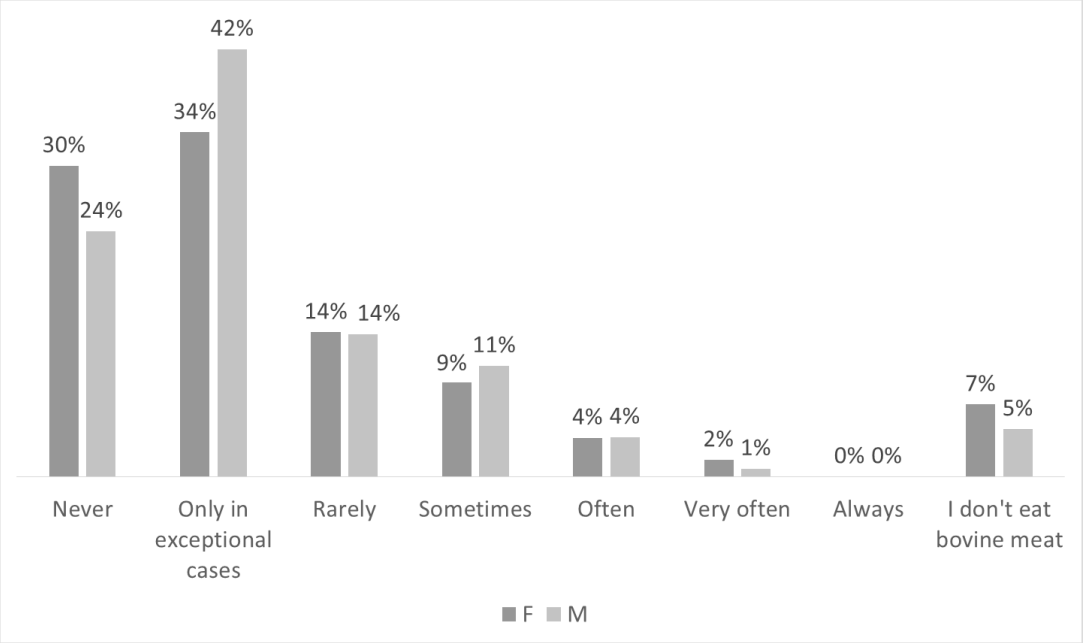


SHEEP MEAT

Cooking habits



Consumption of raw bovine meat



Manuscript 4

Bovine meat versus pork in *Toxoplasma gondii* transmission: a quantitative risk assessment model.

Simone Belluco[1,2,#,*], Ilaria Patuzzi[1,3,#], Antonia Ricci[1]

[#]These authors contributed equally to the present work.

[1]*Department of Food Safety, Istituto Zooprofilattico Sperimentale delle Venezie, Legnaro, Italy.*

[2]*Department of Animal Medicine, Production and Health, Università di Padova, Padova, Italy.*

[3]*Department of Information Engineering, Università di Padova, Padova, Italy.*

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Bovine meat versus pork in *Toxoplasma gondii* transmission: a quantitative risk assessment model

Simone Belluco[1,2,#,*], Ilaria Patuzzi[1,3,#], Antonia Ricci[1]

[#]These authors contributed equally to the present work.

[1]*Department of Food Safety, Istituto Zooprofilattico Sperimentale delle Venezie, Legnaro, Italy.*

[2]*Department of Animal Medicine, Production and Health, Università di Padova, Padova, Italy.*

[3]*Department of Information Engineering, Università di Padova, Padova, Italy.*

[*]Corresponding author

Simone Belluco

Istituto Zooprofilattico Sperimentale delle Venezie

Viale dell'Università

35020 Legnaro (PD)

sbelluco@izsvenezie.it

Abstract

Toxoplasma gondii is a widespread zoonotic parasite with a high seroprevalence among the human population and the ability to infect almost all warm blooded animals. Humans can acquire Toxoplasma infection from different transmission routes and food plays a critical role as it accounts for about half of the total number of infections. Within the food category, meats are of the utmost importance, as meat can contain bradyzoites inside tissue cysts, which can potentially cause infection after ingestion if parasites are not inactivated through freezing or cooking before consumption. In Italy, the most commonly consumed meat animal species are bovines and pigs. However, *T. gondii* prevalences and meat consumption habits for meat of these animal species are very different. There is debate within the scientific community concerning which of these meat species is the main source of meat-derived human Toxoplasma infection. The aim of this work was to build a quantitative risk assessment model to estimate the yearly probability of acquiring toxoplasmosis infection due to consumption of bovine meat and pork in Italy, taking into account different food habits. The model was fitted with the data obtained from relevant literature describing: bradyzoite concentrations, portion size, dose-response curves, prevalence of *T. gondii* in bovines and pigs, meat consumption and meat preparation habits. The model estimated the risk of acquiring *T. gondii* infection from bovine meat consumption was 0.034% and from pork consumption, it was 0.019%. Alternative handling scenarios were considered.

Keywords: Beef, veal, pork, risk assessment, toxoplasmosis, meat.

1. Introduction

T. gondii is one of the most successful parasites at the worldwide level (Flegr, 2013). Oral infectivity has been demonstrated to be a key adaptation, giving a selective advantage over closely related parasites and ancestral strains (Su, 2003). The ability of *T. gondii* to circumvent sexual propagation and to infect hosts directly through ingestion of tissue cysts explains the importance of meat as a risk factor for foodborne *T. gondii* infection, as first observed in 1965 (Desmonts et al., 1965). This particular horizontal route of transmission accounts for almost 60% of *T. gondii* infections (WHO, 2014).

