Alma Mater Studiorum – Università di Bologna

DOTTORATO DI RICERCA IN

SCIENZE VETERINARIE

Ciclo XXXVI

Settore Concorsuale: 07/H3- Malattie infettive e parassitarie degli animali

Settore Scientifico Disciplinare: VET/06 Parassitologia e Malattie Parassitarie degli Animali

EPIDEMIOLOGICAL STUDIES ON *TOXOPLASMA GONDII* IN A ONE HEALTH PERSPECTIVE

Presentata da: **Dott. Filippo Maria Dini**

Coordinatore Dottorato

Prof.ssa Carolina Castagnetti

Supervisore

Prof.ssa Roberta Galuppi

Co-Supervisore

Prof.ssa Monica Caffara

Esame finale anno 2024

Abstract:

The One Health approach emphasizes the need for interdisciplinary collaboration to address complex challenges spanning human health, animal well-being, and the environment. Recent global issues like environmental shifts, biodiversity decline, and the emergence of infectious diseases necessitate holistic and collaborative strategies. Effective monitoring of toxoplasmosis and *Toxoplasma gondii* diffusion is crucial. The objective of the present Ph.D. thesis was to investigate with a One Health approach the epidemiological patterns of *T. gondii* infection in Italy, to better understand the transmission dynamics of the parasite, following different research lines. The results of a retrospective analysis in animals and human showed the widespread distribution of *T. gondii* in the study area, with specific antibodies found in various animal species and human populations, indicating its constant presence across diverse environments. The environment plays a significant role in *T. gondii*'s epidemiology, as it serves as a natural reservoir for its infectious form, the oocysts. Migratory aquatic birds, rodents, wolves, and wild boars were investigated as sentinels of the environmental contamination by oocysts, highlighting the potential transmission across geographic areas and infection risks for wildlife in natural settings. The study also provided insights into seroprevalence in wolves. Dogs, subjected to serological investigations as intermediate hosts of veterinary importance, exhibited risk factors for *T. gondii* infection, such as cohabitation with cats, coprophagy behaviours, and continuous outdoor. Correlation between serological evidence of exposure to *T. gondii* and pathological anxiety in large-size dogs was observed, and the consumption of raw meat was associated with a higher risk of infection in these animals. The consumption of raw or undercooked meat is a significant risk factor for *T. gondii* infection in human, although in cattle the parasite is rarely directly detected in muscle. Results of the investigations conducted in this thesis, demonstrate the dynamic nature of *T. gondii* infection in cattle, characterized by new infections and declining antibody levels over the production cycle. The study also describes a co-infection between *T. gondii* and *Sarcocystis hominis* in bovine eosinophilic myositis, using innovative detection methods. In the final part of the Thesis, a comprehensive genotyping of *T. gondii* in Italy reveals the predominance of Type II strains, particularly in cases of ovine abortion and fatal toxoplasmosis among captive *Lemur catta*. This approach enhances our understanding of the parasite's genetic diversity and transmission patterns, vital for effective management of its impact on human and animal health in Italy.

INDEX

CHAPTER 1- *Toxoplasma gondii:* Uniting Health and Ecosystems, a holistic perspective

1.1 Taxonomy, Genetic intricacy and Population Structure

Toxoplasma gondii is a protist parasite found within the subphylum Apicomplexa, a taxonomic category encompassing over 6000 members, known for their endoparasitic nature and distinguished by the presence of an apical complex in their cellular structure. Infections caused by apicomplexan parasites impose a substantial burden on both public and animal health globally, with genera such as *Plasmodium*, *Babesia, Cryptosporidium*, *Sarcocystis*, and *Toxoplasma*, and also have significant economic implications in livestock industry due to parasites *Eimeria*, *Besnoitia*, *Neospora*, and *Theileria*, among others (Swapna and Parkinson, 2017).

Within the subphylum Apicomplexa, *T. gondii* falls under the Coccidia subclass, alongside other parasites characterized by life cycles which include merogony, gametogony, and sporogony phases. Initially, *T. gondii* was believed to exclusively parasitize extraintestinal tissues of a wide array of warm-blooded hosts. It wasn't until 1970 when Frenkel and his collaborators identified the parasite as an intestinal coccidium within cats, featuring an oocyst stage reminiscent of *Isospora* sp. (Frenkel et al., 1970). Within the Coccidia subclass, *T. gondii* is classified under the family Sarcocystidae, which includes other genera of cyst-forming parasites with heteroxenous life cycles. These life cycles include both sexual and asexual replication stages, the first lasting in the elimination of oocysts in the environment. Notable genera within this family include *Neospora*, *Besnoitia*, and *Hammondia* (Tenter et al., 2002). *T. gondii* stands as the sole species within its genus.

In the life cycle of *T. gondii*, akin to other apicomplexan parasites, there is a predominant haploid state, with a brief diploid phase occurring during the sexual stage within the definitive host's intestine. The formation of sporozoites through postzygotic meiosis adheres to classical Mendelian laws, as elucidated (Dubey, 2022). Consequently, most phases of the life cycle are characterized by allelic homozygosity, which not only facilitates genetic recombination but also supports direct assessments of population-level heterozygosity.

It's worth noting that for many apicomplexan parasites like *Cryptosporidium* and *Eimeria*, the sexual phase is considered obligatory. However, in the case of the *Toxoplasma* and

Neospora genera, zoites possess the ability to perpetuate asexual replication indefinitely, as outlined by Beck et al. (2009).

The haploid genome of *T. gondii* is composed of 13 chromosomes and boasts an excess of 8300 identified protein-coding genes, with a total genome size exceeding 65 million base pairs (Mb). This extensive genomic information has been reported in various studies (Khan et al., 2005; Reid et al., 2012; Lorenzi et al., 2016; Xia et al., 2021).

Comparative genomic investigations involving *T. gondii* and multiple members of the Apicomplexa subphylum have unveiled distinctions that set *T. gondii* apart from its closest relatives. These differences are notably attributed to the tandem amplification and diversification of specific gene groups engaged in host-parasite interactions. They also play a pivotal role in defining variations among the 16 significant clades within the species. These determinant groups include genes encoding for micronemes (MIC, involved in host cell attachment), dense granular (GRA) and rhoptries (ROP) (involved in the modulation of host immunity) secretory proteins, as well as members of the SAG1-related sequence (SRS) super family of surface adhesins (adherence and immune evasion) (Lorenzi et al., 2016).

Before methods reliant on the characterization of specific genetic markers were established, *T. gondii* isolates were primarily categorized based on their virulence in outbred mice. Additionally, observed differences in the expression of polymorphic antigenic peptides and zymodemes among *T. gondii* strains indicated variances in particular genes. These early findings were used to formulate initial population structures (Dardé et al., 1992; Bohne et al., 1993; Meisel et al., 1996).

Concurrently, during the 1980s and 1990s, molecular methods has been used for discerning genetic distinctions among *T. gondii* strains and correlating them with the observed virulence in mice, as documented by Sibley and Boothroyd (1992) and Howe and Sibley (1995). Among these approaches, the Restriction Fragment Length Polymorphism (RFLP) method, focused exclusively on the Surface Antigen-2 (SAG2) gene, enabled pioneering researchers to initially outline a clonal population structure consisting of three genetic types (I, II, and III) that exhibited associations with the virulence observed in mouse isolates derived from human patients (Howe and Sibley, 1995). The authors of these studies proposed that type I isolates were uniformly lethal to mice, regardless of the administered dose, while types II and III generally lacked virulence.

Subsequent to these developments, multilocus typing methods unveiled strains that carried alleles matching types I, II, or III, akin to those present in the three primary lineages. However, these alleles segregated differently across the analyzed loci. These strains were recognized as the outcome of recombination events between strains originating from the major lineages and were defined "recombinant strains." Furthermore, strains featuring unique polymorphisms at certain loci, distinct from those in the predominant lineages, were also identified and categorized as "atypical" (Ajzenberg et al., 2004). The ToxoDB database (http://toxodb.org/toxo/) plays a pivotal role in this context, allowing for the identification, compilation, and assignment of a distinct code for all deposited isolates and genetic variants. This coding is determined by the combination of alleles from 11 PCR-RFLP markers and is referred to as the ToxoDB genotype number (#). Additionally, specific designations related to origin can be attributed to particular genetic profiles defined by 15 microsatellite markers (e.g., Africa 1, Caribbean 3).

The genome-wide polymorphism rate among the three primary lineages has been estimated at approximately 1%. This polymorphism is marked by an extensive bi-allelism distinguishing type I, II, and III SNPs, as reported in various studies (Grigg et al., 2001; Khan et al., 2005; Boyle et al., 2006; Sibley and Ajioka, 2008). The origin of this clonality, the low genetic diversity within each lineage, and the limited divergence between them have been attributed to a relatively recent emergence and expansion from a common ancestor, occurring just 10,000 years ago (Su et al., 2003). This phenomenon is further compounded by a significant reduction in sexual reproduction, with a predominantly asexual mode of propagation (Sibley and Ajioka, 2008).

The prevailing assumption is that the genetic population of *T. gondii* primarily results from infrequent but pivotal meiotic/genetic crosses between highly similar parental strains. The substantial expansion takes place through asexual reproduction, facilitated by direct oral infection among diverse intermediate hosts. The unique capacity of a single zoite to engage in complete sexual development and self-fertilization within feline hosts, coupled with the limited number of cats simultaneously harbouring multiple strains, restricts the opportunities for genetic exchange (Boyle et al., 2006; Sibley and Ajioka, 2008; Wendte et al., 2010). Nevertheless, this theory does not fully explain the situation in the South American model, where a significantly higher prevalence of infection, along with an increased diversity of wild

felids, may have led to more frequent recombination events, resulting in a notably diverse non-clonal population, as highlighted by Bertranpetit et al. (2017).

In the wake of significant advancements in molecular typing techniques, there have been numerous comprehensive efforts to elucidate the population structure of the parasite. Khan et al. (2007) conducted a phylogenetic analysis of intron sequences by pooling strains of *T. gondii* from Europe, North and South America, leading to the identification of 11 haplogroups. These included the three major clonal lineages, which were subsequently renamed as haplogroups 1, 2, and 3, predominantly distributed in North America and Europe. Additionally, they identified other haplogroups representing successful recombinant/atypical strains that had primarily clonally expanded throughout South America.

Later on, the same authors (Khan et al., 2011) re-evaluated the population structure of *T. gondii* in North America, by sequence-based phylogenetic and population analyses. Their work resulted in the delineation of a new clonal lineage, labelled as lineage 12, encompassing American strains that had previously been classified as atypical by PCR-RFLP typing. On a different front, Pena et al. (2008) concentrated their efforts on the Brazilian scenario, which mirrors tropical conditions. They analyzed a substantial number of Brazilian isolates using PCR-RFLP typing, leading to the identification of four clonal lineages that had successfully expanded (BrI, BrII, BrIII, and BrIV), alongside a substantial group of divergent and highly diverse strains. Furthermore, Su et al. (2012) identified 16 distinct haplogroups, organized into six major clades (A-F), characterizing isolates from around the world. These findings were largely in line with the earlier results reported by Khan et al. (2007; 2011).

Notably, Africa and Asia, in comparison with other continents, remained relatively uncharted and were underrepresented in the mentioned phylogenetic analyses. In a recent extensive review, du Plooy et al. (2023) showed that *T. gondii* type II and III isolates are also prevalent in African regions, particularly type III, coexisting with other less common genotypes identified by microsatellite markers as Africa 1 (haplogroup 6) and Africa 3 (haplogroup 14). In the case of Asian strains, research carried out by Chaichan et al. (2017) uncovered 36 distinct PCR-RFLP genotypes, the majority of which belonged to type I, II, III, or the Chinese 1 clonal lineage (ToxoDB #9, haplogroup 13).

In North America and Europe, the population structure of *T. gondii* is predominantly characterized by the presence of three dominant clonal lineages (I, II, and III). These lineages

coexist with relatively fewer isolates that exhibit greater genetic diversity. There is also a fourth clonal lineage, as mentioned earlier (lineage 12), which is primarily confined to North America, where it's more commonly found in wild animals. In contrast, South America displays a significantly greater degree of genetic diversity, following an epidemic structure. Here, the population features a few major clonal complexes alongside numerous less closely related isolates. The African and Asian scenarios represent a blend of both patterns. These regions harbour a considerable number of isolates belonging to the type I, II, and III clonal lineages, alongside other clonal groups that have emerged from the expansion of recombinant or atypical isolates. However, these clonal groups exhibit a lesser degree of divergence compared to South America (Lorenzi et al., 2016; Su et al., 2012). Utilizing phylogenetic and geostatistical approaches, Bertranpetit et al. (2017) hypothesized that *T. gondii* might have originated in South America and then spread through North America, Asia, Europe, and finally Africa via various migration routes. This dispersal could be closely linked to the co-evolution of members of the Felidae family and humans, potentially contributing to the distinct population structures observed between South America and other continents.

1.2 Unravelling *T. gondii*'s Epidemiological Triumph: From its Invasive Phases to Diverse Transmission Routes

In the intricate heteroxenous life cycle of *T. gondii*, three distinct invasive stages have been identified: tachyzoites, bradyzoites within tissue cysts, and sporozoites in the sporulated oocysts.

The structure of zoites (general term used to refer to any stage of *T. gondii* life cycle) in general is very complex and present a series of structures that play a pivotal role in the invasion and pathogenesis of the infection. In the following sections, a summarized overview of the main structures of *T. gondii* zoite that are involved in the invasive phase will be presented.

A complex of membranes, referred to as the pellicle, encloses the entire protozoan body. It comprises an external plasma membrane and, beneath it, two closely juxtaposed membranes that constitute the inner membrane complex. This inner complex is absent from the most apical region, where the conoid is situated, and in farthest posterior portion of the cell (Porchet and Torpier, 1977).

The three infective stages exhibit a distinct specialization of their anterior area, housing the apical complex, which plays a pivotal role in initiating the host cell infection process. This complex consists of the conoid and two sets of secretory organelles: the micronemes and the rhoptries. Micronemes are the most abundant ones. They are the initial organelles to release their protein content, a crucial component for the movement of the protozoan and its interaction with the host cell's membrane. The proteins within the micronemes, are referred to as MICs. This group encompasses proteins with properties resembling perforins, adhesins, and serine proteases (subtilisins). They are more abundant in sporozoites while bradyzoites have a lower count, and tachyzoites fall in between. (Reviewed by Attias et al., 2020).

Rhoptries, larger than micronemes, are the second group of apical secretory organelles. The most basal structure, wider and imparting a spongy appearance, houses proteins referred to as ROPs, which are involved in subverting host cell functions. In contrast, the anterior portion, also known as the neck, is dedicated to concentrating proteins associated with host cell invasion, known as Rhoptry Neck Proteins (RONs). The secretion of rhoptries plays an important role in the formation of the moving junction for *T. gondii* invasion and the constitution of the parasitophorous vacuole (PV) membrane (Bradley and Sibley, 2007; Boothroyd and Dubremetz, 2008).

The third group of secretory organelles are the dense granules, distributed throughout the protozoan body, and contain a large number of proteins (known as GRAs) that are involved in the assembly of a network of tubules and filamentous structures with the PV. Their number is higher in sporozoites (Paredes-Santos et al., 2012). Despite these cytoplasmic organelles being present in all three invasive stages, they exhibit distinct variations in their structure, function, and roles within the life cycle.

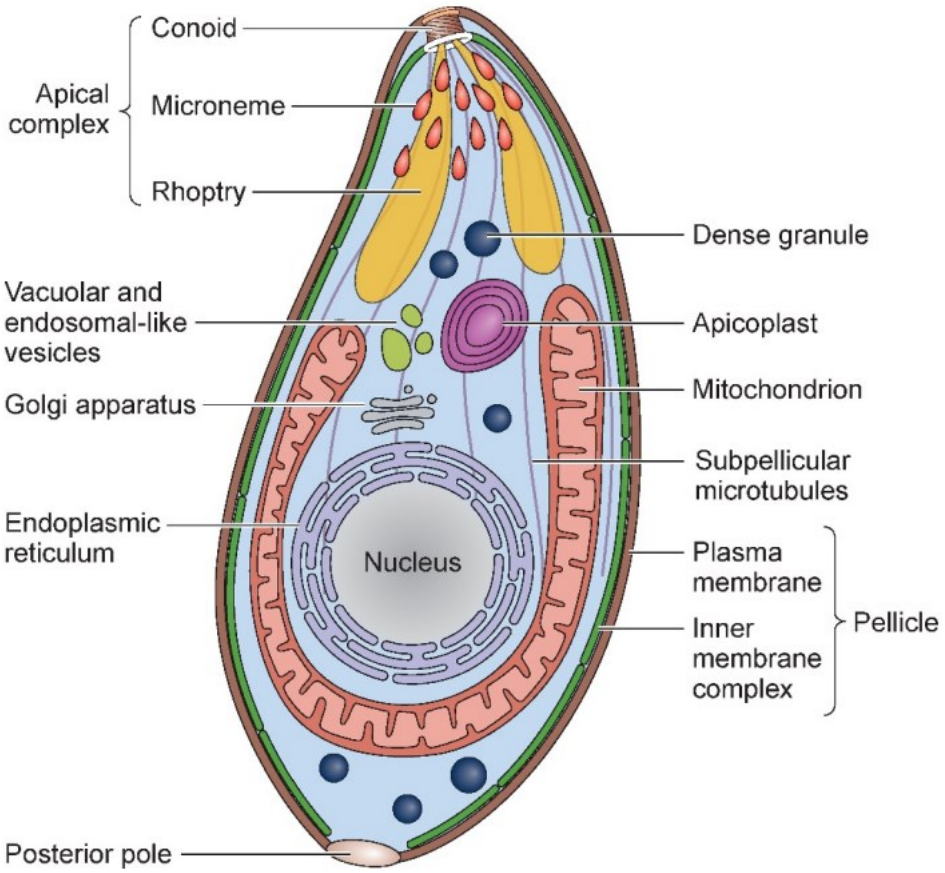


Figure 1 Longitudinal section view of the tachyzoite form of Toxoplasma gondii *indicating the main structures and organelles, from Sanchez and Besteiro (2021).*

The following sections outline the key characteristics associated with each of these infective forms.

Tachyzoites (Figure 2a) represent the swiftly multiplying phase of *T. gondii*, driving the acute stage of infection, host-wide dissemination, and tissue harm. These tachyzoites take on a distinctive crescent shape, measuring approximately 2 × 6 μm, with a remarkable capacity to infiltrate nearly all types of nucleated cells. They thrive within the PV. Within this vacuole, the parasite undergoes repeated asexual replication through endodyogeny until it ruptures the host cell, leading to its egression and the invasion of neighbouring cells. This continuous cycle of lysis and the accompanying immunopathological consequences are responsible for the manifestation of acute clinical disease, as documented in studies by Dubey et al. (1998) and Dubey (2022).

Bradyzoites (Figure 2b), in contrast, are the parasite's slow-replicating stage. Their primary role is to facilitate the chronicity of the infection, residing predominantly within tissue cysts, especially in immune privileged host tissues like central nervous system (CNS), eye, and skeletal muscles. These tissue cysts undergo growth while remaining intracellular, with bradyzoites multiplying via endodyogeny to form several hundreds of them. The size and shape of these cysts vary, depending on the time since their formation and the invaded tissue. For instance, in the brain, cysts often take on a spheroidal shape and rarely reach a diameter of 70 μm; whereas intramuscular cysts are elongated, sometimes reaching a length of 100 μm. The tissue cyst wall is characterized by its elasticity, and thickness of less than 0.5 μm, as reported by Dubey et al. (1998) and Dubey (2022).

Sporozoites (Figure 2c), are enclosed within the sporulated oocysts, which represent the environmentally resistant stage of *T. gondii*. Initially, oocysts are excreted into the environment unsporulated through the faeces of the definitive host (limited to members of the Felidae family) as noted by Martorelli et al. (2019). The process of sporulation takes place within 1 to 5 days after excretion, contingent on factors like aeration and temperature. This transformation involves sporogony, after which the oocysts become infective and environmentally resistant. The oocyst stage of *T. gondii* is *Isospora*-like, characterized by its spherical shape and an approximate diameter of 11 × 13 μm. These oocysts contain two ellipsoidal sporocysts, each housing four sporozoites along with a residual sporocystic body inside them as described by Dubey et al. (1998) and Dubey (2022).

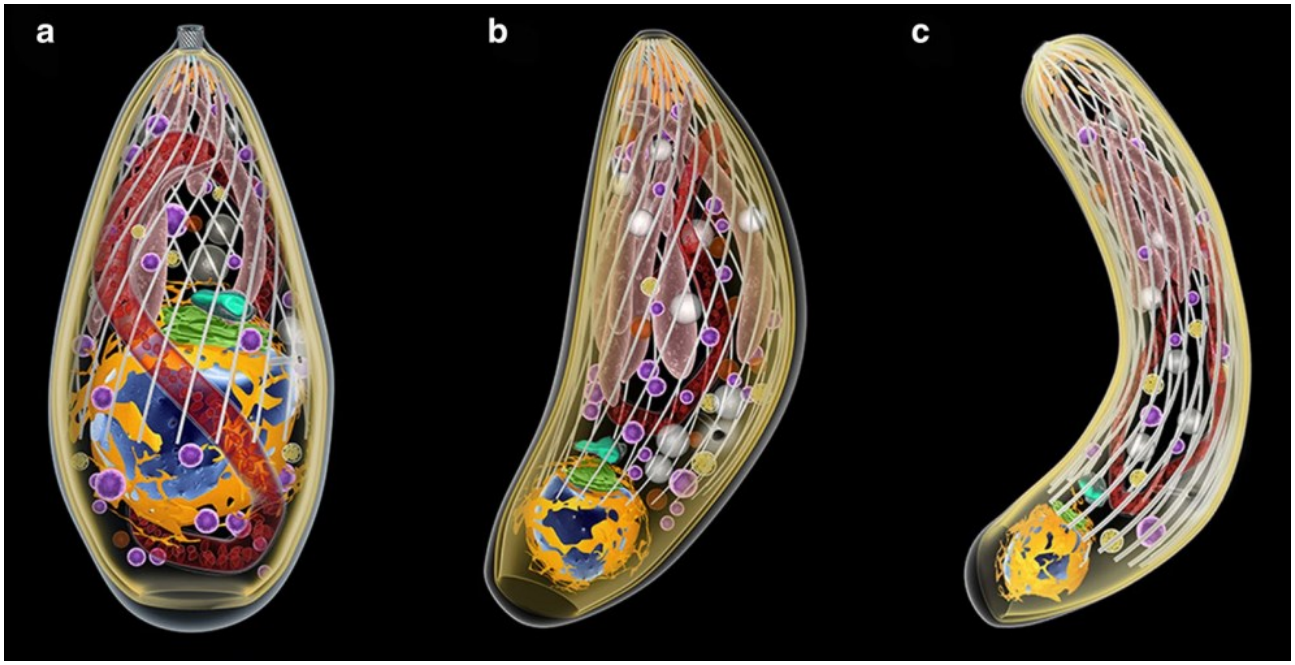


Figure 2: three infective stages of T. gondii, *including the tachyzoite (a), bradyzoite (b), and sporozoite (c). From Attias et al., 2020.*

The intricate life cycle of *T. gondii* is characterized as facultative heteroxenous, involving almost all warm-blooded animals, which serve as intermediate hosts (IH). This category includes various livestock species and humans. In contrast, definitive hosts (DH) are exclusively members of the Felidae family, encompassing both wild and domestic felines. The life cycle can be distinctly partitioned into three stages: an enteroepithelial sexual stage, an exogenous phase in the environment, and an extraintestinal asexual stage.

- **Enteroepithelial sexual stage**

Toxoplasma gondii exclusively perform the sexual reproduction of its life cycle within the small intestine of the felids definitive host (Tenter et al., 2000, Martorelli et al., 2019). Following the ingestion of tissue cysts or oocysts by cats, proteolytic enzymes in the stomach and small intestine break down the tissue cyst wall. This process allows to release bradyzoites (from tissue cysts), or sporozoites (from oocysts), both haploid, that infiltrate the epithelial cells of the small intestine, starting the asexual reproduction of multiple generations of *T. gondii*. Within the intestinal epithelial cells, five distinct asexual forms of *T. gondii* (types A to E) emerge, engaging in a continuous cycle of asexual replication through merozoite-schizont divisions via schizogony before transitioning to gametogony. Each schizogonic cycle gives rise several merozoites that will be released to readily invade new enterocytes, exponentially

enhancing the number of parasites (Dubey and Frenkel, 1972). Between three to fifteen days following the initial feline infection, schizonts and merozoites are primarily located in the ileum section of the intestine. During this period, some of them begin the process of differentiation into gametes. After the fertilization of haploid macrogametes (generated from a single merozoite) by haploid microgametes (formed by a schizogonic division), resulting in the formation of diploid zygotes, an oocyst wall develops around the parasite. The lysis of epithelial cells then enables the release of unsporulated oocysts into the intestinal lumen (Tenter et al., 2000; Dubey, 2022).

- **Exogenous stage in the environment**

Oocysts excreted in felid feces, are initially unsporulated and non-infectious. However, under favourable conditions of aeration, humidity, and temperature, sporogony takes place in the environment within a period ranging from 1 to 5 days. This process involves meiosis (postzygotic) and sporulation, ultimately resulting in the production of two sets of four haploid sporozoites. These sporozoites are enclosed within a second set of walled structures known as sporocysts. Once sporulated, these oocysts become infectious for both the intermediate hosts (IH) and the definitive hosts (DH) (Dubey, 2022; Ramakrishnan et al., 2019).

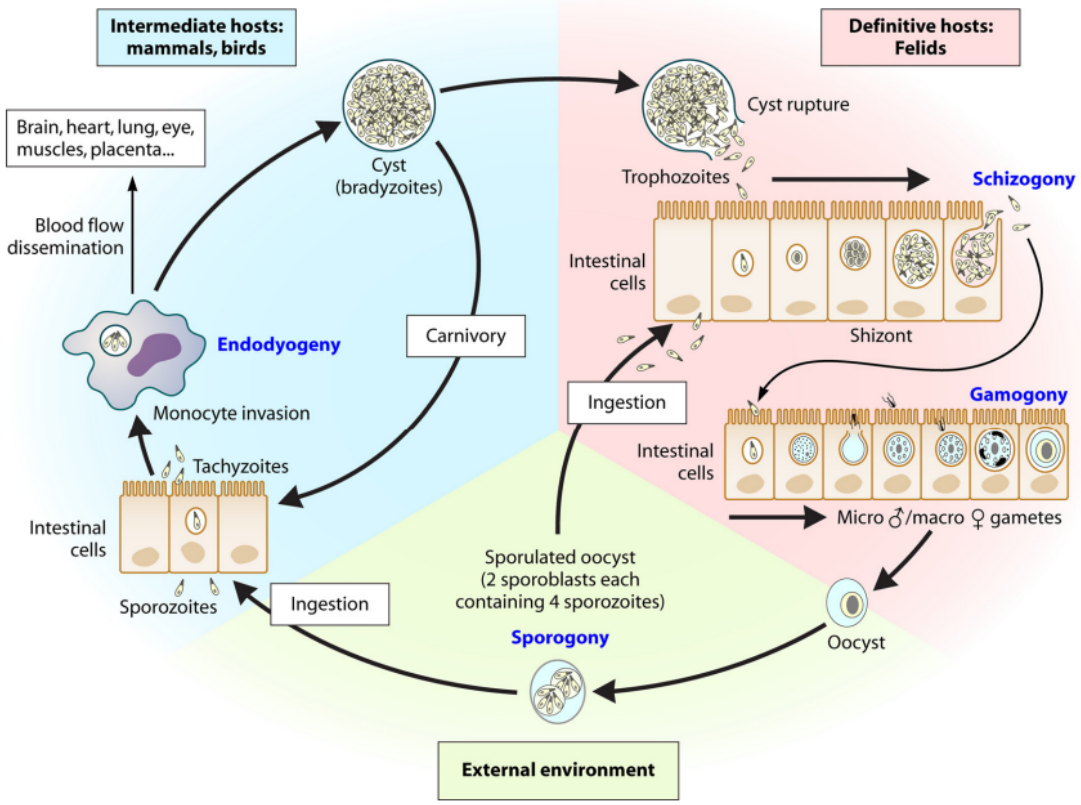


Figure 3 Life cycle of Toxoplasma gondii. Shown are the biology, infection, and replication of the three infective stages of the parasites in their respective hosts (Robert-Gangneux and Dardé, 2012)

- **Extraintestinal Asexual Stage**

Within a new host, after the ingestion of tissue cysts or oocysts, both walls are broken by digestive enzymes, releasing bradyzoites or sporozoites, respectively. These newly liberated bradyzoites or sporozoites employ a unique gliding mechanism for movement. In this gliding process, micronemal proteins are the first to be secreted, playing a crucial role in the protozoan's motility by facilitating gliding and the initial adhesion to the host cell surface. The gliding motility is a result of a complex assembly of proteins that are anchored to an actin-myosin motor situated between the plasma membrane and the inner pellicle. The complex interaction of proteins leads to the identification and attachment of the zoite to receptors of the host cell's plasma membrane (Opitz and Soldati, 2002; Frénal et al., 2017, Kato, 2018; Attias et al., 2020).

This interaction ultimately leads to internalization. This process can take place virtually in any nucleated cell, with a particular affinity for macrophages, epithelial cells, muscle cells, and neurons. Initially, *T. gondii* attaches the surface of the potential host cell and subsequently, by secreting proteins localized in the apical organelles (micronemes and rhoptries), orchestrates the reorientation of its apical side, thereby initiating the internalization process. Subsequently, proteins from the basal section of the rhoptry are secreted, leading to alterations in the host cell's behaviour and the formation of the membrane of PV which serves as the habitat for the protozoan's survival and multiplication. These changes also extend to modifications in the host cell cytosol, including the inhibition of the fusion between host cell lysosomes and the PV membrane (Attias et al., 2020). This initial phase of penetration takes place within the enterocytes of the intestinal epithelium, serving as the primary site of infection in an intermediate host, and then penetrate to the lamina propria; here they undergo asexual replication via endodyogeny within various nucleated cells, eventually developing into tachyzoite forms. Tachyzoites widely disseminate throughout the host's body and subsequently transform into bradyzoites. These bradyzoites persist within tissue cysts, often found in a diverse array of organs, resulting in a chronic infection that may persist for the host's lifetime. Bradyzoites exhibit slow replication through endodyogeny while residing inside tissue cysts. It's worth noting that bradyzoites appear to be less infective than sporozoites, as detailed in some studies (Tenter et al., 2000, Dubey, 2022).

Toxoplasma gondii has adapted to two primary routes: the oocyst-oral route in intermediate hosts and the tissue cyst-oral route in carnivores, particularly cats. Interestingly, *T. gondii* oocysts are less infective and less pathogenic for cats compared to other hosts, in contrast to bradyzoites, as highlighted by Dubey et al. (1996; 2006). Hence, *T. gondii* can be transmitted from definitive hosts to intermediate hosts, from intermediate hosts to definitive hosts, and even between definitive and intermediate hosts (Fig. 4). The cycle's continuity isn't solely dependent on the presence of a specific host species, it can persist indefinitely through the transmission of tissue cysts between intermediate hosts, even in the absence of definitive hosts, and by the transmission of oocysts between definitive hosts, even in the absence of intermediate hosts, as elucidated by Tenter et al. (2000).

As previously outlined, the three biological stages – tachyzoites, bradyzoites, and sporozoites – exhibit several degrees of infectivity for both intermediate and definitive hosts. These hosts can acquire the infection through one of several pathways (Fig. 4).

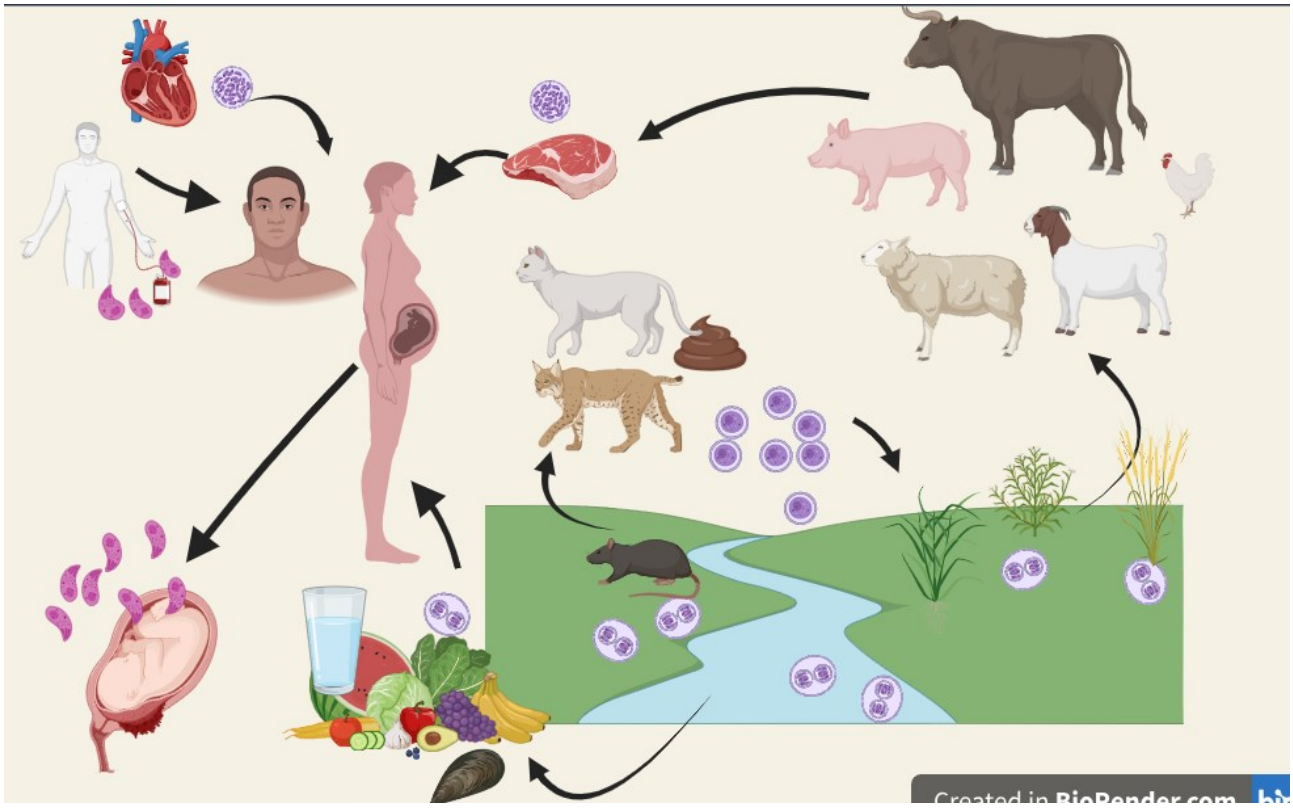


Figure 4 oxoplasma gondii transmission routes. Created with Biorender

- Fecal route: horizontal transmission by oral ingestion of sporulated oocyst from the environment (soil, contaminated water, vegetables).

- Carnivorism: horizontal transmission by oral ingestion of tissue cysts contained in raw or undercooked meat or viscera of intermediate hosts

- Transplacental route: vertical transmission by tachyzoites transference from a pregnant host to the foetus (congenital toxoplasmosis).

- Iatrogenic transmission: transmission by tachyzoites via transfusion of packed leukocytes or laboratory accidents, as well as transmission by tachyzoites/bradyzoites via transplantation (e.g., solid organs).

The prevalence of *T. gondii* infection and the primary transmission route within each intermediate host (IH) are influenced by a multitude of factors: the presence of felids in the environment, prevailing climate conditions that support sporulation and oocyst survival in the environment, the susceptibility of different host species to *Toxoplasma* infection (with some species exhibiting higher resistance), as well as the dietary habits and feeding behaviours of the host species, as highlighted by Robert-Gangneux and Dardé (2012).

On a global scale, it is estimated that approximately one-third of the human population is infected with *T. gondii*, though these values can vary significantly from one country to another, ranging from 10% to 80%. The lowest seroprevalence rates are typically observed in countries such as North America, South East Asia, and northern Europe (ranging from 10% to 30%). Intermediate values are commonly found in central and southern European countries (ranging from 30% to 50%). Conversely, the highest rates of infection are observed in regions of Latin America and tropical African countries (Pappas et al., 2009).

Significant attention is warranted with regard to meat-producing animals due to the zoonotic nature of the parasite. It is well-established that human infections are predominantly acquired, especially in some areas of Europe, through the consumption of raw or undercooked meat that contains viable *T. gondii* tissue cysts (Cook et al., 2000, Opsteegh et al., 2011b, Belluco et al., 2018). In the European Union (EU), *Toxoplasma gondii* is recognized as a specific risk to food safety, as outlined in the EFSA reports (2007, 2011,) and by De Berardinis et al. (2017). In the USA, it ranks as the second leading cause of foodborne illnesses, according to Scallan et al. (2011).

Given that farm animals simultaneously serve as a significant source of infection for humans and act as reservoirs of *T. gondii* for wildlife predators, there is a growing concern in this regard. Extensive efforts are being made to enhance our understanding of the real prevalence, primary risk factors, economic implications, characterization of circulating strains in livestocks, development of vaccines, and even the quest for "*Toxoplasma*-free" meat (Hiszczyńska-Sawicka et al., 2014; Djokić et al., 2016; Stelzer et al., 2019).