T. gondii infection in immunocompetent individuals is considered of minor importance due to the absence or mildness of associated symptoms, although the long-term effect of tissue cysts, particularly when located in the brain region, are poorly understood. On the other hand, infection in pregnant women can be a severe event causing death of the foetus or important congenital malformations (Montoya and Liesenfeld, 2004). The limited proportion of individuals at risk of disease among the human population is probably the reason why control programs along the meat chain are lacking in most countries and prevention relies only on the education of people in at-risk categories and, in some countries, on serological monitoring during pregnancy. Pregnant women are counselled about hygienic precautions in the case of cat ownership and about the avoidance of high-risk foods. However, it has been reported that not all medical professionals are aware of the most important risk factors for *T. gondii* infection (Kravetz and Federman, 2005).

Moreover, the role of meats of different animal origins is debated among experts. Sheep meat is historically recognized as a highly contaminated meat source (Desmonts et al., 1965), together with goat meat and pork, whereas the role of meat from bovines is not always acknowledged (Dubey and Jones, 2008; Kijlstra and Jongert, 2008).

Due to the null prevalence rate determined in the US after a nation-wide study involving more than two thousand beef samples, the consumption of raw/undercooked beef was not considered as a risk factor for Toxoplasma infection (Dubey et al., 2005). In contrast, starting from an estimated 2% *T. gondii* prevalence in cattle, a QMRA model developed in the Netherlands indicated beef was the most important meatborne source of *T. gondii* infection (Opsteegh et al., 2011). Epidemiological evidence is not always consistent, but consumption of raw/undercooked beef has been recognized as a significant risk factor (Baril et al., 1999; Cook et al., 2000).

The ingestion of raw/undercooked pork is generally emphasized as an important risk factor, due to the prevalence rates in pigs being higher than in bovines (Belluco et al., 2016) and to the isolation of infective parasites from pork meat (Dubey et al., 2005). Bovine meat and pork are the most commonly consumed meats in Italy (Leclercq et al., 2009), but evidence about their importance as risk factors for human Toxoplasma infection in Italy are lacking.

The aim of this study was to quantify the yearly probability that an Italian consumer would be infected with *T. gondii* due to the consumption of fresh or previously frozen meat from pigs and cattle. Quantification took into account parasite prevalence and concentration in meat, consumption data, consumption habits and the dose-response relationship.

2. Materials and methods

2.1 Model building

This model was inspired by the structure of the Quantitative Microbial Risk Assessment model for meatborne *T. gondii* infection developed in the Netherlands (Opsteegh et al., 2011) and modified according to available data. The model design with input parameters and intermediate outputs is depicted in Figure 1.

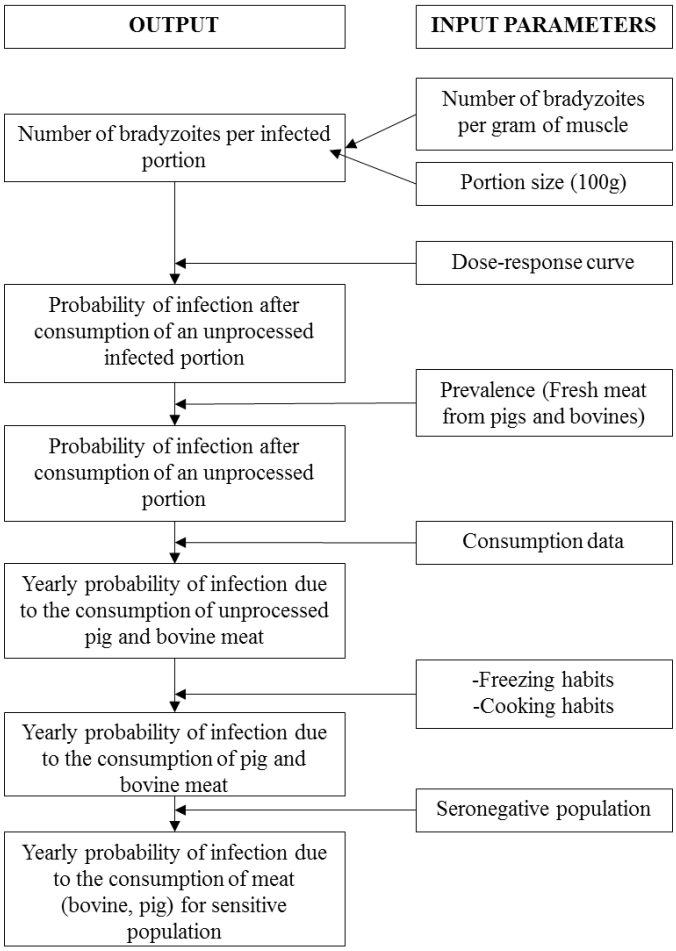

Figure 4: structure of the *T. gondii* risk assessment model presented in this work

2.2 Data sources and calculations

2.2.1 Selection of meat species and types

Meats were selected for inclusion in the model according to data availability. Commonly consumed livestock in Italy comprises cattle and calf, pigs and poultry, whereas meat from sheep, goats and horses follows more local, smaller consumption patterns. Consumption data were obtained from the 2005-06 Italian National Food Consumption Survey INRAN-SCAI (Leclercq et al., 2009). Poultry were not included in the model because of the low expected prevalence and because poultry meat is generally consumed well-cooked. Horse, goat and sheep meat were not included because their consumption data were available at an aggregate level in the “other meat” category. The final model included consumption data related to fresh and previously frozen unprocessed meat from cattle and pigs, and excluded all data related to

offal and to meat products (e.g. salami, ham, bacon-style meats, fermented sausages, dried meat and preserved meat).