- **Environmental and Foodborne Transmission in Humans**

Water

In recent times, the significance of waterborne infections associated with *T. gondii* oocysts has grown, primarily due to the documented occurrence of widespread outbreaks (Bowie et al., 1997; Shapiro et al., 2019). Water sources such as irrigation systems, rivers, lakes, beaches, coastal areas, as well as wastewater and groundwater, are susceptible to contamination with these environmentally resistant oocysts. Furthermore, oocysts have shown the ability to withstand various decontamination procedures involving chemical agents, such as sodium hypochlorite and chlorine (Wainwright et al., 2007; Mirza Alizadeh et al., 2018). In aquatic environments, oocysts can maintain their viability for extended periods, for instance, up to 18 months at 4 °C after exposure to 2% sulfuric acid (Shapiro et al., 2019), or for 15 and 54 months at temperatures of 20–25 °C and 4 °C, respectively, in freshwater. In artificial seawater with a salinity of 15 ppt, they can persist for approximately 6 months under the same temperature conditions (Hohweyer et al., 2013). The transmission patterns of *T. gondii* oocysts to humans have predominantly been characterized through toxoplasmosis outbreaks, with Brazil, in particular, experiencing multiple oocyst-related outbreaks where water sources were identified as common exposure sources (Ferreira et al., 2018). Several contributing factors to oocyst transmission patterns in Brazil are likely representative of other regions with epidemic and/or endemic *T. gondii* occurrences, including inadequate infrastructure for water and sewage treatment, a significant portion of the population living in impoverished and underserved conditions, and limited access to healthcare (Shapiro et al., 2019).

Fresh produces

In recent years, there has been a rising number of *T. gondii* infection cases associated with the consumption of fresh vegetables (Pinto et al., 2019). The contamination of fresh vegetables with *T. gondii* oocysts can occur during their growth in soil or when contaminated water is used for

irrigation or washing. Since there are no regulations requiring the testing of fresh produce for parasite contamination, and it is not mandatory, the increasing popularity of consuming raw and ready-to-eat vegetables could potentially expose consumers to oocysts unintentionally. This is because most post-harvest processing methods do not guarantee complete removal or effective inactivation of oocysts (Mirza Alizadeh et al., 2018, Lass et al., 2019, López Ureña et al., 2022).

In Europe, Lass et al. (2012) documented the presence of *T. gondii* on vegetables from stores and home gardens in Poland, with a contamination rate of 9.7%. In their study, the fresh produce was washed, and the eluate was concentrated using a flocculation method. Subsequently, they employed real-time PCR targeting the B1 gene to specifically detect *T. gondii*. Caradonna et al. (2017) investigated the prevalence of *T. gondii* in ready-to-eat packaged mixed salads, using microscopy examination and PCR detection. The PCR results revealed that 0.8% of the pooled ready-to-eat salads tested positive for *T. gondii*, and a significant oocyst burden was found in those pooled samples. In both of these studies, the general procedure for the recovery and detection of the parasite followed three key steps: (i) washing the samples; (ii) concentrating the parasites through methods such as filtration and centrifugation; and (iii) employing PCR detection or microscopy examination. (EFSA, 2018)

Molluscan shellfish

Toxoplasma gondii oocysts can also enter the marine environment through improper disposal of sewage, inefficient treatment plants, water discharge, and water runoff (Barhoumi et al., 2014), and they can cause infections in marine animals and the contamination of marine fauna (Shapiro et al., 2015). Consistently, oocysts have been detected in wild and commercial bivalve molluscs in several countries. Bivalves continuously filter large volumes of water and concentrate microorganisms. They can retain viable *T. gondii* oocysts for 85 days following uptake (Linsday et al., 2004). Thus, they are considered good biological indicators of parasitic contamination of aquatic environments and could pose another risk for consumers when consumed undercooked or raw (Hohweyer et al., 2013; Palos-Ladeiro et a., 2013). Consumption of raw shellfish products was recognised as a risk factor for *T. gondii* infection in the USA (Jones et al., 2009). A survey of Mediterranean mussels (*Mytilus galloprovincialis*) collected from eight different sites on the west coast of Turkey found 9.4% (n = 795) to be positive for *T. gondii* using a PCR assay (Aksoy et al., 2014). A study looking at the presence of

T. gondii in a range of different farmed shellfish in Italy (Putignani et al., 2011) found the presence of positive DNA samples using a nested PCR assay and a fluorescent amplicon generation real-time PCR assay using the B1 target in 17% of *Crassostrea gigas* and 4% of *Tapes decussates*. *Toxoplasma gondii* DNA was detected in 43 out of 409 (10.5%) *Mytilus galloprovincialis* from southern Italy, revealing the presence of five distinct genotypes including one corresponding to type I and four atypical genotypes (Santoro et al., 2020).

Dairy products

The ingestion of unpasteurized milk, whey, and fresh cheese derived from animals infected with *T. gondii* can serve as a potential pathway for the transmission of the parasite to humans (Boughattas, 2017). Human infection and disease have been linked to the consumption of raw milk from goats infected with the parasite (Sacks et al., 1982). In milk, the parasite's tachyzoite stage is most likely to be present, as these are directly excreted into the milk and are relatively delicate compared to other stages in the parasite's life cycle. It is generally believed that *T. gondii* tachyzoites do not survive pasteurization and are vulnerable to the acidic environment of gastric secretions (EFSA, 2018). Various techniques, including PCR, tissue culture, and in vivo bioassays, have been used to detect *T. gondii* in raw milk from infected animals (Dehkordi et al., 2013). A recent study conducted in southern Italy sampled 21 milk samples from three different sheep farms and identified one milk sample as positive using a PCR assay (Vismarra et al., 2017). A previous study in Italy, focusing on goat milk, revealed that 13% of the 77 samples tested positive using a *T. gondii*-specific PCR test (Mancianti et al., 2013). Despite tachyzoites being considered relatively fragile when compared to other stages involved in transmission, a recent study (Koethe et al., 2017) demonstrated that *T. gondii* tachyzoites were capable of surviving for at least one hour in gastric fluids when mixed with various volumes of cow's milk samples that were experimentally spiked with the parasite. This milk-gastric fluid mixture increased the overall pH, which enabled the tachyzoites to survive long enough to potentially pass through the stomach and gain entry to the intestine, where they could infect the host (EFSA, 2018).

Meat

The primary livestock species, including cattle, small ruminants, pigs, poultry, and horses, serve as sources for meat-borne toxoplasmosis. Assessing the prevalence of *Toxoplasma* in meat-producing animals can offer insights into the risk to humans. Many studies, such as Tenter et al. (2000), employ indirect detection methods like serology to estimate the seroprevalence of *T. gondii*. However, serology can only be indicative of the risk of human infection if there exists a correlation between seroprevalence and the presence of tissue cysts in meat. Opsteegh et al. (2016) demonstrated a strong correlation between the detection of antibodies to *T. gondii* and the direct detection of the parasite in pigs, small ruminants, and chickens. However, the predicting value of seroprevalence for DNA detection in cattle muscles was found to be low (Opsteegh et al., 2011a). While serology can assist in identifying a risk to consumers with certain species, such as pigs, small ruminants, and chickens, its utility may be limited with other animals like horses and cattle. Furthermore, tissue cysts have been identified in seronegative pigs (4.9%), sheep and goats (1.8% and 2.0%), and chickens (1.8%), signifying that a negative serological result does not necessarily guarantee the absence of *T. gondii* in the meat (EFSA, 2018).

From a public health perspective, the lack of information regarding the prevalence of *T. gondii* tissue cysts in horses and cattle is a significant data gap, given that beef is a major meat source in many European countries, and horse meat is consumed in some regions often undercooked or raw (EFSA, 2018). This information is crucial for evaluating studies that rely on serology to detect *T. gondii* in meat samples. Numerous serological studies have been conducted (as reviewed by Tenter et al., 2000), and seroprevalence can vary widely, ranging from a few percent to over 80% in pigs and small ruminants, depending on the husbandry system. This suggests that pork and mutton are important sources of *Toxoplasma* infection for humans (EFSA, 2018). In the Netherlands and Italy, quantitative risk assessments were conducted for meat-borne toxoplasmosis, revealing that beef, rather than pork or mutton, contributed to the majority of the predicted human cases (Opsteegh et al., 2011b, Bellucco et al., 2018). In Table 1 are presented the principle detection methods in for *Toxoplasma gondii* in foods.

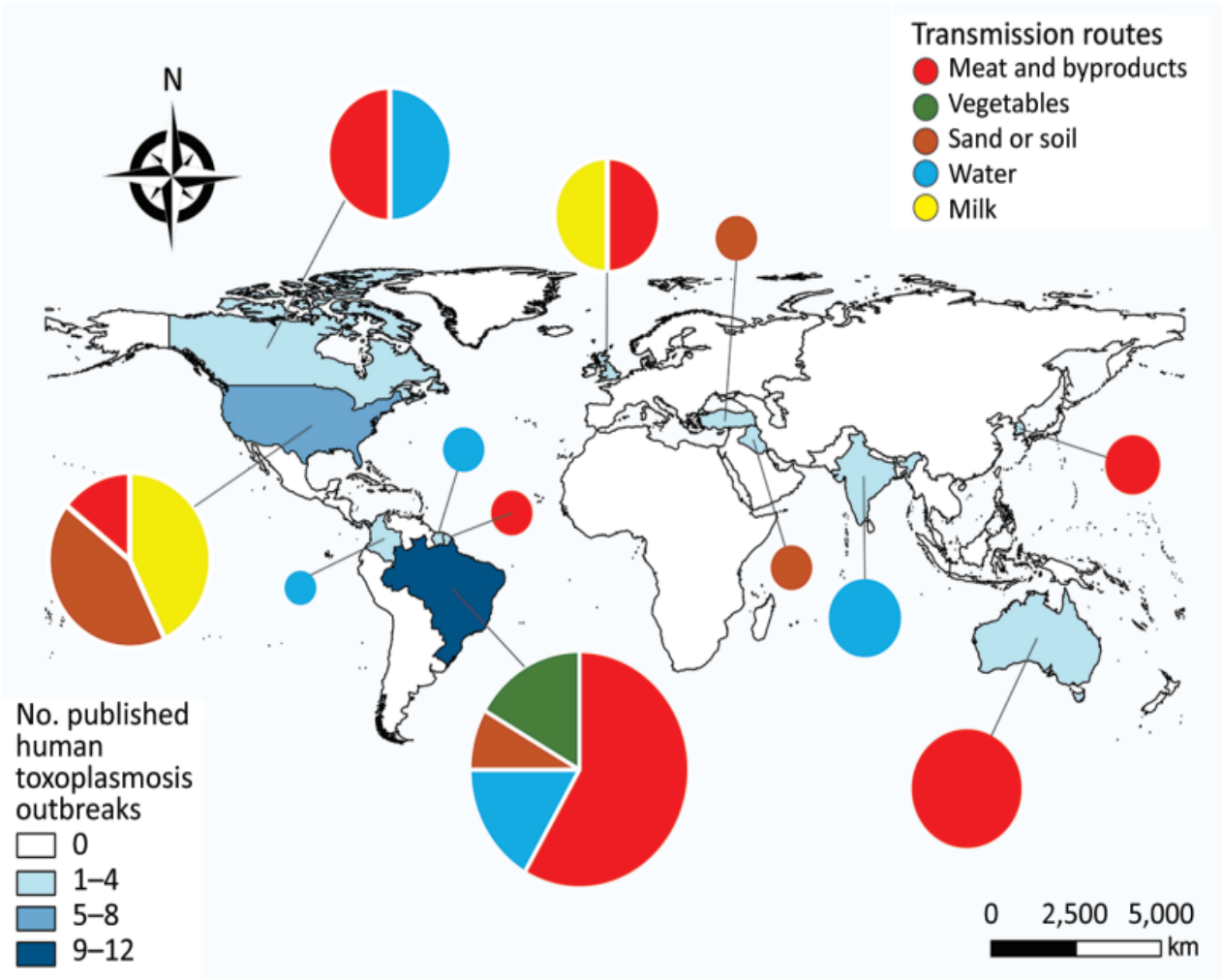


Figure 5 most probable transmission routes on documented human outbreaks of toxoplasmosis (from Pinto-Ferreira et al., 2019).

Table 1. Summary overview of the main detection methods for Toxoplasma gondii in food products (EFSA, 2018).

Detection Method	Type of food	Direct/indirect	Demonstration of viability/infectivity	Comments
Cat bioassay	Meat, shellfish, milk products	Direct	Yes	Seronegative cats fed test samples of food and their faeces checked for oocysts, and blood for seroconversion. Cats can be fed large quantities of food
Mouse bioassay	Meat, shellfish, milk products	Direct	Yes	Homogenates of food samples are inoculated into mice followed by clinical monitoring and demonstration of T. gondii in body tissues and seroconversion
PCR	Meat, shellfish, milk products	Direct	No	The B1 gene and the 529 bp repeat element are the most common targets. Various systems are used; conventional, nested and real-time PCR. A magnetic capture-based PCR technique detects 1 tissue cyst in 100 g of meat. In addition, sporulated oocysts can be identified using RT-PCR
Loop-mediated isothermal amplification (LAMP)	Meat, fresh produce	Direct	No	Unlike PCR, amplification products from LAMP cannot be sequenced. Recent adaptation to a lateral-flow dipstick method for rapid results
Microscopy	Meat, fresh produce	Direct	No	Detection based on morphology and staining using specific conjugated antibodies. Limited sensitivity for direct use on food samples, but useful to confirm infection on mouse and cat bioassays. Technique is labour intensive and

				requires an experienced technician
In vitro culture	Liquid samples where tachyzoites or bradyzoites may be present, e.g. meat homogenates or milk samples	Direct	Yes	Tachyzoites and bradyzoites (tissue cysts) may be cultured in a wide variety of cell lines with vero cells being commonly used. In vitro cultures are mostly used to prepare antigen or for strain isolation after bioassays. Not common to use directly on food samples
Specific antibodies	Liquid samples from meat juices where antibodies may be present. Blood samples from food animals	Indirect	No	The detection of specific antibodies in food animals confirms the animal has been infected with T. gondii and has had an immune response to the parasite. The correlation of seropositivity and the presence of tissue cysts vary according to different livestock species

1.3 *Toxoplasma gondii* Pathogenesis: Diverse Infection Patterns and Clinical Consequences Arising from Specific Host-Parasite Interactions

The outcome of a *T. gondii* infection is subjected to variation, contingent on the genetic background and immune status of the host, as well as the genotype of the parasite involved in the infection. Host species, and even subspecies, can exhibit different resistance or susceptibility to infection, as exemplified in the case of rodents, as reported by Hassan et al. (2019) and Mukhopadhyay et al. (2020). There appears to be a trend wherein hosts that have evolved alongside the parasite over time tend to develop greater resistance to the disease. A prime example is the lethal impact of the infection on Australian marsupials, whose evolutionary history has largely occurred in the absence of felids (Innes, 1997).

The genotype of *T. gondii* strains to which the host is exposed also plays a crucial role. For instance, type I strains are more prevalent in North and Southeast Asia, where *Mus musculus castaneus* and *M. musculus musculus* are the dominant mice subspecies. Type I strains are non-lethal to these mice but exhibit extreme virulence toward *M. m. domesticus*, the primary subspecies in Europe and North America. In contrast, type II and III strains, predominant in Europe and North America, generally do not cause mortality in *M. m. domesticus* (Shwab et al., 2014; Mukhopadhyay et al., 2020).

Certain animals display a higher degree of resistance to *Toxoplasma* infection, often experiencing inapparent infections or mild, transient symptoms during the acute phase, despite chronic infection could persist throughout their lifetimes. This group encompasses species such as cattle, pigs, and humans, among others. This resilience can be attributed to the prolonged co-evolution of these species with the domestic cat, stemming from their domestication by humans. Essentially, an ongoing evolutionary arms race characterized by mutual selection pressures between the parasite's virulence factors and the host's immune defences could account for the varying susceptibility to toxoplasmosis among different species, as suggested by some authors (Gazzinelli et al. 2014; Mukhopadhyay et al., 2020).

The host's immune status significantly influences the development of the disease. *Toxoplasma gondii* is widely recognized as the most prevalent opportunistic pathogen in patients with AIDS. Notably, *Toxoplasma* encephalitis stands out as the most frequently observed neurological disorder in HIV-infected patients in affluent nations, and it is also noted

as one of the primary neurological disorders affecting individuals with HIV in African regions (Howlett et al., 2019). Moreover, numerous reports have documented cases of infection following immunosuppressive treatments or transplantation procedures (Collazos, 2003; Ajzenberg et al., 2002b, 2009). Additionally, immune response imbalances during pregnancy emerge as one of the main contributing factors to the diverse clinical manifestations of *Toxoplasma* infection during pregnancy (Dos Santos et al., 2023).

In order to streamline the intricate web of pathological pathways resulting in various clinical manifestations, we've outlined the three primary pathological patterns that may emerge post-infection: acquired acute infection, congenital infection and chronic infection. Embracing the One Health approach, we aim to harmonize the pathological aspects observed in both human and animal cases, while also pinpointing species-specific distinctions in certain instances.

- **Acquired acute infection:** Natural infections primarily occur through the consumption of meat containing tissue cysts or the ingestion of oocyst-contaminated food or water. In the initial phase of infection (known as the acute stage), which occurs within 4 to 12 days after ingestion, bradyzoites or sporozoites invade the intestinal epithelial cells, multiply, and then locally spread to mesenteric lymph nodes and various organs via the lymphatic and bloodstream routes (parasitemia) (Dubey, 2022). This acute phase may manifest with non-specific clinical signs like low-grade fever, difficult breathing, joint pain, fatigue, or swollen lymph nodes. The invasion of the intestinal epithelium progresses to cause enteritis and necrotic lesions in the intestine and mesenteric lymph nodes. The necrotic damage is a result of the intracellular growth of tachyzoites and can also extend to various organs, such as the lungs, liver, eyes, heart, or adrenal glands, following the widespread dissemination of the parasite, particularly in visceral tissues.

In the case of individuals with weakened immune systems, the initial phase of enteroepithelial invasion and subsequent dissemination can lead to aggravated outcomes affecting vital organs, such as toxoplasmic pneumonitis, encephalitis, or myocarditis, and in severe instances, it can result in a fatal acute toxoplasmosis accompanied by multiorgan failure and death.

There are three primary clinical scenarios of immunosuppression to consider: certain viral infections that compromise the immune system (e.g., human immunodeficiency virus

[HIV] in humans, feline immunodeficiency virus [FIV] and feline leukemia virus [FeLV] in cats), organ transplantation, and the use of immunosuppressive chemotherapy in the treatment of malignant diseases (Davidson et al., 1993; Wang et al., 2017, Calero-Bernal and Gennari, 2019).

In addition to the risk posed by primary infection in immunocompromised individuals, there is a higher likelihood of disease reactivation due to the rupture of tissue cysts from a previous asymptomatic infection (Ajzenberg et al., 2009; Wang et al., 2017). Cerebral toxoplasmosis is a frequent cause of extensive brain lesions in AIDS patients and presents a potentially life-threatening danger to other immunocompromised patients, typically associated with the reactivation of a latent previous cerebral toxoplasmosis (Wang et al., 2017; Schlüter and Barragan, 2019).

- **Congenital infection**

In pregnant individuals who contract the infection, a process known as vertical transmission could occurs, wherein tachyzoites are transferred to the foetus, resulting in congenital transmission. Parasitaemia during pregnancy can lead to placentitis, with tachyzoites breaching the placental blood barrier and invading foetal organs, thereby compromising the normal developmental process (Dubey, 2022; Schlüter and Barragan, 2019). Similar to the manifestations seen in a chronically reactivated infection, congenital infection primarily affects the central nervous system of the foetus (Wang et al., 2017; Schlüter and Barragan, 2019).

Congenital infection is characterized by a wide array of forms, dependent on the gestational stage when the infection occurs. These manifestations range from early embryonic death with reabsorption in the case of early gestation, to stillbirth or neonatal death during mid-gestation, and in some instances, the birth of transplacentally infected offspring during late gestation (Dubey, 2022; Khan and Khan, 2018).

Toxoplasma gondii induces distinct histological lesions in both the placenta and the foetus. In the placenta typically multifocal necrosis and mineralization of cotyledonary villi were observed (Dubey, 1989). In the foetus, they commonly entail infiltrations of various immune cells, often accompanied by necrosis affecting multiple organs (Dubey, 2022).

Research on *T. gondii* vertical transmission has primarily focused on humans and small ruminants due to its elevated prevalence and significant impact in these species.

In humans, congenitally infected infants commonly exhibit symptoms such as hydrocephalus or microcephalus, cerebral calcifications, retinochoroiditis, and long-term debilitating consequences. Retinochoroiditis or neurological complications may also manifest later in life (Dubey and Jones, 2008; Singh et al., 2016; Daher et al., 2021). Ocular toxoplasmosis stands out as one of the most common clinical manifestations of *T. gondii* infection congenitally acquired in chronic stage. It is primarily characterized by necrotizing retinitis with secondary choroiditis (retinochoroiditis), typically adjacent to a pigmented retinochoroidal scar. While it frequently occurs in congenital infections and among immunocompromised hosts, it can also affect immunocompetent patients (Butler et al., 2013).

Similarly, to what happens in humans, in small ruminants' infection during pregnancy often leads to the expulsion of small mummified foetuses or the birth of weak lambs, depending on whether the infection occurs during mid or late gestation, respectively (Stelzer et al., 2019; Dubey et al., 2020a).

Cases of congenital transmission associated with toxoplasmosis reactivation during pregnancy have been documented in women (Ladas et al., 1999; Silveira et al., 2003; Garweg et al., 2005). While recrudescence is common in successive goat pregnancies, its significance remains a topic of debate in pregnant sheep (Dubey, 1982; Trees and Williams, 2005).

On the other hand, cattle and horses are generally considered highly resistant to clinical toxoplasmosis, with limited reports of reproductive failure, while pigs fall within an intermediate range of susceptibility (Canada et al., 2002; Dubey, 2022; Sah et al., 2019; Nayeri et al., 2021). There are other animal species where transplacental transmission of *T. gondii* can occur, but its clinical significance is comparatively less notable. These species include cats and dogs (Bresciani et al., 2009; and Calero Bernal and Gennari, 2019), rodents (Freyre et al., 2001; Mercier et al., 2013), and white-tailed deer (Dubey et al., 2008).

- **Chronic infection**

Following the acute phase, the host typically gains control over the infection by developing humoral and cellular immunity against the parasite, leading to inflammation and the initiation of the chronic phase. Subsequently, as the inflammatory stage subsides the process of clearing the infection commences and tachyzoites migrate from predominantly visceral tissues to immunoprivileged organs such as the brain, eyes, and muscle tissues. In these organs, they undergo a transformation into encysted bradyzoites remaining protected from the host's immune response (Dubey, 2022). Nevertheless, reports of cyst ruptures, resulting in clusters of tissue cysts being observed in brain tissues and even a reactivation of the disease, are documented (Ferguson et al., 1989; Ajzenberg et al., 2009; Cerutti et al., 2020). The precise mechanism behind relapses, often linked to immunosuppressive conditions remains largely elusive (Dubey, 2022).

Toxoplasma gondii is typically classified as a primarily neurotropic pathogen, indicating its strong preference for the central nervous system in comparison to other organs (Schlüter and Barragan, 2019), in fact it's essential to conduct a thorough examination of the central nervous system (CNS) localization of *T. gondii* in the chronic phase. Upon successfully breaching the blood-brain barrier, a cascade of responses is triggered, including the host's immune reaction and factors related to intracellular neuronal homeostasis. As a result, *T. gondii* tachyzoites undergo a transition into bradyzoite cysts, which represent a defining feature of the chronic phase of the infection. The immune system maintains control over these intraneuronal cysts, yet it does not entirely destroy them (Blanchard et al., 2019; Matta et al., 2021). Despite the relatively slow replication of bradyzoites, their growth impacts the integrity of neuronal structures and disrupts their connectivity. Additionally, these tissue cysts induce a brain-specific immune response (Matta et al., 2021).

Astrocytes, microglia, and neurons, play a role in the intracerebral immune response by producing cytokines, chemokines, and expressing immune-regulatory cell surface molecules, such as major histocompatibility (MHC) antigens (Blanchard et al., 2019; Matta et al., 2021). Additionally, circulating immune cells are recruited to the CNS infection site, contributing to the overall immune response against the infection (Harker et al., 2013; Torgerson et al., 2015).

The release of various cytokines, including interleukins (IL12, IL1β, IL-6), inducible nitric oxide synthase (iNOS), along with tumor necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ), which activates IFN-inducible GTPases, inhibit *T. gondii* replication (Daher et al., 2021). Infiltrating CD4+ and CD8+ T cells primarily release IFN-γ (Matta et al., 2021); as a result, the immune response leads to brain inflammation, causing ventricular dilatation and disrupting the structure and connectivity of neurons (Hermes et al., 2008; Xiao et al., 2016). These morphological changes entail alterations in fiber density, a loss of fiber continuity and, a reduction in dendritic spines leading to a decrease in network activity (Daher et al., 2021).

The presence of bradyzoite cysts in neural tissues has been demonstrated to induce changes in behaviour, as indicated by Webster et al. (2013). *Toxoplasma gondii* appears to modify the behaviour of rodents increasing their vulnerability to predation by cats. This alteration involves reducing neophobia and diminishing predator vigilance behaviours (Webster, 1994, 2001, 2007; Webster et al., 1994; Berdoy et al., 1995; Lamberton et al., 2008). These analogous effects, which can be considered nonadaptive or residual manipulations according to Flegr et al. (2011), encompass a range of behavioural consequences in *T. gondii*-infected humans. These consequences span from subtle shifts in personality traits to more severe outcomes, including an increased susceptibility to schizophrenia, (Flegr 2013a; b), and an association with a higher risk of developing various neuropsychiatric disorders, as noted by Milne et al. (2020a).

This association has also been explored in other animal species during natural infections, particularly in wildlife. An intriguing phenomenon observed in wild red foxes known as "Dopey Fox Syndrome" (DFS) is characterized by unusual behavioural traits similar to those exhibited by infected rodents and has been directly linked to *T. gondii* infection (Milne et al. in 2020b). A recent study by Mayer et al. (2020) reported that wolves testing positive for *T. gondii* displayed a greater propensity to make high-risk decisions, such as dispersing and assuming leadership roles within packs. This behaviour resulted in an increase in spatial overlap and disease transmission between wolves and cougars, which are the definitive hosts of the parasite.

1.4 Diagnosis: clinical and epidemiological importance

The diagnosis of *T. gondii* infection holds significant importance for the surveillance, prevention, and management of toxoplasmosis. This is particularly relevant in cases of abortion or pregnancy-related complications, where a wide range of viral, bacterial, and parasitic pathogens could potentially be involved, especially in livestock's. As a result, laboratory analyses play an important role in the diagnosis of *T. gondii*, and these analyses can be categorized as follows (Liu et al., in 2015)

- **Direct Diagnosis:**

  - Microscopic Techniques: involve the detection of the parasite's oocysts by light microscopy. Although being conventionally employed, shows low sensitivity and the need of skilled personnel to ensure reliable detection outcomes. Oocysts can be observed in fecal samples from felids, as well as in water, soil, or food stuff e.g., vegetables or fruits. Oocysts may even be found in aerosols following filtration and centrifugation processes (Lass et al., 2009, 2017; Sroka et al., 2010; Mancianti et al., 2015; Caradonna et al., 2017; López Ureña et al., 2022). Alternatively, direct observation of tachyzoites is feasible in various tissues and body fluids from infected hosts, such as broncho-alveolar lavage (BAL), cerebrospinal fluid (CSF), aqueous humor (AH), vitreous humor (VH), amniotic and peritoneal/ascitic fluids, or skin aspirates, among others (De Salvador-Guillouët et al., 2006; Stajner et al., 2013; Pena et al., 2014).

  - Bioassays: The use of bioassays involving laboratory animals, such as mice and cats, has traditionally been regarded as the gold standard for diagnosing toxoplasmosis (Ghosn et al., 2003; Costache et al., 2013), and represents the most commonly employed approaches to establish the viability of *T. gondii* in positive matrices. Cats, in particular, serve as the most sensitive bioassay model for detecting *T. gondii* in meat due to their capacity to consume significantly larger quantities of tissues (500 g or more) and subsequently excrete millions of oocysts after ingesting just one bradyzoite (Dubey, 2022). Nevertheless, the complexity, cost and time-intensive nature of this technique have led to the initial preference for serological or molecular methods in *Toxoplasma* diagnosis. However, it's important to recognize that bioassays in mice remain an

invaluable means to obtaining *T. gondii* isolates, as emphasized in several studies (Su and Dubey, 2020; Dubey et al. 2020c).

- Molecular diagnosis:

  - **PCR**: The first PCR method for *T. gondii* detection, targeting the B1 gene, was established in 1989 (Burg et al., 1989). In their pioneering investigation into the applicability of the B1 gene for diagnosing human toxoplasmosis, they achieved the successful amplification and identification of DNA from a single organism directly from a crude cell lysate. This remarkable level of sensitivity allowed them to detect the B1 gene within purified DNA samples containing as few as 10 parasites, even in the presence of 100,000 human leukocytes. Subsequently, this method found widespread use in prenatal diagnosis of congenital toxoplasmosis and the diagnosis of *T. gondii* infection in immunocompromised patients, as indicated by Parmley et al. (1992), Ho-Yen et al. (1992), and Lamoril et al. (1992). In 2000, Homan et al. made a significant contribution by identifying a novel 529 bp fragment, which is found in repetitions ranging from 200 to 300-fold within the *T. gondii* genome. This 529 bp segment served as the basis for the development of an exceptionally sensitive and specific PCR method for diagnostic purposes. Sequencing of this gene revealed a highly conserved nucleotide sequence among various *T. gondii* strains and isolates, as demonstrated by Reischl et al. (2003). Notably, PCR using the 529 bp repeat element was reported to be 10 to 100 times more sensitive than the B1 gene, as highlighted by Homan et al. (2000) and Reischl et al. (2003). Additionally, a few studies have explored the use of multicopy ITS-1 and 18S rDNA as alternative targets, demonstrating a sensitivity level similar to that of the B1 gene, as observed in the research conducted by Hurtado et al. (2001) and Calderaro et al. (2006). To further improve sensitivity and specificity, nested PCR techniques built upon the B1 gene, the 529 bp repeat element, and ITS-1 sequences have been devised, as documented by Fallahi et al. (2014) and Jones et

al. (2000). An interesting observation from Fallahi et al. (2013) is that, when compared to B1-nested PCR, 529 bp-nested PCR exhibited the ability to detect *T. gondii* DNA even in seronegative samples. In nested PCR, two sets of primers are employed across two sequential PCR reactions, with the products of the first reaction serving as templates for the second. When targeting a specific gene, nested PCR outperforms conventional PCR in terms of sensitivity, a fact well-documented by Liu et al. (2015). The 529 bp repeat element-nested PCR boasts a detection limit of 640 fg of parasite DNA, whereas the rate for B1-nested PCR stands at 5.12 pg (Fallahi et al., 2014). Additionally, the nested PCR approach targeting the B1 gene proves to be more sensitive than targeting the ITS-1 sequence, according to Jones et al. (2014). It is important to emphasize that, even in the context of highly specific PCR assays, it remains crucial to verify the sequence of the PCR product to ensure adequate diagnostic specificity, as highlighted by Liu et al. (2015).

- **qPCR:** Real-time PCR, also known as quantitative PCR, possesses the capability to identify and quantify low concentrations of target DNA as well as determine the initial number of specific template DNA copies. During each cycle of amplification, the resulting product is measured using probes or intercalating dyes, and its quantity can be ascertained by comparing it to a known standard concentration (Liu et al., 2015). This real-time PCR technique has proven effective in the detection of *T. gondii* DNA in various human samples, including blood, cerebrospinal fluid, aqueous humor, and amniotic fluid, as outlined by Kompalic-Cristo et al. (2007) and Nogui et al. (2009). Moreover, it has found application in assessing the progression of toxoplasmosis and the effectiveness of treatment, as it can estimate the severity of *T. gondii* infection, as noted by Menotti et al. (2003). When it comes to diagnosing congenital toxoplasmosis, the real-time PCR assay featuring the B1 gene is considered the top-performing method in comparison to conventional PCR and nested-PCR (Teixeira et al., 2013). Real-time PCR

offers the advantage of being a rapid, closed-tube system, thereby minimizing the potential risk of contamination and delivering reproducible quantitative results (Liu et al., 2015). Opsteegh et al. (2010) have introduced a sequence-specific magnetic capture technique for the extraction of *T. gondii* DNA from substantial tissue samples. This innovative method addresses the challenge of the uneven distribution of *T. gondii* tissue cysts and the small sample size. When coupled with real-time PCR, this approach has been applied to meat samples (Opsteegh et al., 2010, 2019). This combination enhances the probability of detecting the parasite's DNA in large muscle samples, particularly in cattle, where tissue cyst distribution poses a detection challenge.

- **LAMP**: Loop-mediated isothermal amplification (LAMP) is a distinctive DNA amplification method operating under constant temperature conditions. It utilizes four primers designed to recognize six distinct regions on the target DNA (Notomi et al., 2000). In comparison to conventional PCR, LAMP demonstrates slightly higher sensitivity but falls slightly short of the sensitivity of real-time PCR (Lin et al., 2012). LAMP assays have been developed for various target genes, including *T. gondii* SAG1, the 529-bp repetitive element, B1, SAG2, GRA1, oocyst wall protein (OWP) genes, and 18S rRNA, tailored for use with veterinary and medical samples, as well as water samples (Liu et al., 2015). An application of LAMP centered on the SAG1 gene demonstrated its ability to detect *T. gondii* in the blood of experimentally infected pigs as early as 2 days post-infection, suggesting that LAMP could serve as a valuable tool for early toxoplasmosis diagnosis, especially in settings lacking sophisticated and costly equipment (Wang et al., 2015). The B1- and OWP-LAMP assays, with a detection limit of 0.1 tachyzoites DNA, were shown to be effective in water samples, highlighting LAMP as a rapid, specific, and highly sensitive method for detecting *Toxoplasma* contamination in environmental samples (Sotiriadou et al., 2008). It's important to note that LAMP appears to be particularly sensitive to contamination, emphasizing the need for

rigorous quality control measures to eliminate the risk of false positives (Liu et al., 2015).

o Histopathology and immunohistochemistry assessments: have been used as complementary techniques in various clinical scenarios for diagnosing toxoplasmosis. This is particularly important in cases of abortions in sheep and goats, where the preferred materials for histological examination are the placenta and fetal brain (Uggla et al., 1987; Pereira-Bueno et al., 2004; Partoandazanpoor et al., 2020; Dubey et al., 2020b).

o Direct serological antigenic assays: given the challenging interpretation of serological outcomes based on the detection of specific antibodies (indirect diagnosis) for ascertaining the timing of infection (recent or chronic), a valuable alternative method involves detecting circulating antigens in the serum (direct diagnosis). The sandwich enzyme-linked immunosorbent assay (sandwich ELISA) has been specifically developed for this purpose. This assay entails utilizing a well-coated with a specific antibody, which is exposed to the serum sample, along with an enzyme-conjugated antibody designed to recognize the antibody-antigen complex that forms (Liu et al., 2015). While not widely used, several intriguing cases and applications have been documented in the literature (Attallah et al., 2006; Dautu et al., 2008).

• Indirect Diagnosis

o Serological Assays: Serological tests are indispensable not only for specific diagnoses but also for epidemiological investigations. These serology methods have been employed on both adult and fetal serum samples, as well as on other fluids that may contain antibodies, such as fetal fluids (e.g., thoracic fluids) or meat juices (Ranucci et al. 2012; Vismarra et al., 2016; Gazzonis et al., 2020). Several serological assays have been developed, including the dye test (DT), modified agglutination test (MAT), enzyme-linked immunosorbent assay (ELISA), immunosorbent agglutination assay (ISAGA), indirect fluorescent antibody test (IFAT), indirect hemagglutination assays (IHA), latex agglutination test (LAT), and Western-Blot (WB), aimed at detecting different classes of antibodies (Liu et al., 2015). Nonetheless, in both clinical and scientific reports across various hosts, ELISA, IFAT, and MAT are the most widely employed techniques (Calero-Bernal and Gennari, 2019, Dubey et al., 2020a, c; Nayeri et al., 2021).

- **MAT** serves as a highly sensitive serological approach for detecting *T. gondii* IgG antibodies in both herds and wild animals. This method primarily identifies antibodies present in animal tissue fluid, serum, or plasma, and stands out as the most widely employed and cost-effective diagnostic method for detecting *T. gondii* infection. Notably, it is also one of the simplest techniques available and doesn't require specialized equipment (de Barros et al., 2022).

- **ELISA** has been used as de most reliable, convenient, cost-effective, and widely used method for identifying exposure to *T. gondii* in hosts. It boasts the advantage of requiring only a small sample volume and can be partially automated, rendering it suitable for extensive screening. Additionally, various ELISA variants can differentiate between different immunoglobulin classes, thereby facilitating the determination of the infection stage (de Barros et al., 2022). Diverse methodologies for ELISA standardization have been established, involving a range of antigens (such as native, recombinant, and chimeric), secondary antibodies, and antibody binding reagents (Ferra et al., 2015). There are commercially available ELISA multispecies kits designed for detecting *T. gondii* antibodies in domestic animals, simplifying the process of conducting large-scale and regular screening.

- **IFAT** is a straightforward assay employed for the identification of IgG and IgM antibodies against *T. gondii* in both humans and animals (Miller et al., 2002). Commercially, there are fluorescence-labelled antibodies accessible for different species. Nevertheless, this test need the use of a fluorescence microscope for examination, and the results are visually interpreted, but can be affected by individual variability. Finding species-specific conjugates may pose challenges, but for some species it has been used as the serologic gold standard test, thanks to his specificity and sensitivity (Dubey, 2022).