2.2.2 Number of bradyzoites per unprocessed meat portion

Few studies are available in the literature estimating the concentration of bradyzoites in meat. To our knowledge, data are available for sheep heart (Opsteegh et al., 2010), goat muscle (Juránková et al., 2013) and pig muscle (Juránková et al., 2014). As our model included only pigs and cattle, we chose to use the bradyzoites concentrations as estimated for pigs, for both meat categories. For our purposes, only the subgroup of data referring to muscle tissues were retained. Selected data were obtained from different tissues (left forelimb, right forelimb, left hindlimb, right hindlimb and dorsal muscle) isolated from five pigs, experimentally infected with *T. gondii (Juránková et al., 2014)*. The selected data were used to fit a distribution using *@Risk 6 Software*. A Weibull distribution (Weibull (0.85115; 54.584)) was selected according to the Kolmogorov-Smirnov statistic among a set of possible solutions.

The number of bradyzoites per gram was extrapolated to the portion size to obtain the number of parasites per meat portion. The portion size was 100 g, as suggested by Italian National Guidelines (SINU, 2014).

2.2.3 Dose-response curve

The definition of a dose-response curve for *T. gondii* infection in humans is challenging. However, the Dutch QMRA model (Opsteegh et al., 2011) and also a recent paper defining a dose-response curve for *T. gondii* in humans (Guo et al., 2015b) agreed in using data obtained from an investigation carried out in a mouse model where the infectivity of different *T. gondii* strains was assessed. The difference in the final dose-response curves produced by the two studies is explained by the application of a scaling factor. Opsteegh and colleagues applied the original mouse-based curve, whereas Guo and colleagues defined a scaling factor of 0.003 (beta-Poisson model) to account for differences between humans and mice. Our model (the base model; see below) was fitted using the final curve obtained by Guo through the beta-Poisson model.

2.2.4 Prevalence of T. gondii in pigs and cattle

Prevalence estimates vary across countries and years and are influenced by farming systems and the presence of other risk factors (Belluco et al., 2016; Guo et al., 2015a). To account for potential differences, we chose to use estimates from a recent meta-analysis of *T. gondii* prevalences in common livestock species, selecting the estimates resulting from studies carried out in Europe: 2.2% (CI 95% 0-8.7%) for cattle and 8.7% (CI 95% 2.7-17.2%) for pigs (Belluco et al., 2016).

2.2.5 Meat consumption data

Meat consumption data were obtained from the 2005-06 Italian National Food Consumption Survey INRAN-SCAI. This cross sectional survey was carried out on randomly selected households. The final study sample comprised 3328 respondents (1501 males and 1822 females) of all ages belonging to 1329 households (Leclercq et al., 2009). Mean (SD), median, 95^{th} and 99^{th} percentiles for consumption data (grams/day) were available in detail according to residency (geographical area) and sex (Input data in Appendix A). The survey reported

consumption data both for the total population and for only the actual consumers (respondents who actually consumed the food item). The latter estimate was selected for inclusion in our model. The most appropriate continuous distributions using known quantiles were calculated for bovine meat and pork ("beef and veal not preserved excluding offal" and "pork not preserved, excluding offal" within the "meat, meat products and substitutes category"), and separately for male and female consumers. This analysis was performed in the *R* environment using the *rriskDistributions* package (R Core Team, 2012). The resulting distributions were selected according to the Kolmogorov-Smirnov statistic among a set of possible solutions. The estimated number of meat portions consumed per year was calculated by multiplying values resulting from these distributions (g/day) by 365.25 and dividing them by the recommended portion size (100g) (SINU, 2014).

2.2.6 Meat consumption habits survey

Data on meat consumption habits of the Italian population were acquired through an *ad hoc* designed survey. Briefly, a questionnaire comprising 23 multiple-choice questions, nine about socio-demographic features and 14 about food manipulation habits, was created. The questionnaire was first validated on a subsample of 25 individuals and modified according to feedback received. The survey then investigated the frequency of raw/undercooked meat consumption and the frequency of consumption of frozen meat for different meat categories (beef and veal, pork, pork sausages, horse meat, ovine meat). Raw/undercooked meat was defined with photographs showing meat which had undergone various degrees of cooking. A temperature of 60° was considered effective for parasite inactivation (Dubey et al., 1990) and thus, qualitative data was obtained to describe the impact of cooking on *T. gondii* inactivation. Freezing was considered as an effective strategy for parasite inactivation at temperature <8 °C for one hour.

The questionnaire was built through the online free tool Google Forms and disseminated through social networks and mailing lists of consumers available at the Istituto Zooprofilattico Sperimentale delle Venezie for survey purposes.

A descriptive analysis of data about the frequency of raw/undercooked meat consumption and the frequency of previously frozen meat consumption acquired through the *ad hoc* survey was carried out. Thus, the resulting information was translated into *@Risk* distributions (one for each meat species) to model the set of possible values and corresponding probabilities.

Seroprevalence

Because *T. gondii* infection produces long-life immunity, a seropositive subject is no longer susceptible to the infection. A seroprevalence estimate of 24.4% (Mosti et al., 2013) was used to obtain the probability of new infections in the Italian population. Thus, the final probability of infection was multiplied by a factor of 0.756.