The binding affinity (avidity) of specific antibodies to antigens undergoes changes during the course of infection, with avidity values initially being low and subsequently increasing with the progression of the infection. IgG avidity ELISA tests have the capability to differentiate between high and low avidity IgG proteins in serum, enabling the distinction

between recent and chronic infections, as demonstrated in several studies (Villard et al., 2013; Caballero-Ortega et al., 2008).

- **Typing Techniques**

To achieve genotype analysis, different approaches have been proposed: PCR-RFLP (Sibley and Boothroyd, 1992; Howe and Sibley, 1995; Howe et al., 1997; Biñas and Johnson, 1998; Su et al., 2006), gene sequencing (multilocus genes sequencing and whole genome sequencing (Su et al., 2012; Lorenzi et al., 2016), and microsatellite sequences analysis (Ajzenberg et al., 2002a, 2010; Joeres et al., 2023). These Genotyping techniques, to be effective required a high amount of parasite's DNA, often deriving from culture isolates. Isolating *T. gondii* from clinical and non-clinical samples presents a complex challenge. For this reason, serotyping offers an alternative method for characterizing *T. gondii* strains, eliminating the need for parasite isolation (Sousa et al., 2023). This technique relies on the recognition of strain-specific polymorphic peptides by antibodies (Konga et al., 2003; Sousa et al., 2008, 2009; Maksimov et al., 2012). The section will not delve further into genotyping techniques since they will be the focal point of Chapter 7.

CHAPTER 2: Thesis objective: elucidate the epidemiological characteristics of *Toxoplasma gondii* in Northern Italy within the context of a One Health approach.

Toxoplasma gondii stands as one of the most widely distributed and successful opportunistic parasites globally. Its significance extends to both medical and veterinary domains. It has the capacity to infect virtually all warm-blooded animal species, making it a substantial concern not only for public health but also for the livestock industry and wildlife conservation programs. Although toxoplasmosis typically remains latent and asymptomatic, it can manifest as severe clinical conditions in individuals with compromised immune systems and pregnant hosts (Dubey, 2022).

Toxoplasmosis imposes a significant health burden on a staggering one-third of the world's population. Nonetheless, toxoplasmosis remains classified as a neglected disease, primarily because it often shows a subclinical course in both animals and humans upon infection. Moreover, in intermediate hosts designated for meat production, the presence of the parasite does not manifest visually, resulting in the development of microscopic cysts within various organs. Consequently, the control of the foodborne transmission of the disease is challenging, and currently, it lacks regulation in Europe, especially from an inspection perspective.

The primary objective of this Ph.D. thesis was to delve into the epidemiological patterns of toxoplasmosis in Northern Italy. This study aimed to comprehensively explore various aspects of the parasite's infection dynamics in several host species, adopting a one-health perspective.

The initial research phase involved performing a retrospective survey to investigate the parasite's distribution in the Emilia-Romagna region, specifically the Province of Bologna (Emilia-Romagna Region), as detailed in Chapter 3. This survey relied on the examination of serological data collected from multiple diagnostic centres within the area of interest.

The research subsequently directed its attention towards three primary thematic areas. In line with the One Health framework, three distinct categories of hosts were deliberately selected, each assuming unique epidemiological roles in the context of *Toxoplasma* infection:

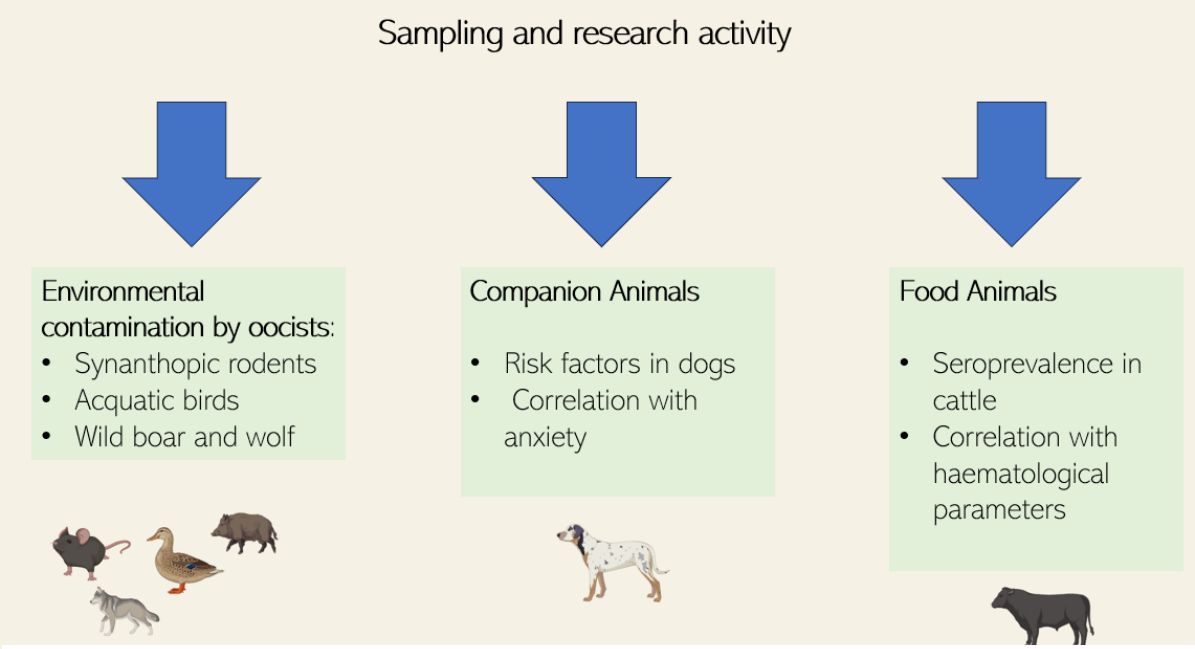


Figure 6: three main areas of interest considered in the sampling

- ✓ Wildlife, which functioned as indicators of environmental contamination via oocysts and thereby offered insights into the spatial dispersion of the parasite within natural ecosystems;
- ✓ Companion animals, notably canines, in which toxoplasmosis could bear clinical relevance;
- ✓ Animals intended for human consumption, specifically bovines, for which toxoplasmosis demonstrate limited clinical significance but held importance in terms of meat-borne transmission in humans (Fig. 6).

Regarding the first research area (Chapter 4), among the various wildlife species examined, the initial investigation centered on synanthropic rodents. Within these species, which have coevolved with human habits and tend to prefer urban and peri-urban areas over completely wild environments, the presence of protozoa belonging to the Sarcocystidae family was investigated (figure 7).

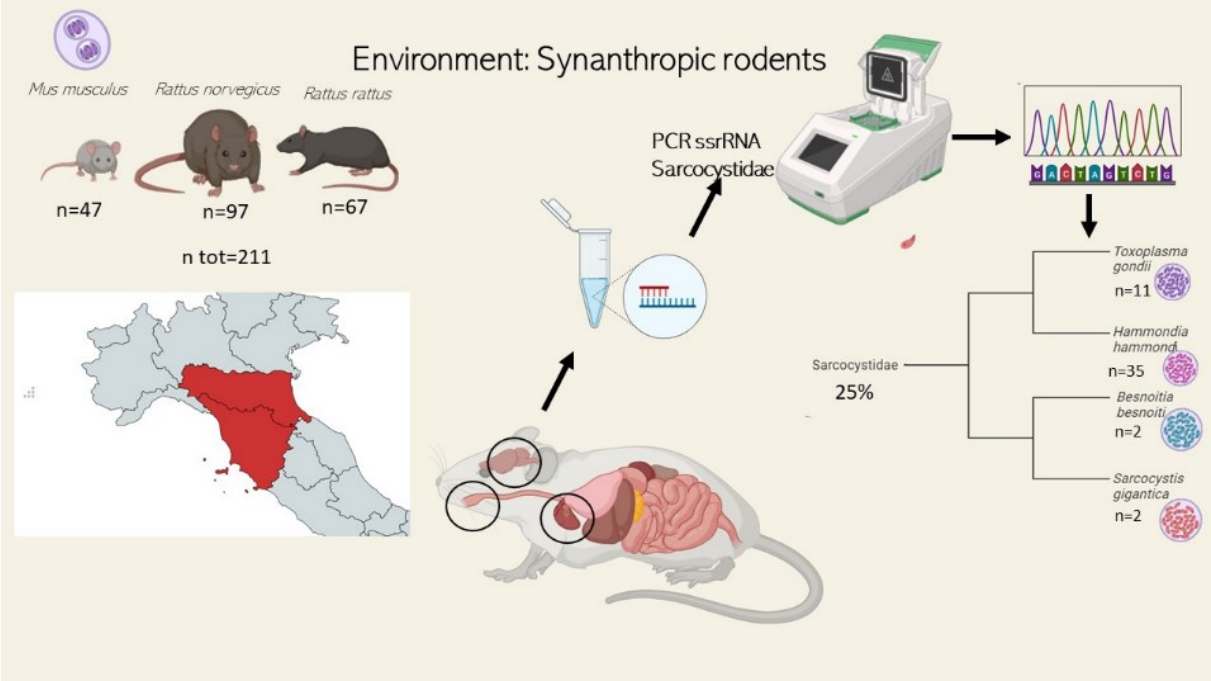


Figure 7 graphical characterization of Chapter 4.1

In this context, another cohort of animals sampled belong to migratory aquatic avian species, collected during the hunting season, with the primary objective of *T. gondii* detection through molecular methodologies. The rationale for focusing on these species is grounded in their distinctive ecological and behavioural attributes, which exhibit strong associations with aquatic ecosystems. Positive findings in these avian subjects may serve as sentinel indicators of contamination within aquatic ecological contexts. Furthermore, considering their migratory nature, they present the potential to act as reservoirs for infections originating from their respective source regions (figure 8).

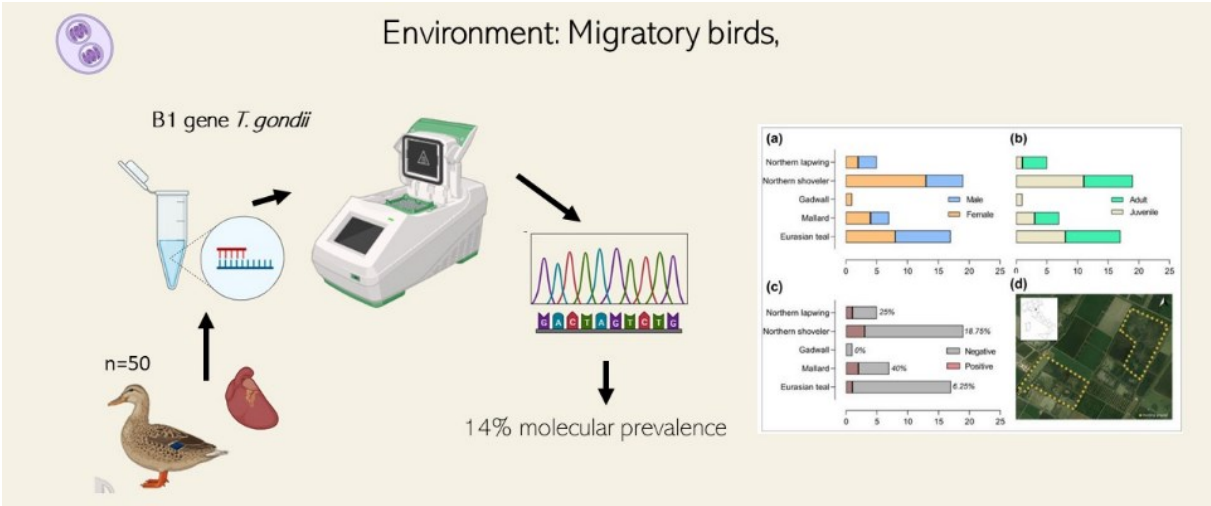


Figure 8 graphical characterization of Chapter 4.2

The final research investigation outlined in Chapter 4 pertains to a serological study of *T. gondii* in two pivotal species within the wild ecological balance: wild boar and wolves. While both can become infected through carnivorous pathways, potentially not exclusively signifying contamination from oocysts within a particular territory, their significance in this context lies in the crucial predator-prey dynamics inherent in the *T. gondii* life cycle, particularly in natural environments. In these cases, seroprevalence serves the purpose of providing data on past exposure to the parasite, which is valuable for epidemiological purposes (figure 9)

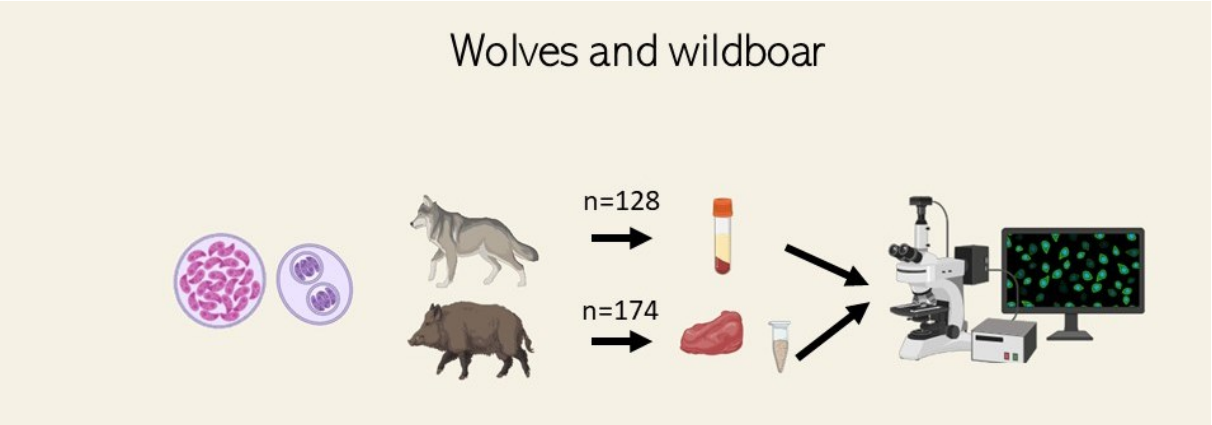


Figure 9 graphical characterization of Chapter 4.3

In Chapter 5, the research exclusively focused on dogs as companion animals potentially susceptible to clinical forms of toxoplasmosis. An initial project entailed the examination of risk factors for seropositivity in dogs with various lifestyles and roles,

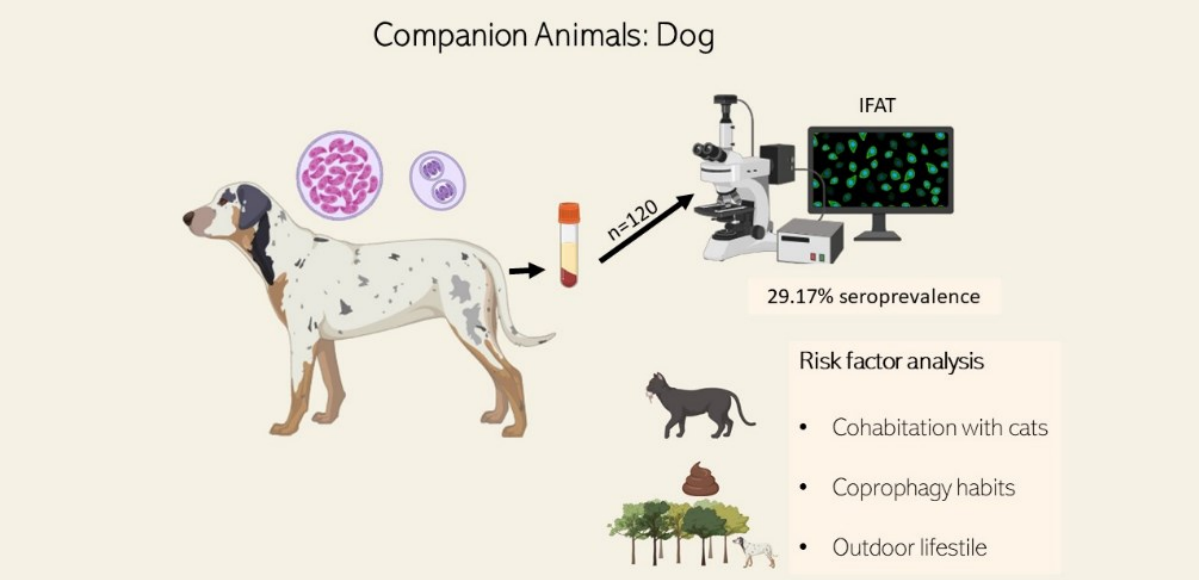


Figure 10 graphical characterization of Chapter 5.1

specifically companion dogs, guard dogs, hunting dogs, and truffle-hunting dogs. Through the collection of anamnestic data, this investigation aimed to identify risk factors associated with the development of antibody titers against *T. gondii* in these different categories of dogs (figure 10).

A second research consisted in a double-blinded study finalized to explore the correlation between serological evidence of exposure to *T. gondii* and pathological anxiety in companion dogs (figure 11).

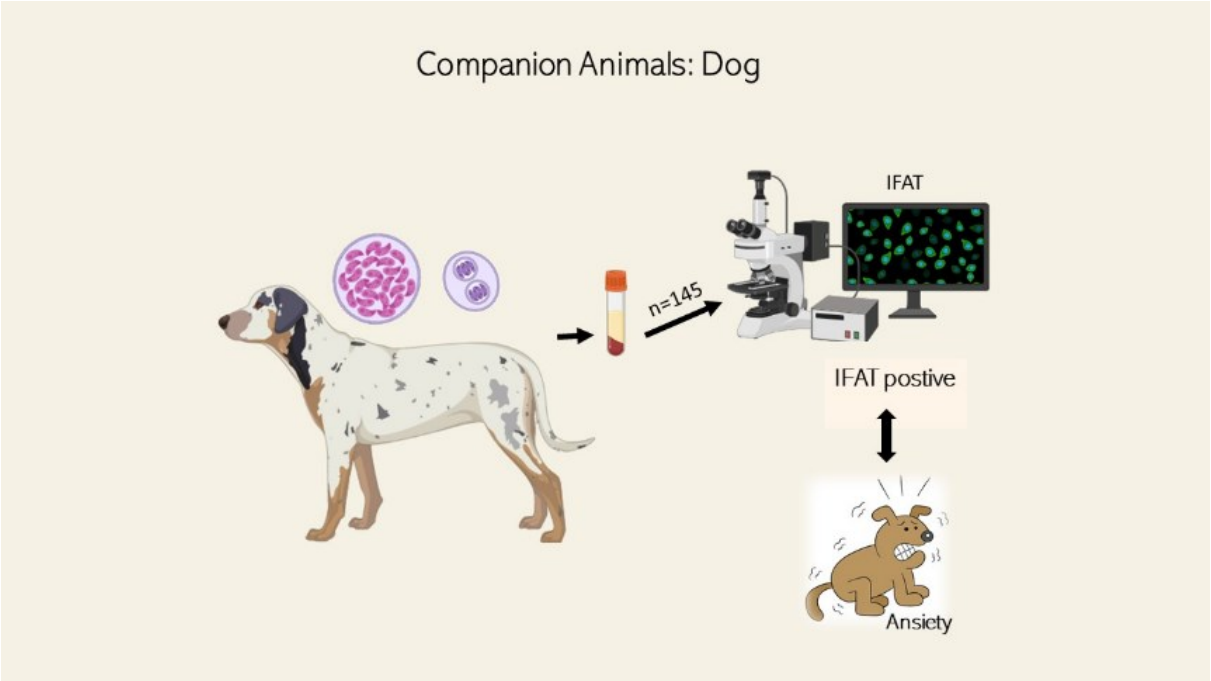


Figure 11 graphical characterization of Chapter 5.2

The investigations concerning the bovine species, as outlined in Chapter 6, focused on beef cattle. The study evaluated the antibody trends over consecutively samplings in subjects naturally exposed to the parasite in a feedlot setting. Additionally, a correlation was established between seropositivity, antibody titers, and hematological variations (figure 12). Within the scope of this research, another study unveiled a novel coinfection between *Sarcocystis hominis* and *Toxoplasma gondii* in a case of Bovine Eosinophilic Myositis (BEM), marking the first instance of such a co-occurrence. This diagnostic investigation utilized skeletal muscle tissue as well as meat juice as matrices for direct diagnosis, as a novel matrix for the diagnosis of these tissue forming apicomplexan.