2.3 Yearly probability of infection

The final base model was obtained by integrating the previously described data to obtain an estimate of the distribution of the yearly probabilities of acquiring *T. gondii* infection from both pork and bovine meat consumption. The final distributions were obtained by a Monte Carlo

simulation with 200000 iterations performed with *@Risk 6 Software*. For each iteration, the model extracts a value from the bradyzoite concentration distribution, and from this value, the dose-response equation calculates the probability of infection for an infected meat portion. The resulting probability is then multiplied by the prevalence (0.022 for cattle, 0.087 for pigs). In the following steps, the model extracts a male or female profile from the consumption distribution and values corresponding to number of meat portions consumed per year, freezing habits and cooking habits. The last two input parameters are expressed in terms of frequency of effective cooking and freezing which induce parasite death. The value resulting from the multiplication of these three quantities by the previously calculated factor (i.e. probability x prevalence) is then inserted in a binomial model to calculate, for each iteration, the probability that at least one positive (infection causing) event per year occurs. This probability is finally multiplied by 0.74, which is the proportion of the Italian population susceptible to *Toxoplasma gondii* infection, to produce the yearly probability of infection.

Finally, the yearly probability of infection was multiplied by the total Italian population according to the 2011 census data (ISTAT, 2016) to obtain an estimate of the number of new toxoplasmosis cases according to the model. Two values were selected for study; the total present population and the adjusted population after removal of children (<5 years) and aged people (>65 years).

As congenital toxoplasmosis is the most severe consequence of parasite infection, especially when acquire during the first trimester, the model was modified to obtain an estimate of the probability that a pregnant woman could acquire infection during pregnancy or during the first trimester. To obtain these estimates data inputted in the base model were limited to consumption data reported by women, limited to a period of nine or three months, assuming a seronegativity at the beginning of this period, and to meat preparation habits reported by women aged between 20 and 50 years. Finally, the model also took into account the probability that vertical transmission from mother to foetus occurs. The probability of vertical transmission was 9% in the first trimester, 31% in the second and 59% in the third (Montoya and Liesenfeld, 2004). The resulting probability was multiplied by the number of children born alive in Italy in 2014 (494,550) to obtain the predicted yearly number of vertically transmitted cases.

2.4 Alternative scenarios

Alternative scenarios were considered to account for the uncertainty of some parameters. In particular, the following three modifications were considered to assess their impact on the final estimates:

- Meat consumption and preparation habits were restricted by geographic region to only the northeast of Italy. This was because most survey respondents lived in the Veneto region and a more precise estimate could be obtained with this subset of data.
- The prevalence of *T. gondii* in cattle was lowered to 0.5% as suggested by a previous model (Opsteegh et al., 2011). This accounted for the potential overestimation of prevalence due to the frequent recourse to PCR in primary studies contributing to the prevalence estimate included in our model.

- An unscaled mouse dose-response curve was applied to account for the uncertainty in the calculation of the scaling factor and to obtain results comparable with the Dutch model (Opsteegh et al., 2011).

2.5 Sensitivity analysis

To investigate the elements which most affected the total probability estimate, a sensitivity analysis was performed using *@Risk 6 Software,* separately for pork and bovine meat. A regression analysis was carried out. Sampled input variable values were regressed against output values, leading to a measurement of sensitivity by input variable. With this analysis, regression coefficients were calculated between the output values and each set of sampled input values.

3. Results

3.1 Meat consumption habits survey

A total of 313 responded to the survey about meat consumption habits, 184 (59%) women and 129 (41%) men. The majority of respondents were aged between 20 and 40 years (71%) and none was below 20 years. Due to the snowballing distribution of the survey, a large proportion of respondents were from the northeast part of Italy. Details about sex, age and residency distribution are shown in Figure 2.

The distribution of respondents according to meat consumption and preparation habits is shown in Figures 3 and 4, respectively. Freezing habits are almost comparable between the two meat categories but a noticeable difference exists in the case of cooking habits (Figure 3). About 70% of the respondents consume bovine meat raw or undercooked at least once out of 5 times, whereas in the case of pork, less than 20% of respondents declared this habit. Detailed results of the discrete distributions are reported in Appendix B.

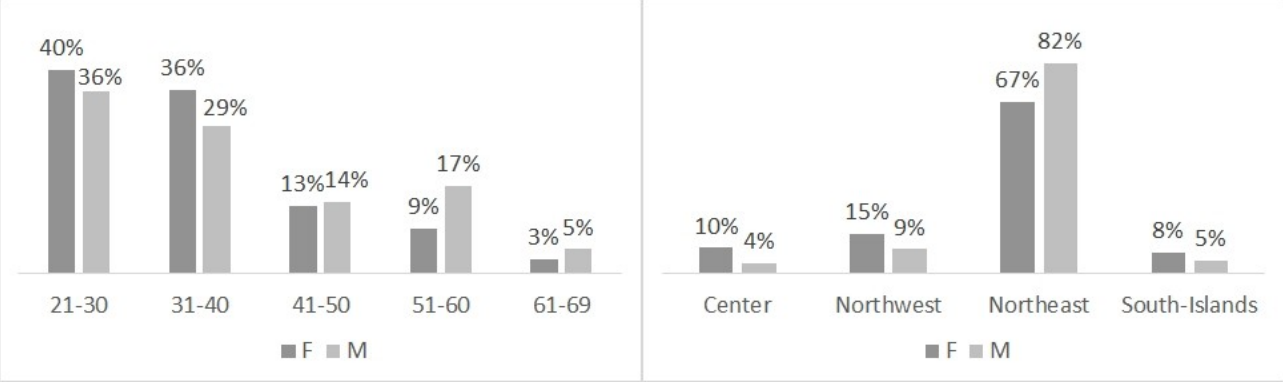

Figure 5: age (left panel) and geographical (right panel) sex based distribution of respondents to the survey on meat consumption habits. Northwest (Piemonte, Lombardia, Liguria), Northeast (Veneto, Friuli Venezia Giulia, Trentino-Alto Adige, Emilia Romagna), Center (Toscana, Marche, Lazio), South and Islands (Campania, Calabria, Puglia, Sicilia)

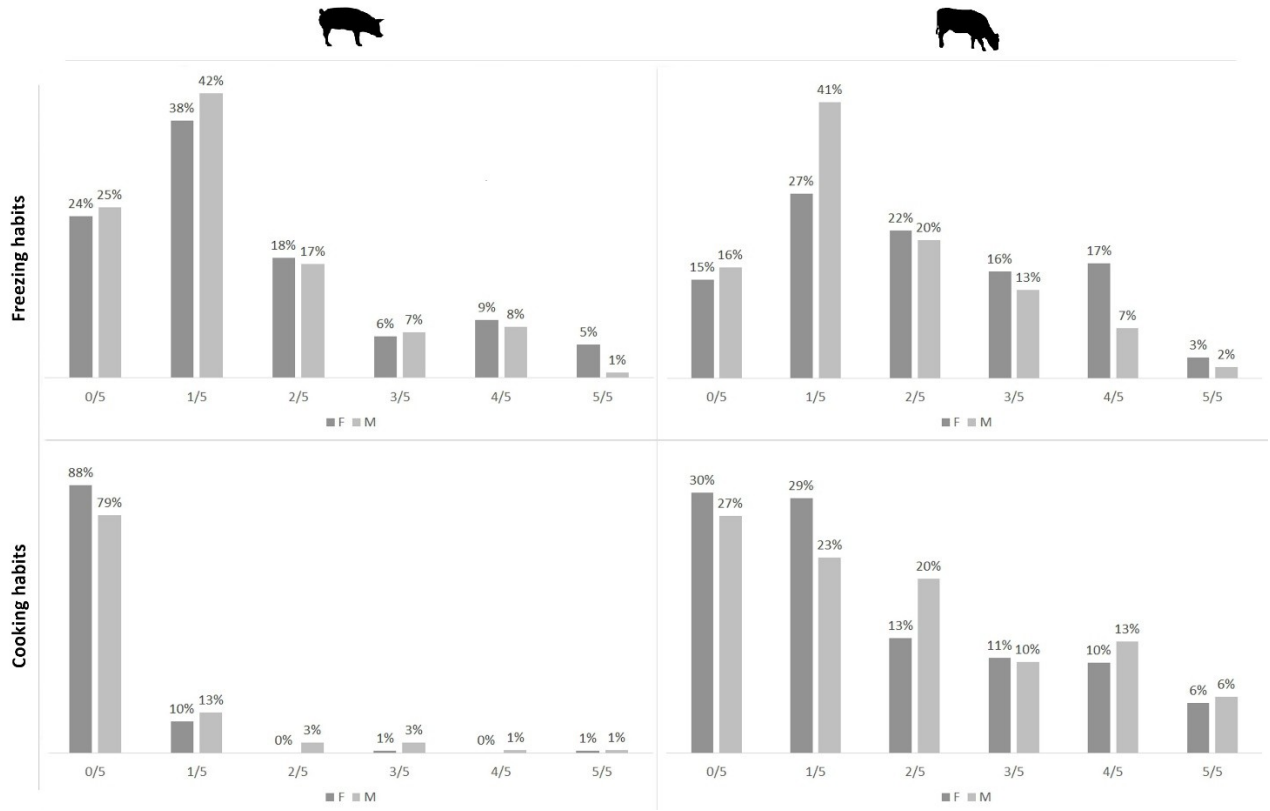

Figure 6: distribution of respondents according to freezing and cooking habits regarding pork (left) and bovine meat (right) consumption.

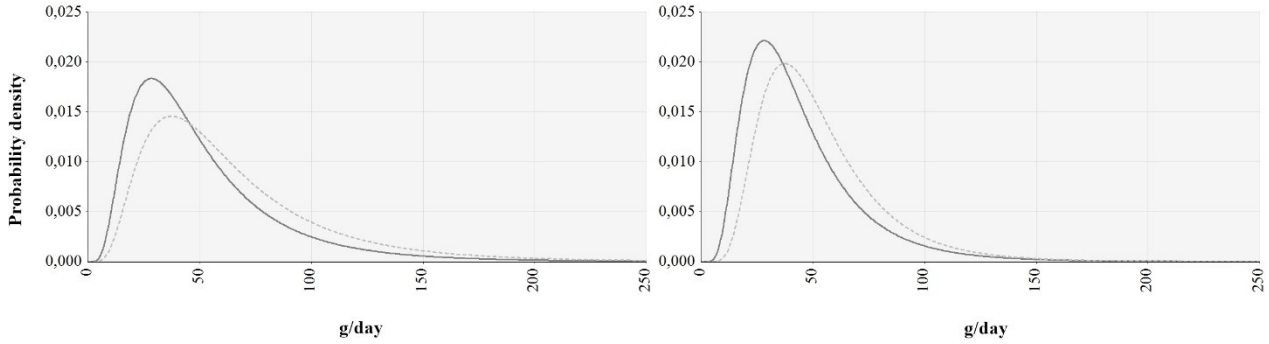

Figure 4: distribution of pork (left panel) and bovine meat (right panel) consumption habits according to sex. Female=full line, Male=dotted line.