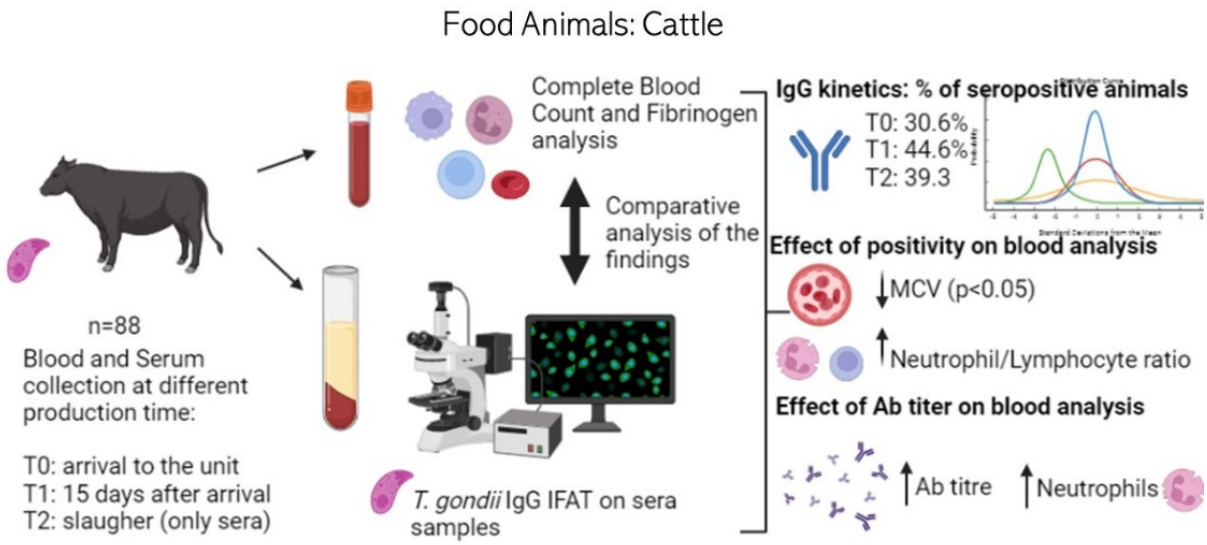


Figure 12 graphical characterization of Chapter 6

Finally, in the seventh chapter, the research aimed at molecularly characterizing strains of *T. gondii* collected over the three years of the candidate's doctoral research to identify the circulating variants in the geographical area of interest. Three different genotyping techniques were applied to DNA-positive *T. gondii* samples, and complete genotyping profiles were obtained from some of them. This provided, for the first time in Italy, a comprehensive characterization of *T. gondii* strains (figure 13).

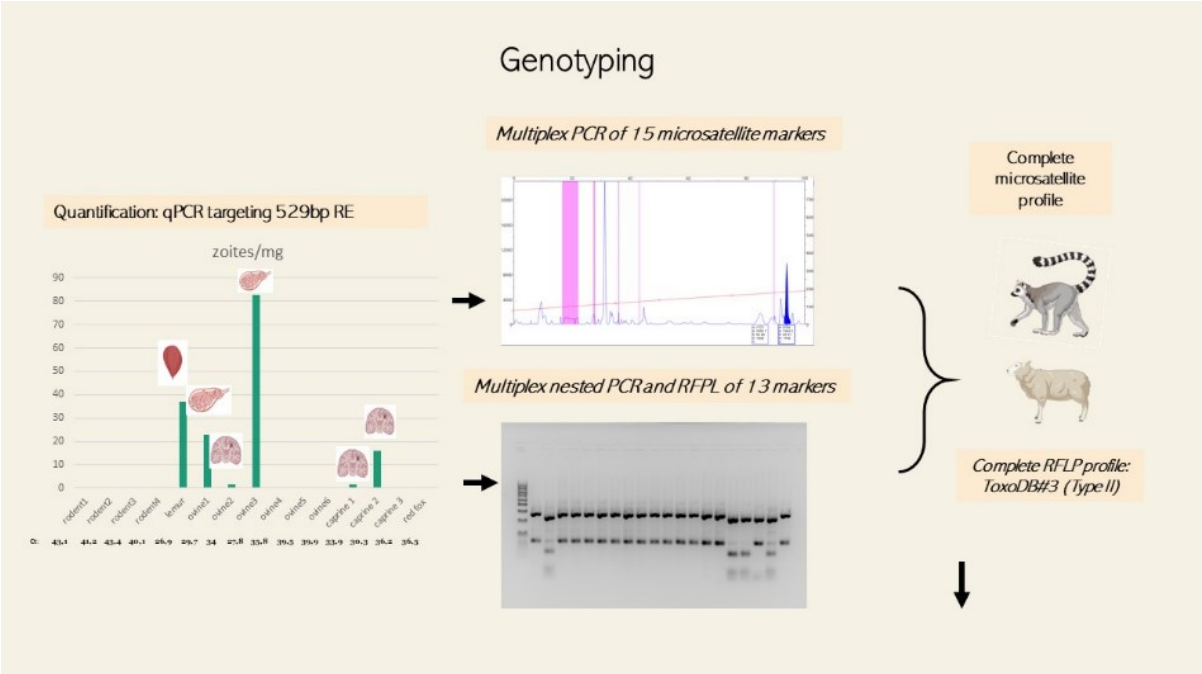


Figure 13 graphical characterization of chapter 7

REFERENCES

Ajzenberg D, Bañuls AL, Dardé ML. (2002a). Microsatellite analysis of *Toxoplasma gondii* shows considerable polymorphism structured into two main clonal groups. *Int J Parasitol.* 32: 27–38. 10.1016/S0020-7519(01)00301-0

Ajzenberg D, Cogné N, Paris L, Bessières MH, Thulliez P, Filisetti D, Pelloux H, Marty P, Dardé ML. (2002b) Genotype of 86 *Toxoplasma gondii* isolates associated with human congenital toxoplasmosis, and correlation with clinical findings. *J Infect Dis.* 186 (5) :684-689. doi: 10.1086/342663.

Ajzenberg D, Bañuls AL, Su C, Dumètre A, Demar M, Carme B, Dardé ML. (2004) Genetic diversity, clonality and sexuality in *Toxoplasma gondii*. *Int J Parasitol*. 34 (10): 1185- 1196. doi:10.1016/j.ijpara.2004.06.007.

Ajzenberg D, Collinet F, Mercier A, Vignoles P, Dardé ML. (2010). Genotyping of *Toxoplasma gondii* isolates with 15 microsatellite markers in a single multiplex PCR assay. *J Clin Microbiol.* 48: 4641–5. doi:10.1128/JCM.01152-10

Ajzenberg D, Yera H, Marty P, Paris L, Dalle F, Menotti J, Aubert D, Franck J, Bessières MH, Quinio D, Pelloux H, Delhaes L, Desbois N, Thulliez P, Robert-Gangneux F, KauffmannLacroix C, Pujol S, Rabodonirina M, Bougnoux ME, Cuisenier B, Duhamel C, Duong TH, Filisetti D, Flori P, Gay-Andrieu F, Pratlong F, Nevez G, Totet A, Carme B, Bonnabau H, Dardé ML, Villena I. (2009). Genotype of 88 *Toxoplasma gondii isolates* associated with toxoplasmosis in immunocompromised patients and correlation with clinical findings. *J Infect Dis*. 199 (8): 1155-1167. doi: 10.1086/597477.

Aksoy U, Marangi M, Papini R, Ozkoc S, Delibas SB and Giangaspero A. (2014). Detection of *Toxoplasma gondii* and *Cyclospora cayetanensis* in *Mytilus galloprovincialis* from Izmir Province coast (Turkey) by real time PCR/High-Resolution Melting analysis (HRM). *Food Microbiol*. 44: 128–135. doi: 10.1016/j.fm.2014.05.012.

Attallah AM, Ismail H, Ibrahim AS, Al-Zawawy LA, El-Ebiary MT, El-Waseef AM. (2006). Immunochemical identification and detection of a 36-kDa *Toxoplasma gondii* circulating antigen in sera of infected women for laboratory diagnosis of toxoplasmosis. *J Immunoassay Immunochem*. 27 (1) :45-60. doi:10.1080/15321810500403748.

Barhoumi B; Lemenach K; Devier MH; Ameur WB; Etcheber H; Budzinski H; Cachot J; Driss MR. (2014). Polycyclic Aromatic Hydrocarbons (PAHs) in Surface Sediments from the Bizerte Lagoon, Tunisia: Levels, Sources, and Toxicological Significance. *Environ. Monit. Assess.* 186: 2653–2669. doi: 10.1007/s10661-013-3569-5.

Beck HP, Blake D, Dardé ML, Felger I, Pedraza-Díaz S, Regidor-Cerrillo J, Gómez-Bautista M, Ortega-Mora LM, Putignani L, Shiels B, Tait A, Weir W. (2009). Molecular approaches to diversity of populations of apicomplexan parasites. *Int J Parasitol*. 39 (2): 175-189. doi: 10.1016/j.ijpara.2008.10.001.

Belluco S, Simonato G, Mancin M, Pietrobelli M, Ricci A. (2018). *Toxoplasma gondii* infection and food consumption: A systematic review and meta-analysis of case-controlled studies. *Crit Rev Food Sci Nutr.* 58 (18): 3085-3096. doi: 10.1080/10408398.2017.1352563.

Berdoy M, Webster JP, Macdonald DW. (1995). Parasite altered behaviour: is the effect of *Toxoplasma gondii* on *Rattus norvegicus* specific? *Parasitology* 111: 403–409 doi: 10.1017/s0031182000065902.

Bertranpetit E, Jombart T, Paradis E, Pena H, Dubey J, Su C, Mercier A, Devillard S, Ajzenberg D. (2017). Phylogeography of *Toxoplasma gondii* points to a South American origin. *Infect Genet Evol.* 48: 150-155. doi: 10.1016/j.meegid.2016.12.020.

Biñas, M., & Johnson, A. M. (1998). A polymorphism in a DNA polymerase alpha gene intron differentiates between murine virulent and avirulent strains of *Toxoplasma gondii*. *Int. J. Parasitol*. 28 (7): 1033–1040. https://doi.org/10.1016/s0020-7519(98)00052-6

Blanchard N; Dunay IR; Schlüter D. (2015). Persistence of *Toxoplasma gondii* in the central nervous system: A fine-tuned balance between the parasite, the brain and the immune system. *Parasite Immunol*. 37: 150–158 doi: 10.1111/pim.12173.

Bohne W, Gross U, Heesemann J. (1993). Differentiation between mouse-virulent and -avirulent strains of *Toxoplasma gondii* by a monoclonal antibody recognizing a 27-kilodalton antigen. *J Clin Microbiol*. 31: 1641-1643. doi: 10.1128/JCM.31.6.1641-1643.1993.

Boothroyd JC, Dubremetz JFF. (2008). Kiss and spit: the dual roles of *Toxoplasma* rhoptries. *Nat Rev Microbiol.* 6: 79–88. doi: 10.1038/nrmicro1800.

Boughattas S. (2017). *Toxoplasma* infection and milk consumption: meta-analysis of assumptions and evidences. *Crit Rev Food Sci Nutr*. 57: 2924–2933. doi: 10.1080/10408398.2015.1084993.

Bowie WR; King AS; Werker DH; Isaac-Renton JL; Bell A; Eng SB; Marion SA. (1997). Outbreak of Toxoplasmosis Associated with Municipal Drinking Water. The BC *Toxoplasma* Investigation Team. *Lancet*. 350, 173–177. doi: 10.1016/s0140-6736(96)11105-3.

Boyle JP, Rajasekar B, Saeij JPJ, Ajioka JW, Berriman M, Paulsen I, Roos DS, Sibley LD, White MW, Boothroyd JC. (2006). Just one cross appears capable of dramatically altering the population biology of a eukaryotic pathogen like *Toxoplasma gondii*. *Proc Natl Acad Sci U S A*. 103: 10514-10519. doi: 10.1073/pnas.0510319103

Bradley PJ, Sibley LD. (2007). Rhoptries: an arsenal of secreted virulence factors. *Curr Opin Microbiol*. 10: 582–7. doi: 10.1016/j.mib.2007.09.013.

Bresciani K D, Costa AJ, Toniollo GH, Luvizzoto MC, Kanamura CT, Moraes FR, Perri SH, Gennari SM. (2009). Transplacental transmission of *Toxoplasma gondii* in reinfected pregnant female canines. *Parasitol. Res*. 104: 1213–1217. doi: 10.1007/s00436-008-1317-5

Burg JL, Grover CM, Pouletty P, Boothroyd JC. (1989). Direct and sensitive detection of a pathogenic protozoan, *Toxoplasma gondii*, by polymerase chain reaction. *J Clin Microbiol*. 27: 1787-1792. doi: 10.1128/JCM.27.8.1787-1792.1989.

Butler NJ, Furtado JM, Winthrop KL, Smith JR. (2013). Ocular toxoplasmosis II: clinical features, pathology and management. *Clin Exp Ophthalmol*. 41: 95-108. doi: 10.1111/j.1442-9071.2012.02838.x.

Caballero-Ortega H, Quiroz-Romero H, Olazarán-Jenkins S, Correa D. (2008). Frequency of *Toxoplasma gondii* infection in sheep from a tropical zone of Mexico and temporal analysis of the humoral response changes. *Parasitology*. 135: 897-902. doi: 10.1017/S0031182008004460.

Calderaro A, Piccolo G, Gorrini C, Peruzzi S, Zerbini L, Bommezzadri S, Dettori G, Chezzi C. (2006). Comparison between two real-time PCR assays and a nested-PCR for the detection of *Toxoplasma gondii*. *Acta Biomed*. 77: 75–80.

Calero-Bernal R, Gennari SM. (2019). Clinical Toxoplasmosis in Dogs and Cats: An Update. *Front. Vet. Sci*. 6: 54. doi: 10.3389/fvets.2019.00054

Canada N, Meireles CS, Rocha A, da Costa JM, Erickson MW, Dubey JP. (2002). Isolation of viable *Toxoplasma gondii* from naturally infected aborted bovine fetuses. *J Parasitol.* 88: 1247-1248. doi:10.1645/0022-3395(2002)088

Caradonna T, Marangi M, Del Chierico F, Ferrari N, Reddel S, Bracaglia G, Normanno G, Putignani L, Giangaspero A. (2017). Detection and prevalence of protozoan parasites in ready-to-eat packaged salads on sale in Italy. *Food Microbiol*. 67: 67-75. doi:10.1016/j.fm.2017.06.006.

Cerutti A, Blanchard N, Besteiro S. (2020). The Bradyzoite: A Key Developmental Stage for the Persistence and Pathogenesis of Toxoplasmosis. *Pathogens* (Basel, Switzerland), 9: 234. doi: 10.3390/pathogens9030234

Chaichan P, Mercier A, Galal L, Mahittikorn A, Ariey F, Morand S, Boumédiène F, Udonsom R, Hamidovic A, Murat JB, Sukthana Y, Dardé ML. (2017). Geographical distribution of *Toxoplasma gondii* genotypes in Asia: A link with neighbouring continents. *Infect Genet Evol*. 53: 227-238. doi:10.1016/j.meegid.2017.06.002.

Collazos J. (2003). Opportunistic infections of the CNS in patients with AIDS: diagnosis andmanagement. *CNS Drugs*. 17: 869-887. doi:10.2165/00023210-200317120-00002.

Cook AJ, Gilbert RE, Buffolano W, Zufferey J, Petersen E, Jenum PA, Foulon W, Semprini AE, Dunn DT. (2000). Sources of *Toxoplasma* infection in pregnant women: European multicentre case-control study. *European Research Network on Congenital Toxoplasmosis*. *BMJ.* 321: 142-147. doi: 10.1136/bmj.321.7254.142.

Costache CA, Colosi HA, Blaga L, Györke A, Paştiu AI, Colosi IA, Ajzenberg D. (2013). First isolation and genetic characterization of a *Toxoplasma gondii* strain from a symptomatic human case of congenital toxoplasmosis in Romania. *Parasite*. 20: 11. doi:10.1051/parasite/2013011.

Daher D, Shaghlil A, Sobh E, Hamie M, Hassan ME, Moumneh MB, Itani S, El Hajj R, Tawk L, El Sabban M, El Hajj H. (2021). Comprehensive Overview of *Toxoplasma gondii*-induced and associated diseases. *Pathogens*.10: 1351. doi:10.3390/pathogens10111351.

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

Dautu G, Ueno A, Miranda A, Mwanyumba S, Munyaka B, Carmen G, Kariya T, Omata Y, Saito A, Xuan X, Igarashi M. (2008). *Toxoplasma gondii*: detection of MIC10 antigen in sera of experimentally infected mice. *Exp Parasitol.* 118: 362-371. doi:10.1016/j.exppara.2007.09.010.

de Barros R A M, Torrecilhas A C, Marciano M A M, Mazuz M L, Pereira-Chioccola V L, Fux B. (2022). Toxoplasmosis in human and animals around the world. diagnosis and perspectives in the One Health Approach. *Acta tropica*. 231: 106432. 10.1016/j.actatropica.2022.106432

De Berardinis A, Paludi D, Pennisi L, Vergara A. (2017). *Toxoplasma gondii*, a foodborne pathogen in the swine production chain from a European perspective. *Foodborne Pathog Dis.* 14: 637-648. doi:10.1089/fpd.2017.2305.

De Salvador-Guillouët F, Ajzenberg D, Chaillou-Opitz S, Saint-Paul MC, Dunais B, Dellamonica P, Marty P. (2006). Severe pneumonia during primary infection with an atypical strain of *Toxoplasma gondii* in an immunocompetent young man. *J Infect.* 53: e47-50. doi: 10.1016/j.jinf.2005.10.026

Dehkordi FS, Haghighi Borujeni MR, Rahimi E and Abdizadeh R. (2013). Detection of *Toxoplasma gondii* in raw caprine, ovine, buffalo, bovine, and camel milk using cell cultivation, cat bioassay, capture ELISA, and PCR methods in Iran. *Foodborne Pathog. Dis*. 10: 120–125. doi: 10.1089/fpd.2012.1311.