3.2 Models

3.2.1 Base model

The base model estimated a mean yearly probability of *T. gondii* infection of 0.019% caused by pork and 0.034% caused by bovine meat. This predicts a mean number of 8,460 and 15,151 infections per year from pork and bovine meat, respectively, among the Italian population between 5 and 65 years of age (Table 1). The contribution from bovine meat consumption to human infections resulted to be 1.8 times higher than contribution form pork consumption. The mean yearly probability of congenital *T. gondii* infection was 0.0218% caused by pork and 0.0718% caused by bovine meat, with the estimated number of newborns with congenital

toxoplasmosis being 10 and 32, respectively, among all newborns in Italy (based on live births in 2014; ISTAT).

Limiting the risk to three months, which were assumed to correspond to the first trimester of pregnancy, resulted in mean yearly probabilities of 0.0072% and 0.0239% for pork and bovine meat, respectively, with the predicted number of cases being 1 and 3, respectively.

Results of the sensitivity analysis for the base model are reported in Figure 5, all other results of sensitivity evaluations are available in Appendix C.

3.2.2 Alternative scenarios

When consumption and preparation habits were considered only for the northeast part of Italy, variations were observed in the relative contribution of the two meat species considered. In this case, the number of *T. gondii* infections attributed to bovine meat was 2.3 times higher than the number caused by pork (Table 1).

In the scenario considering a 0.5% prevalence value of *T. gondii* infection in cattle, a switch of the most important meat category from bovine meat to pork was observed. In this case, pork accounted for 2.4 times more cases of Toxoplasma infection than bovine meat (Table 1).

The third alternative scenario used an unscaled mouse dose-response relationship in which original data from a mouse infection model were incorporated. In this case, the relative contribution of pork and bovine meat was similar to that calculated by our base model (Table 1). However, a marked increase in the estimated number of *T. gondii* infections per year was predicted.

Scenario		Mean probability of infection	Mean number of infections per year	Mean number of infections per year (age adjusted)
Base	Pork	0.019%	11426	8460
	Bovine meat	0.034%	20461	15151
Northeast	Pork	0.014%	1664	1219
	Bovine meat	0.032%	3801	2784
Cattle 0.5%	Pork	0.019%	11426	8460
	Bovine meat	0.008%	4657	3448
Unscaled mouse dose-response	Pork	2.973%	1797357	1330892
	Bovine meat	6.712%	4058234	3005007

Table 1: results of the base model and the alternative scenarios. Adjustment was made to consider only the population between 5 and 65 years.

Scenario	Base	Northeast	Cattle 0.5%	Unscaled mouse dose-response
Minimum	0	0	0	0
Maximum	0.138729	0.1012829	0.1386718	1.50873
Mean	0.000527	0.00046639	0.000266009	0.09685402
Std Deviation	0.001415	0.001178448	0.001106178	0.1519947

Variance	2E-06	1.38874E-06	1.22363E-06	0.02310238
Skewness	13.49048	11.02676	22.89889	2.379472
Kurtosis	628.6025	411.8377	1585.34	9.465725
5% Perc	0	0	0	0
50% Perc	9.73E-05	8.60914E-05	2.38293E-05	0.02954687
95% Perc	0.002369	0.00211899	0.001173283	0.4353082

Table 2: output parameters of the base model and of alternative scenarios.

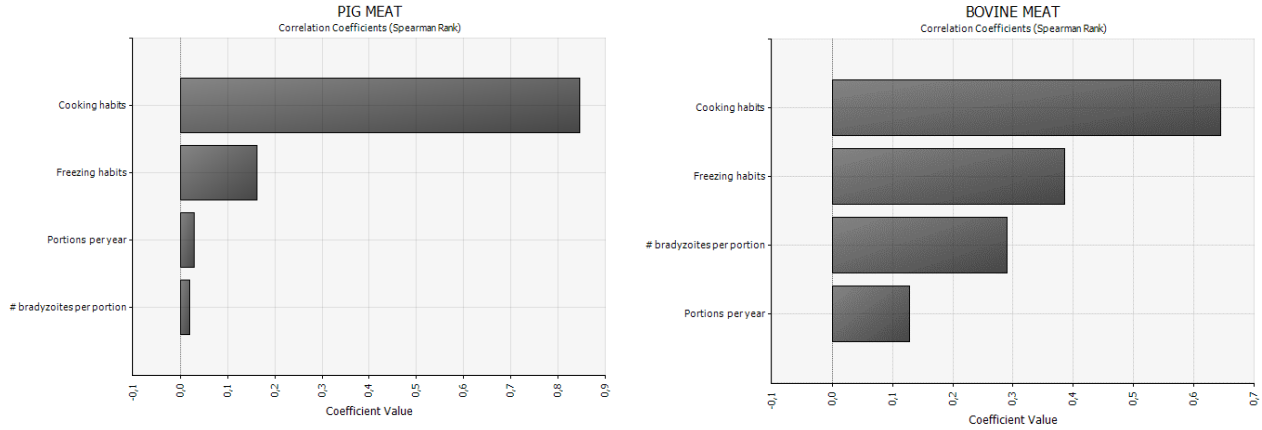

Figure 5: output of the sensitivity analysis carried out on the base model for pork (left panel) and bovine meat (right panel).

4. Discussion

The present study aimed to estimate the yearly probability of an Italian consumer acquiring *T. gondii*, through the consumption of pork or bovine meat. This parasite can be acquired by food other than pork and bovine meat, and also by different routes, and thus, our result was not expected to explain the whole seroprevalence rate estimated for the entire Italian population. The model resulted in a yearly probability of infection of 0.019% and 0.034% for pork and bovine meat, respectively. This would result in 8,460 and 15,151 new cases of Toxoplasma infection caused by pork and bovine meat, respectively, among the Italian population between five and 65 years of age.