Djokić V, Blaga R, Aubert D, Durand B, Perret C, Geers R, Ducry T, Vallee I, DjurkovićDjaković O, Mzabi A, Villena I, Boireau P. (2016). *Toxoplasma gondii* infection in pork produced in France. *Parasitology*. 143 (5): 557-567. doi:10.1017/S0031182015001870.

Dos Santos PV, de Toledo D N M, de Souza D M S, Menezes T P, Perucci LO, Silva Z M, Teixeira D C, Vieira EWR, de Andrade-Neto V F, Guimarães N S, Talvani A. (2023). The imbalance in the relationship between inflammatory and regulatory cytokines during gestational toxoplasmosis can be harmful to fetuses: A systematic review. *Front. Immunol.* 14: 1074760. https://doi.org/10.3389/fimmu.2023.1074760.

du Plooy I, Mlangeni M, Christian R, Tsotetsi-Khambule A M (2023). An African perspective on the genetic diversity of *Toxoplasma gondii*: A systematic review. *Parasitology*. 150: 551–578. doi: 10.1017/S0031182023000252.

Dubey JP. (1982) Repeat transplacental transfer of *Toxoplasma gondii* in dairy goats. *J Am Vet Med Assoc.* 180 (10): 1220-1221.

Dubey JP. (2022) Toxoplasmosis of animals and humans. CRC Press, Boca Raton, FL, USA.

Dubey JP, Frenkel JK. (1972). Cyst-induced toxoplasmosis in cats. *J Protozool*. 19: 155–77.

Dubey JP, Jones JL. (2008). *Toxoplasma gondii* infection in humans and animals in the United States. *Int J Parasitol.* 38: 1257-1278. doi: 10.1016/j.ijpara.2008.03.007.

Dubey JP, Murata FHA, Cerqueira-Cézar CK, Kwok OCH, Su C. (2020a). Economic and public health importance of *Toxoplasma gondii* infections in sheep: 2009-2020. *Vet Parasitol*. 286: 109195. doi: 10.1016/j.vetpar.2020.109195.

Dubey JP, Murata FHA, Cerqueira-Cézar CK, Kwok OCH. (2020b). Public health and economic importance of *Toxoplasma gondii* infections in goats: The last decade. *Res Vet Sci.* 132: 292-307. doi: 10.1016/j.rvsc.2020.06.014.

Dubey JP, Cerqueira-Cézar CK, Murata FHA, Kwok OCH, Hill D, Yang Y, Su C. (2020c). All about *Toxoplasma gondii* infections in pigs: 2009-2020. *Vet Parasitol.* 288: 109185. doi: 10.1016/j.vetpar.2020.109185.

Dubey JP, Lindsay DS, Speer CA. (1998). Structures of *Toxoplasma gondii* tachyzoites, bradyzoites, and sporozoites and biology and development of tissue cysts. *Clin Microbiol Rev*. 11:267-299.

Dubey JP, Su C, Cortés JA, Sundar N, Gomez-Marin JE, Polo LJ, Zambrano L, Mora LE, Lora F, Jimenez J, Kwok OC, Shen SK, Zhang X, Nieto A, Thulliez P. (2006). Prevalence of Toxoplasma gondii in cats from Colombia, South America and genetic characterization of T. gondii isolates. Vet Parasitol. 141(1-2):42-47. doi: 10.1016/j.vetpar.2006.04.037.

Dubey J P, Velmurugan G V, Ulrich V, Gill J, Carstensen M, Sundar N, Kwok O C, Thulliez P, Majumdar D, Su C. (2008). Transplacental toxoplasmosis in naturally-infected white-

tailed deer: Isolation and genetic characterisation of *Toxoplasma gondii* from foetuses of different gestational ages. Int. J Parasitol. 38: 1057–1063. doi: 10.1016/j.ijpara.2007.11.010

European Food Safety Authority (EFSA). (2007). Surveillance and monitoring of *Toxoplasma* in humans, food and animals. EFSA J. 583:1-64. doi: 10.2903/j.efsa.2007.583

European Food Safety Authority (EFSA). (2011). Scientific Opinion on the public health hazards to be covered by inspection of meat (swine). EFSA J. 9(10):2351. doi: 10.2903/j.efsa.2011.2351.

European Food Safety Authority (EFSA). (2018). Public health risks associated with food-borne parasites. EFSA J. 16(12) :5495. doi: 10.2903/j.efsa.2018.5495

Fallahi Sh, Kazemi B, Seyyed tabaei S J, Bandehpour M, Lasjerdi Z, Taghipour N, Zebardast N, Nikmanesh B, Omrani V F, Ebrahimzadeh F. (2014). Comparison of the RE and B1 gene for detection of *Toxoplasma gondii* infection in children with cancer. *Parasitol. Int*. 63: 37–41. doi: 10.1016/j.parint.2013.08.005

Ferguson D J, Hutchison W M, Pettersen E. (1989). Tissue cyst rupture in mice chronically infected with *Toxoplasma gondii*. An immunocytochemical and ultrastructural study. *Parasitol. Res*. 75(8): 599–603.

Ferra B, Holec-Gąsior L, Kur J. (2015). Serodiagnosis of *Toxoplasma gondii* infection in farm animals (horses, swine, and sheep) by enzyme-linked immunosorbent assay using chimeric antigens. *Parasitol. Int*. 64: 288–294. doi:10.1016/j.parint.2015.03.004.

Ferreira FP, Caldart ET, Freire RL, Mitsuka-Bregano R, de Freitas FM, Miura AC. (2018). The effect of water source and soil supplementation on parasite contamination in organic vegetable gardens. *Rev. Bras. Parasitol*. Vet. 27: 327–337 doi: 10.1590/S1984-296120180050.

Flegr J. (2013b). How and why *Toxoplasma* makes us crazy. *Trends Parasitol*. 29 : 156–163. doi :10.1016/j.pt.2013.01.007

Flegr, J. (2013a) Influence of latent *Toxoplasma* infection on human personality, physiology and morphology: Pros and cons of the *Toxoplasma*–human model in studying the manipulation hypothesis. *J. Exp. Biol*. 216: 127–133 doi: 10.1242/jeb.073635.

Flegr J, Lenochová P, Hodný Z, Vondrová M. (2011). Fatal attraction phenomenon in humans: cat odour attractiveness increased for toxoplasma-infected men while decreased for infected women. PLOS Negl. Trop. Dis. 5: e1389. doi :10.1371/journal.pntd.0001389

Frénal K, Dubremetz JF, Lebrun M, Soldati-Favre D. (2017). Gliding motility powers invasion and egress in Apicomplexa. Nat Rev Microbiol. 15:645–60. doi: 10.1038/nrmicro.2017.86.

Frenkel JK, Dubey JP, Miller NL. (1970). Toxoplasma gondii in cats: fecal stages identified as coccidian oocysts. *Science*. 167:893-896. doi: 10.1126/science.167.3919.893.

Freyre A, Correa O, Falcón J, Mendez J, González M, Venzal J M. (2001). Some factors influencing transmission of *Toxoplasma* in pregnant rats fed cysts. *Parasitol. Res*. 87: 941–944. doi :10.1007/s004360100486

Garweg JG, Scherrer J, Wallon M, Kodjikian L, Peyron F. (2005). Reactivation of ocular toxoplasmosis during pregnancy. *BJOG.* 112:241-242. doi: 10.1111/j.1471-0528.2004.00302.x.

Gazzinelli RT, Mendonça-Neto R, Lilue J, Howard J, Sher A. (2014). Innate resistance against *Toxoplasma gondii*: an evolutionary tale of mice, cats, and men. *Cell Host Microbe*. 15:132-138. doi: 10.1016/j.chom.2014.01.004.

Ghosn J, Paris L, Ajzenberg D, Carcelain G, Dardé ML, Tubiana R, Bossi P, Bricaire F, Katlama C. (2003). Atypical toxoplasmic manifestation after discontinuation of maintenance therapy in a human immunodeficiency virus type 1-infected patient with immune recovery. *Clin Infect Dis.* 37:e112-114. doi: 10.1086/378126.

Grigg ME, Boothroyd JC. (2001). Rapid identification of virulent type I strains of the protozoan pathogen *Toxoplasma gondii* by PCR-restriction fragment length polymorphism analysis at the B1 gene. *J Clin Microbiol*. 39:398-400. doi: 10.1128/JCM.39.1.398-400.2001.

Grigg ME, Ganatra J, Boothroyd JC, Margolis TP. (2001). Unusual abundance of atypical strains associated with human ocular toxoplasmosis. *J Infect Dis*. 184:633-639. doi:10.1086/322800.

Harker KS; Ueno N; Wang T; Bonhomme C; Liu W; Lodoen MB. (2013). *Toxoplasma gondii* modulates the dynamics of human monocyte adhesion to vascular endothelium under fluidic shear stress. *J. Leukoc. Biol*. 93: 789–800. doi: 10.1189/jlb.1012517.

Hassan MA, Olijnik AA, Frickel EM, Saeij JP. (2019). Clonal and atypical *Toxoplasma* strain differences in virulence vary with mouse sub-species. *Int J Parasitol*. 49:63-70. doi: 10.1016/j.ijpara.2018.08.007

Hermes G, Ajioka JW, Kelly KA, Mui E, Roberts F, Kasza K, Mayr T, Kirisits MJ, Wollmann R, Ferguson DJ, Roberts CW, Hwang JH, Trendler T, Kennan RP, Suzuki Y, Reardon C, Hickey WF, Chen L, McLeod R. (2008). Neurological and behavioral abnormalities, ventricular dilatation, altered cellular functions, inflammation, and neuronal injury in brains of mice due to common, persistent, parasitic infection. *J Neuroinflammation*. 5:48. doi: 10.1186/1742-2094-5-48.

Hiszczyńska-Sawicka E, Gatkowska JM, Grzybowski MM, Długońska H. (2014). Veterinary vaccines against toxoplasmosis. *Parasitology*. 141: 1365-7138. doi: 10.1017/S0031182014000481.

Hohweyer J; Dumètre A; Aubert D; Azas N; Villena I. (2013). Tools and Methods for Detecting and Characterizing *Giardia*, *Cryptosporidium*, and *Toxoplasma* Parasites in Marine Mollusks. J. Food Prot. 76:1649–1657. doi: 10.4315/0362-028X.JFP-13-002.

Howe DK, Honoré S, Derouin F, Sibley D. (1997). Determination of genotypes of *Toxoplasma gondii* strains isolated from patients with toxoplasmosis. *J Clin Microbiol.* 35: 1411–4. 10.1128/jcm.35.6.1411-1414.1997

Howe DK, Sibley LD. (1995). *Toxoplasma gondii* comprises three clonal lineages: correlation of parasite genotype with human disease. *J Infect Dis*. 172: 1561-1556. doi: 10.1093/infdis/172.6.1561.

Howlett W. P. (2019). Neurological disorders in HIV in Africa: a review. African health sciences, 19: 1953–1977. doi:10.4314/ahs.v19i2.19

Ho-Yen DO, Joss AW, Balfour AH, Smyth ET, Baird D, Chatterton JM. (1992), Use of the polymerase chain reaction to detect *Toxoplasma gondii* in human blood samples. *J Clin Pathol*. 45:910–3. doi: 10.1136/jcp.45.10.910.

Hurtado, A., Aduriz, G., Moreno, B., Barandika, J., & García-Pérez, A. L. (2001). Single tube nested PCR for the detection of *Toxoplasma gondii* in fetal tissues from naturally aborted ewes. Vet. Parasitol.102: 17–27. https://doi.org/10.1016/s0304-4017(01)00526-x

Innes EA. (1997). Toxoplasmosis: comparative species susceptibility and host immune response. Comp Immunol Microbiol Infect Dis. 20:131-138. doi: 10.1016/s0147-9571(96)00038-0

Joeres M, Cardron G, Passebosc-Faure K, Plaut N, Fernández-Escobar M, Hamilton C, O'Brien-Anderson L, Calero-Bernal R, Galal L, Lutterman C, Maksimov P, Conraths FJ, Dardé ML, Ortega-Mora L, Jokelainen P, Mercier A, Schares G. (2023). A ring trial to harmonize *Toxoplasma gondii* microsatellite typing: comparative analysis of results and recommendations for optimization. *Eur J Clin Microbiol Infect Dis* 42:803–818. doi:10.1007/s10096-023-04597-7

Jones JL, Dargelas V, Roberts J, Press C, Remington JS and Montoya JG. (2009). Risk factors for *Toxoplasma gondii* infection in the United States. *Clin. Inf. Dis*. 49: 878–884. doi: 10.1086/605433.

Jones C D, Okhravi N, Adamson P, Tasker S, Lightman S. (2000). Comparison of PCR detection methods for B1, P30, and 18S rDNA genes of *T. gondii* in aqueous humor Invest Ophthalmol Vis Sci. 41:634–644.

Kato K. (2018). How does *Toxoplasma gondii* invade host cells? *J Vet Med Sci.* 80:1702–6. doi: 10.1292/jvms.18-0344.

Khan A, Dubey JP, Su C, Ajioka JW, Rosenthal BM, Sibley LD. (2011). Genetic analyses of atypical *Toxoplasma gondii* strains reveal a fourth clonal lineage in North America. *Int J Parasitol.* 4:645-655. doi: 10.1016/j.ijpara.2011.01.005.

Khan A, Fux B, Su C, Dubey JP, Darde ML, Ajioka JW, Rosenthal BM, Sibley LD. (2007). Recent transcontinental sweep of *Toxoplasma gondii* driven by a single monomorphic chromosome. *Proc Natl Acad Sci U S A*. 104:14872-14877. doi: 10.1073/pnas.0702356104.

Khan A, Taylor S, Su C, Mackey AJ, Boyle J, Cole R, Glover D, Tang K, Paulsen IT, Berriman M, Boothroyd JC, Pfefferkorn ER, Dubey JP, Ajioka JW, Roos DS, Wootton JC, Sibley LD. (2005) Composite genome map and recombination parameters derived from three archetypal lineages of *Toxoplasma gondii*. *Nucleic Acids Res*. 33:2980-2992. doi: 10.1093/nar/gki604.

Khan K, Khan W. (2018). Congenital toxoplasmosis: An overview of the neurological and ocular manifestations. *Parasitol Int.* 67:715-721. doi: 10.1016/j.parint.2018.07.004.

Koethe M, Schade C, Fehlhaber K and Ludewig M (2017). Survival of *Toxoplasma gondii* tachyzoites in simulated gastric fluid and cow's milk. *Vet. Parasitol*. 233: 111–114. doi: 10.1016/j.vetpar.2016.12.010.

Kompalic-Cristo A, Frotta C, Suárez-Mutis M, Fernandes O, Britto C. (2007). Evaluation of a real-time PCR assay based on the repetitive B1 gene for the detection of *Toxoplasma gondii* in human peripheral blood. Parasitol. Res. 101: 619–625. doi: 10.1007/s00436-007-0524-9

Kong JT, Grigg ME, Uyetake L, Parmley S, Boothroyd JC. (2003). Serotyping of *Toxoplasma gondii* infections in humans using synthetic peptides. *J Infect Dis*.187:1484–95. doi: 10.1086/374647

Ladas ID, Rallatos CL, Kanaki CS, Damanakis AG, Zafirakis PK, Rallatos G. (1999). Presumed congenital ocular toxoplasmosis in two successive siblings. *Ophthalmologica*. 213:320-322. doi: 10.1159/000027446.

Lamberton PHL, Donnelly CA, Webster JP (2008). Specificity of the *Toxoplasma gondii*-altered behaviour to definitive versus non-definitive host predation risk. *Parasitology* 135: 1143–1150. doi: 10.1017/S0031182008004666.

Lamoril J, Molina J M, de Gouvello A, Garin Y J, Deybach J C, Modaï J, Derouin F. (1996). Detection by PCR of *Toxoplasma gondii* in blood in the diagnosis of cerebral toxoplasmosis in patients with AIDS. *J.Clin. Path.* 49: 89–92. doi: 10.1136/jcp.49.1.89

Lass A, Pietkiewicz H, Modzelewska E, Dumètre A, Szostakowska B, Myjak P. (2009). Detection of *Toxoplasma gondii* oocysts in environmental soil samples using molecular methods.*Eur J Clin Microbiol Infect Dis.* 28:599-605. doi: 10.1007/s10096-008-0681-5.

Lass A, Szostakowska B, Korzeniewski K, Karanis P. (2017). The first detection of *Toxoplasma gondii* DNA in environmental air samples using gelatine filters, real-time PCR and loopmediated isothermal (LAMP) assays: qualitative and quantitative analysis. *Parasitology*. 144:1791-1801. doi: 10.1017/S0031182017001172.

Lass A, Ma L, Kontogeorgos I, Zhang X, Li X, Karanis P. (2019). First Molecular Detection of *Toxoplasma gondii* in Vegetable Samples in China Using Qualitative, Quantitative Real-

Time PCR and Multilocus Genotyping. *Sci. Rep.* 9: 1–11. doi: 10.1038/s41598-019-54073-6.

Lin Z, Zhang Y, Zhang H, Zhou Y, Cao J, Zhou J. (2012). Comparison of loop-mediated isothermal amplification (LAMP) and real-time PCR method targeting a 529-bp repeat element for diagnosis of toxoplasmosis. *Vet. Parasitol*. 185: 296–300. doi: 10.1016/j.vetpar.2011.10.016

Lindsay DS, Collins MV, Mitchell SM, Wetch CN, Rosypal AC, Flick GJ, Zajac AM, Lindquist A, Dubey J.P. (2004). Survival of *Toxoplasma gondii* Oocysts in Eastern Oysters (*Crassostrea virginica*). *J. Parasitol*. 90: 1054–1057. doi: 10.1645/GE-296R

Liu Q, Wang ZD, Huang SY, Zhu XQ. (2015). Diagnosis of toxoplasmosis and typing of *Toxoplasma gondii*. *Parasit Vectors*. 8:292. doi: 10.1186/s13071-015-0902-6.

López Ureña N M, Chaudhry U, Calero Bernal R, Cano Alsua S, Messina D, Evangelista F, Betson M, Lalle M, Jokelainen P, Ortega Mora L M, Álvarez García G. (2022). Contamination of Soil, Water, Fresh Produce, and Bivalve Mollusks with *Toxoplasma* gondii Oocysts: A Systematic Review. Microorganisms, 10(3): 517. doi:10.3390/microorganisms10030517

Lorenzi H, Khan A, Behnke MS, Namasivayam S, Swapna LS, Hadjithomas M, Karamycheva S, Pinney D, Brunk BP, Ajioka JW, Ajzenberg D, Boothroyd JC, Boyle JP, Dardé ML, DiazMiranda MA, Dubey JP, Fritz HM, Gennari SM, Gregory BD, Kim K, Saeij JP, Su C,White MW, Zhu XQ, Howe DK, Rosenthal BM, Grigg ME, Parkinson J, Liu L, Kissinger JC, Roos DS, Sibley LD. (2016). Local admixture of amplified and diversified secreted pathogenesis determinants shapes mosaic Toxoplasma gondii genomes. *Nat Commun.* 7:10147. doi: 10.1038/ncomms10147.