Interestingly, bovine meat proved to be responsible for twice the number of infections that pork produced. The role of bovine meat is surprising if compared to what is commonly supported within the scientific community, where bovine meat's importance in *T. gondii* transmission is not always acknowledged (Dubey and Jones, 2008; Hofhuis et al., 2011; Kijlstra and Jongert, 2008). However, if this result is compared with recent studies, with different approaches to the topic, the initial surprise can be replaced by increasing certainty that bovine meat is an important source of *T. gondii* infection. *T. gondii* prevalence has been shown to be 2.6% worldwide (2.2% in Europe) (Belluco et al., 2016). A multicentre case-control study carried out in Europe identified the consumption of raw or undercooked bovine meat as a significant risk factor OR 2.4 (1.6-3.4), whereas consumption of raw or undercooked pork was unable to be identified as a risk factor for infection (Cook et al., 2000). This result is not isolated in the scientific literature, as was confirmed by other case-control studies (Baril et al., 1999; Carellos et al., 2014). In addition to this observational evidence, a recent Quantitative Microbial Risk

Assessment Model, estimating the number of *T. gondii* infections in humans in the Netherlands, identified beef as the most important meat source contributing to 68% of human cases (Opsteegh et al., 2011). In contrast, Dubey and colleagues, after a nationwide bioassay-based survey of meat products sold in the USA, estimated the risk of infection due to beef consumption near 0%, due to the inability to find positive meats (Dubey et al., 2005). Obviously, with 0% prevalence, the risk is null, but it is noteworthy that in the present model, the probability estimate for beef is strongly correlated to the prevalence value due to the lower (compared with pork) impact of cooking as a protective factor (Figure 3). In our pork model, cooking habits, and not prevalence, are ranked in first place according to the regression-based sensitivity analysis. This observation confirms the common perception, that pork should be properly cooked, and demonstrates the efficacy of cooking in reducing the probability of *T. gondii* infection. Unfortunately, cooking is not always applied to beef in Italy, as raw or rare beef is considered a delicacy and is consumed in different forms and dishes.

The most severe consequences of *T. gondii* infection occur in women acquiring acute infection during pregnancy. The parasite can cross the placenta with a probability of success that increases with the advance of pregnancy, rising from 9% in the first trimester to 59% in the last one. The severity of clinical manifestations showed an inverse temporal trend, as earlier infections can cause more serious consequences on foetus health (Montoya and Liesenfeld, 2004). Our model, when modified to examine the role of pork and bovine meat in toxoplasmosis in unborn foetuses, showed a very low probability of congenital infection in Italy due to pork (0.0218% - 10 cases) and bovine meat (0.0718% - 32 cases). A three year cohort study carried out in Italy from 2011 to 2013 on 11,147 infants (23.5% from non-native women) resulted in an incidence rate of 0.77% and a probability of congenital toxoplasmosis of 0.06%. All cases of congenital toxoplasmosis were in non-native pregnant women (Capretti et al., 2014). The incidence estimated from our base model was higher compared to the incidence from that cohort study, although the fact that the present model estimates only cases due to consumption of fresh or previously frozen pork or bovine meat meat should be taken into account. However, an overestimation of true rates was expected because pregnant women are likely to have different meat preparation behaviour, with an avoidance of raw meat.

To better investigate the risk difference between bovine meat and pork consumption, beyond the suggestions obtained by sensitivity analysis, alternative scenarios were created. The northeast scenario was restricted to input data belonging to this geographical area (food consumption and meat preparation habits), and confirmed the results of the base model with a slight difference in the relative contributions, even higher for bovine meat in this case.

The second alternative scenario considered a lower *T. gondii* prevalence in cattle (0.5%). This was to account for a potential overestimation of the real prevalence, because the 2.2% prevalence resulted from a meta-analysis of European studies in which biomolecular techniques, unable to differentiate between dead and live parasite, were also used (Belluco et al., 2016). Moreover, *T. gondii* persistence in bovine tissues has been estimated to be low (Dubey and Thulliez, 1993) and the parasite is not necessarily able to survive along the entire production cycle. A prevalence of 0.5% was chosen according to the Dutch model (Opsteegh et

al., 2011) and to be near to 0%, as suggested by Dubey and colleagues (Dubey et al., 2005). Because prevalence, as previously stated, was the top influencer in the model, its 4-fold reduction led to a switch in the relative contribution between pork and bovine meat, with pork now predicted to be 2.4 times more likely to cause infection than bovine meat. This could be a more credible estimate for most experts, but it is not confirmed by relevant epidemiological literature (Baril et al., 1999; Carellos et al., 2014; Cook et al., 2000; Opsteegh et al., 2011), as we described above.

Finally, a different dose-response curve was used, similar to that applied in the Dutch model. This curve was obtained from previous work using a mouse model of infection, is not adapted to humans, and is unscaled, whereas the curve used for our base model was scaled according to previous estimates (Guo et al., 2015b). This unscaled model resulted in similar estimates of the relative contributions of pork and bovine meat to those produced by our base model. However, this unscaled model also produced extremely high yearly probabilities of infection, which are not justified by the epidemiological data on *T. gondii* prevalences in Italians (Mosti et al., 2013). This overestimation is in line with the dutch model, but it was well controlled by our base model, underlining the likelihood that the scaled dose-response curve better models the real scenario.

The higher contribution that bovine meat consumption makes towards *T. gondii* infections in humans could also be explained by some of the data we chose as model inputs. The concentration of bradyzoites in muscles was obtained from an experimental study carried out in pigs (Juránková et al., 2014), since no similar study in cattle was available. However, it is possible that bovine muscle holds a different concentration of bradyzoites than does swine muscle. The dose-response curve by Guo et al. (2015) allowed for a plausible probability estimate and, according to our sensitivity analysis (Figure 5, Appendix C), was ranked as an important influencer only in the scenario where cattle had a lower prevalence. Obviously, as can be seen by the comparison between the base model and the unscaled mouse dose-response model, the dose-response curve greatly affects the predicted number of cases, but not the relative contribution of the two investigated meat species.

In the current study, consumption data was not among the main determinants of model output, whereas meat preparation strategies played an important role. In particular, when the risk of infection from pork was modelled, cooking habits were correlated with predicted *T. gondii* infections. This well explains how, in the presence of a far from negligible *T. gondii* prevalence in meat (8.7% in pork), the consumer phase could well be the main mitigation strategy to reduce the resultant risk of infection. As far as pork is concerned, it is also possible to speculate that survey results could have underestimated the true consumption of undercooked pork. In fact, while bovine meat is voluntarily keep rare in many meat dishes, pork is probably eaten undercooked as an accidental event, leaving the consumer unaware of its real status, and believing that the pork they are consuming is well-cooked.

These previous considerations explain the potential uncertainties of the model. Moreover, it is important to bear in mind that the current study was aimed at estimating the yearly probability of infection due to the consumption of fresh or previously frozen pork and bovine meat meat,

and does not account for the contribution of other important sources of *T. gondii* infection: sheep and goats. In addition, preserved pork meat (ham, salami, etc.) were excluded from both the meat consumption data and meat consumption habits. Thus, the role of pork and all pork-derived products could have been underestimated.

Despite some limitations, the model indicates the potential for bovine meat to act as a source of human toxoplasmosis, even in the scenario where bovine meat has a low prevalence of this parasite. In fact, the model predicts that bovine meat plays a more significant role in human *T. gondii* infections than does pork. Results also highlighted the importance of cooking habits in limiting the number of infections, particularly in the case of pork that has higher *T. gondii* prevalence than other meats. Lowering the *T. gondii* prevalence at farm level and counselling consumers about the important of domestic mitigation strategies (cooking and freezing) are the most suitable prevention options. More studies are warranted in the light of estimating the role of preserved meats, widely consumed in Italy, and to account for different food sources such as sheep, goats, horses and vegetables as transmission routes.

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Appendix A: data used to estimate the distribution of consumption habits used in the model as obtained from INRAN-SCAI .

Meat	Area	Sex	mean	median	p95	p99	DS
Pork	Base	F	39	38	94	140	29
		M	48	47	101	177	36
	Northeast	F	40	38	83	105	24
		M	47	47	99	128	27
Beef	Base	F	52	42	118	179	35
		M	64	54	147	233	44
	Northeast	F	50	41	119	171	35
		M	61	58	139	203	41

Appendix B: data obtained by the survey of meat consumption habits in terms of frequency of the investigated habit on the total number of consumption events. Data are shown per geographic origins and sex of respondents.

Meat	Habits		Base		Northeast	
			F	**M**	**F**	**M**
Pork	Freezing habits	Never	24.1%	25.4%	21.1%	28.9%
		1 of 5	38.3%	42.4%	40.4%	43.3%
		2 of 5	17.9%	16.9%	17.4%	13.4%
		3 of 5	6.2%	6.8%	6.4%	6.2%
		4 of 5	8.6%	7.6%	8.3%	8.2%
		Always	4.9%	0.8%	6.4%	0.0%
	Cooking habits	Never	88.3%	78.5%	88.2%	81.8%
		1 of 5	10.4%	13.2%	11.8%	12.1%
		2 of 5	0.0%	3.3%	0.0%	3.0%
		3 of 5	0.6%	3.3%	0.0%	2.0%
		4 of 5	0.0%	0.8%	0.0%	1.0%
		Always	0.6%	0.8%	0.0%	0.0%
Beef	Freezing habits	Never	14.6%	16.4%	12.8%	18.0%
		1 of 5	27.4%	41.0%	28.4%	37.0%
		2 of 5	22.0%	20.5%	22.9%	24.0%
		3 of 5	15.9%	13.1%	12.8%	11.0%
		4 of 5	17.1%	7.4%	19.3%	9.0%
		Always	3.0%	1.6%	3.7%	1.0%
	Cooking habits	Never	30.1%	27.4%	30.2%	29.4%
		1 of 5	29.5%	22.6%	31.0%	23.5%
		2 of 5	13.3%	20.2%	11.2%	18.6%
		3 of 5	11.0%	10.5%	12.9%	9.8%
		4 of 5	10.4%	12.9%	10.3%	11.8%
		Always	5.8%	6.5%	4.3%	6.9%

Appendix C: outcome of the sensitivity analyses carried out in the base model and in alternative scenario models. Regression coefficients indicate the correlation between input parameters and probability estimates.

Scenario		Cooking habits	Prevalence	Portions per year	Freezing habits	Dose-response
Base	Pork	0.509	0.207	0.094	-0.059	---
	Beef	0.348	0.449	0.268	-0.155	---
Northeast	Pork	0.522	0.210	0.089	-0.058	---
	Beef	0.362	0.452	0.249	-0.159	---
Cattle 0.5%	Pork	0.509	0.207	0.094	-0.059	---
	Beef	0.347	---	0.267	-0.155	0.448
Unscaled mouse dose-response	Pork	0.714	0.199	0.096	-0.082	---
	Beef	0.499	0.471	0.302	-0.218	---