Maksimov P, Zerweck J, Maksimov A, Hotop A, Gross U, Spekker K, Däubener W, Werdermann S, Niederstrasser O, Petri E, Mertens M, Ulrich RG, Conraths FJ, Schares G. (2012) Analysis of clonal type-specific antibody reactions in Toxoplasma gondii seropositive humans from Germany by peptide-microarray. *PLoS One*. 7:e34212. doi: 10.1371/journal.pone.0034212.

Mancianti F, Nardoni S, D'Ascenzi C, Pedonese F, Mugnaini L, Franco F and Papini R. (2013). Seroprevalence, Detection of DNA in Blood and Milk, and Genotyping of *Toxoplasma gondii* in a Goat Population in Italy. *Biomed Res. Int*., 6 pp. doi:10.1155/2013/905326

Mancianti F, Nardoni S, Mugnaini L, Zambernardi L, Guerrini A, Gazzola V, Papini RA. (2015). A retrospective molecular study of select intestinal protozoa in healthy pet cats from Italy. *J Feline Med Surg*. 17:163-167. doi: 10.1177/1098612X14533549.

Martorelli Di Genova B, Wilson SK, Dubey JP, Knoll LJ. (2019). Intestinal delta-6-desaturase activity determines host range for *Toxoplasma* sexual reproduction. *PLoS Biol*. 17:e3000364. doi: 10.1371/journal.pbio.3000364.

Matta SK, Rinkenberger N, Dunay IR, Sibley LD. (2021). *Toxoplasma gondii* infection and its implications within the central nervous system. *Nat Rev Microbiol*. 19:467-480. doi: 10.1038/s41579-021-00518-7.

Meisel R, Stachelhaus S, Mévélec MN, Reichmann G, Dubremetz JF, Fischer HG. (1996). Identification of two alleles in the GRA4 locus of *Toxoplasma gondii* determining a differential epitope which allows discrimination of type I versus type II and III strains. *Mol Biochem Parasitol*. 81:259-263. doi: 10.1016/0166-6851(96)02719-3.

Menotti J, Vilela G, Romand S, Garin Y J, Ades L, Gluckman E, Derouin F, Ribaud P. (2003). Comparison of PCR-enzyme-linked immunosorbent assay and real-time PCR assay for diagnosis of an unusual case of cerebral toxoplasmosis in a stem cell transplant recipient. *J. Clin. Microbiol.* 41: 5313–5316. doi:10.1128/JCM.41.11.5313-5316.2003

Mercier A, Garba M, Bonnabau H, Kane M, Rossi JP, Dardé ML, Dobigny G. (2013). Toxoplasmosis seroprevalence in urban rodents: a survey in Niamey, Niger. *Mem Inst Oswaldo Cruz*.108:399-407. doi: 10.1590/S0074-0276108042013002.

Meyer C J, Cassidy K A, Stahler E E, Brandell E E, Anton C B, Stahler D R, Smith DW. (2022). Parasitic infection increases risk-taking in a social, intermediate host carnivore. *Communications biology*, 5: 1180. doi:10.1038/s42003-022-04122-0

Milne G, Webster JP, Walker M. (2020a). *Toxoplasma gondii*: An Underestimated Threat? *Trends Parasitol*. 36: 12. doi:10.1016/j.pt.2020.08.005

Milne G, Fujimoto C, Bean T, Peters HJ, Hemmington M, Taylor C, Fowkes RC, Martineau HM, Hamilton CM, Walker M, Mitchell JA, Léger E, Priestnall SL, Webster JP. (2020) Infectious Causation of Abnormal Host Behavior: *Toxoplasma gondii* and Its Potential Association With Dopey Fox Syndrome. *Front Psychiatry*. 11:513536. doi: 10.3389/fpsyt.2020.513536.

Mirza Alizadeh, A.; Jazaeri, S.; Shemshadi, B.; Hashempour-Baltork, F.; Sarlak, Z.; Pilevar, Z.; Hosseini, H. (2018). A Review on Inactivation Methods of *Toxoplasma gondii* in Foods. *Pathog. Glob. Health*. 112: 306–319. doi: 10.1080/20477724.2018.1514137.

Mukhopadhyay D, Arranz-Solís D, Saeij JPJ. (2020). Influence of the host and parasite strain on the immune response during *Toxoplasma* infection. *Front Cell Infect Microbiol*. 10:580425. doi: 10.3389/fcimb.2020.580425.

Nayeri T, Sarvi S, Moosazadeh M, Daryani A. (2021). Global prevalence of *Toxoplasma gondii* infection in the aborted fetuses and ruminants that had an abortion: A systematic review and meta-analysis. *Vet Parasitol*. 290:109370. doi: 10.1016/j.vetpar.2021.109370.

Nogui F L, Mattas S, Turcato Júnior G, Lewi D S. (2009). Neurotoxoplasmosis diagnosis for HIV-1 patients by real-time PCR of cerebrospinal fluid. *Braz J Infect Dis*. 13: 18–23. doi:10.1590/s1413-86702009000100006

Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, Hase T. (2000). Loop-mediated isothermal amplification of DNA. *Nucleic Acids Res*. 28(12): E63. doi:10.1093/nar/28.12.e63

Opitz C, Soldati D. (2002). "The glideosome": a dynamic complex powering gliding motion and host cell invasion by *Toxoplasma gondii*. *Mol Microbiol*. 45:597–604. doi: 10.1046/j.1365-2958.2002.03056.x.

Opsteegh M, Maas M, Schares G and Giessen J, (2016). Relationship between seroprevalence in the main livestock species and presence of *Toxoplasma gondii* in meat (GP/EFSA/BIOHAZ/2013/01) An extensive literature review, Final report. *EFSA Supporting Publications*, 13: 294. doi: 10.2903/sp.efsa.2016.EN-996

Opsteegh M, Teunis P, Züchner L, Koets A, Langelaar M, van der Giessen J. (2011a). Low predictive value of seroprevalence of *Toxoplasma gondii* in cattle for detection of parasite DNA. *Int. J. Parasitol*. 41: 343–354. doi.:10.1016/j.ijpara.2010.10.006

Opsteegh M, Prickaerts S, Frankena K, Evers EG. (2011b) A quantitative microbial risk assessment for meatborne *Toxoplasma gondii* infection in The Netherlands. *Int J Food Microbiol*. 150(2-3):103-114. doi: 10.1016/j.ijfoodmicro.2011.07.022

Opsteegh M, Langelaar M, Sprong H, den Hartog L, De Craeye S, Bokken G, Ajzenberg D, Kijlstra A, van der Giessen J. (2010). Direct detection and genotyping of *Toxoplasma gondii* in meat samples using magnetic capture and PCR. *Int. J. Food Microbiol*. 139: 193–201. doi:10.1016/j.ijfoodmicro.2010.02.027

Opsteegh M, Spano F, Aubert D, Balea A, Burrells A, Cherchi S, Cornelissen JBWJ, Dam-Deisz C, Guitian J, Györke A, Innes EA, Katzer F, Limon G, Possenti A, Pozio E, Schares G, Villena I, Wisselink HJ, van der Giessen JWB. (2019). The relationship between the presence of antibodies and direct detection of Toxoplasma gondii in slaughtered calves and cattle in four European countries. *Int J Parasitol*.49:515-522. doi: 10.1016/j.ijpara.2019.01.005. Epub 2019 May 17. PMID: 31108097.

Palos Ladeiro M, Bigot A, Aubert D, Hohweyer J, Favennec L, Villena I, Geffard A. (2013). Protozoa interaction with aquatic invertebrate: interest for watercourses biomonitoring. *Environ Sci Pollut Res Int.* 20:778-89. doi: 10.1007/s11356-012-1189-1.

Pappas G, Roussos N, Falagas M. E. (2009). Toxoplasmosis snapshots: global status of *Toxoplasma gondii* seroprevalence and implications for pregnancy and congenital toxoplasmosis. *Int. J. Parasitol.* 39: 1385–1394. doi:10.1016/j.ijpara.2009.04.003

Paredes-Santos TC, de Souza W, Attias M. (2012). Dynamics and 3D organization of secretory organelles of *Toxoplasma gondii. J Struct Biol*. 177:420–30. doi: 10.1016/j.jsb.2011.11.028.

Parmley SF, Goebel FD, Remington JS. (1992). Detection of *Toxoplasma gondii* in cerebrospinal fluid from AIDS patients by polymerase chain reaction. *J Clin Microbiol*. 30:3000–2.

Partoandazanpoor A, Sadeghi-Dehkordi Z, Ekradi L, Khordadmehr M, Rassouli M, Sazmand A. (2020). Molecular diagnosis and pathological study of *Toxoplasma gondii* in aborted

caprine and ovine fetuses in borderline of Iran-Iraq. *Acta Parasitol*. 65:187-192. doi: 10.2478/s11686-019-00147-4.

Pena HF, Gennari SM, Dubey JP, Su C. (2008). Population structure and mouse-virulence of *Toxoplasma gondii* in Brazil. *Int J Parasitol*. 38:561-569. doi: 10.1016/j.ijpara.2007.09.004.

Pena HF, Moroz LR, Sozigan RK, Ajzenberg D, Carvalho FR, Mota CM, Mineo TW, Marcili A. (2014). Isolation and biological and molecular characterization of *Toxoplasma gondii* from canine cutaneous toxoplasmosis in Brazil. *J Clin Microbiol*. 52:4419-4420. doi:10.1128/JCM.02001-14.

Pereira-Bueno J, Quintanilla-Gozalo A, Pérez-Pérez V, Álvarez-García G, Collantes-Fernández E, Ortega-Mora LM. (2004). Evaluation of ovine abortion associated with Toxoplasma gondii in Spain by different diagnostic techniques. *Vet Parasitol*. 121:33-43. doi: 10.1016/j.vetpar.2004.02.004.

Pinto-Ferreira F, Caldart ET, Pasquali AKS, Mitsuka-Breganó R, Freire RL, Navarro IT. (2019). Patterns of Transmission and Sources of Infection in Outbreaks of Human Toxoplasmosis. *Emerg Infect Dis*. 25:2177-2182. doi: 10.3201/eid2512.181565.

Porchet E, Torpier G. (1977). Freeze fracture study of *Toxoplasma* and *Sarcocystis* infective stages. *Z Parasitenkd*. 54:101–24.

Putignani L, Mancinelli L, Del Chierico F, Menichella D, Adlerstein D, Angelici MC, Marangi M, Berrilli F, Caffara M, di Regalbono DA, Giangaspero A. (2011). Investigation of Toxoplasma gondii presence in farmed shellfish by nested-PCR and real-time PCR fluorescent amplicon generation assay (FLAG). *Exp Parasitol.* 127: 409-17. doi: 10.1016/j.exppara.2010.09.007..

Ramakrishnan C, Maier S, Walker RA, Rehrauer H, Joekel DE, Winiger RR, Basso WU, Grigg ME, Hehl AB, Deplazes P, Smith NC. (2019). An experimental genetically attenuated live vaccine to prevent transmission of *Toxoplasma gondii* by cats. *Sci Rep.* 9: 1474. doi: 10.1038/s41598-018-37671-8.

Ranucci D, Veronesi F, Branciari R, Miraglia D, Moretta I, Fioretti DP. (2012). Evaluation of an immunofluorescence antibody assay for the detection of antibodies against *Toxoplasma*

gondii in meat juice samples from finishing pigs. Foodborne pathogens and disease, 9(1): 75–78. doi: 0.1089/fpd.2011.0930

Reid AJ, Vermont SJ, Cotton JA, Harris D, Hill-Cawthorne GA, Könen-Waisman S, Latham SM, Mourier T, Norton R, Quail MA, Sanders M, Shanmugam D, Sohal A, Wasmuth JD, Brunk B, Grigg ME, Howard JC, Parkinson J, Roos DS, Trees AJ, Berriman M, Pain A, Wastling JM. (2012). Comparative genomics of the apicomplexan parasites *Toxoplasma gondii* and *Neospora caninum*: Coccidia differing in host range and transmission strategy. *PLoS Pathog*. 8:e1002567. doi: 10.1371/journal.ppat.1002567.

Reischl U, Bretagne S, Krüger D, Ernault P, Costa J M, (2003). Comparison of two DNA targets for the diagnosis of Toxoplasmosis by real-time PCR using fluorescence resonance energy transfer hybridization probes. *BMC infectious diseases*, 3: 7. doi: 10.1186/1471-2334-3-7

Robert-Gangneux F, Dardé ML. (2012). Epidemiology of and diagnostic strategies for toxoplasmosis. *Clin. Microbiol. Rev*. 25: 264–296. doi: 10.1128/CMR.05013-11

Sacks J J, Roberto R R, Brooks N F (1982). Toxoplasmosis infection associated with raw goat's milk. JAMA, 248: 1728–1732. doi:10.1001/jama.1982.03330140038029.

Sah RP, Dey AR, Rahman AKMA, Alam MZ, Talukder MH. (2019). Molecular detection of *Toxoplasma gondii* from aborted fetuses of sheep, goats and cattle in Bangladesh. *Vet Parasitol* Reg Stud Reports. 18:100347. doi: 10.1016/j.vprsr.2019.100347.

Sanchez S G, Besteiro S. (2021). The pathogenicity and virulence of *Toxoplasma gondii*. *Virulence*, 12: 3095–3114. doi: 10.1080/21505594.2021.2012346

Santoro M, Viscardi M, Boccia F, Borriello G, Lucibelli MG, Auriemma C, Anastasio A, Veneziano V, Galiero G, Baldi L, Fusco G. (2020). Parasite Load and STRs Genotyping of *Toxoplasma gondii* Isolates From Mediterranean Mussels (*Mytilus galloprovincialis*) in Southern Italy. *Front Microbiol*.11:355. doi: 10.3389/fmicb.2020.00355

Scallan E, Hoekstra RM, Angulo FJ, Tauxe RV, Widdowson MA, Roy SL, Jones JL, Griffin PM. (2011). Foodborne illness acquired in the United States--major pathogens. *Emerg Infect Dis*. 17:7-15. doi: 10.3201/eid1701.p11101.

Schlüter D, Barragan A. (2019). Advances and challenges in understanding cerebral toxoplasmosis. *Front Immunol*. 10:242. doi: 10.3389/fimmu.2019.00242.

Shapiro K, Bahia-Oliveira L, Dixon B, Dumètre A, de Wit LA, VanWormer E, Villena I. (2019). Environmental transmission of *Toxoplasma gondii*: Oocysts in water, soil and food. *Food Waterborne Parasitol.* 15: e00049. doi: 10.1016/j.fawpar.2019.e00049.

Shapiro K, Vanwormer E, Aguilar B, Conrad P.A. (2015). Surveillance for *Toxoplasma gondii* in California Mussels (*Mytilus californianus*) Reveals Transmission of Atypical Genotypes from Land to Sea. *Environ. Microbiol*. 17: 4177–4188. doi: 10.1111/1462-2920.12685

Shwab EK, Jiang T, Pena HF, Gennari SM, Dubey JP, Su C. (2016). The ROP18 and ROP5 gene allele types are highly predictive of virulence in mice across globally distributed strains of *Toxoplasma gondii*. *Int J Parasitol*. 46:141-146. doi: 10.1016/j.ijpara.2015.10.005.

Sibley LD, Ajioka JW. (2008). Population structure of *Toxoplasma gondii*: clonal expansion driven by infrequent recombination and selective sweeps. *Annu Rev Microbiol*. 62:329-351. doi: 10.1146/annurev.micro.62.081307.162925.

Sibley LD, Boothroyd JC. (1992). Virulent strains of *Toxoplasma gondii* comprise a single clonallineage. *Nature*. 359: 82-85. doi: 10.1038/359082a0.

Silveira C, Ferreira R, Muccioli C, Nussenblatt R, Belfort R Jr. (2003). Toxoplasmosis transmitted to a newborn from the mother infected 20 years earlier. *Am J Ophthalmol*. 136: 370-371. doi: 10.1016/s0002-9394(03)00191-0

Singh S. (2016). Congenital toxoplasmosis: Clinical features, outcomes, treatment, and prevention. *Trop. Parasitol*. 6: 113–122. doi: 10.4103/2229-5070.190813.

Sotiriadou I, Karanis P. (2008). Evaluation of loop-mediated isothermal amplification for detection of *Toxoplasma gondii* in water samples and comparative findings by polymerase chain reaction and immunofluorescence test (IFT). Diagn Microbiol Infect Dis. 62: 357–365. doi:10.1016/j.diagmicrobio.2008.07.009

Sousa S, Ajzenberg D, Vilanova M, Costa J, Dardé ML.(2008). Use of GRA6-derived synthetic polymorphic peptides in an immunoenzymatic assay to serotype *Toxoplasma gondii* in human serum samples collected from three continents. *Clin Vaccine Immunol.* 15:1380–6. 10.1128/CVI.00186-08

Sousa S, Ajzenberg D, Marle M, Aubert D, Villena I, da Costa J C, Dardé M L. (2009). Selection of polymorphic peptides from GRA6 and GRA7 sequences of *Toxoplasma gondii* strains to be used in serotyping. Clinical and vaccine immunology : CVI, 16: 1158–1169. doi: 10.1128/CVI.00092-09

Sousa S, Fernandes M, Correia da Costa J M. (2023). Serotyping, a challenging approach for *Toxoplasma gondii* typing. *Front. Med. 10*: 1111509. doi: 10.3389/fmed.2023.1111509

Sroka J, Wojcik-Fatla A, Szymanska J, Dutkiewicz J, Zajac V, Zwolinski J. (2010). The occurrence of *Toxoplasma gondii* infection in people and animals from rural environment of Lublin region - estimate of potential role of water as a source of infection. *Ann Agric Environ Med*. 17:125-132.

Stajner T, Vasiljević Z, Vujić D, Marković M, Ristić G, Mićić D, Pasić S, Ivović V, Ajzenberg D, Djurković-Djaković O. (2013). Atypical strain of *Toxoplasma gondii* causing fatal reactivation after hematopoietic stem cell transplantion in a patient with an underlying immunological deficiency. *J Clin Microbiol*. 51:2686-2690. doi: 10.1128/JCM.01077-13.

Stelzer S, Basso W, Benavides Silván J, Ortega-Mora LM, Maksimov P, Gethmann J, Conraths FJ, Schares G. (2019). *Toxoplasma gondii* infection and toxoplasmosis in farm animals: Risk factors and economic impact. *Food Waterborne Parasitol*. 15: e00037. doi:10.1016/j.fawpar.2019.e00037.

Su C, Evans D, Cole RH, Kissinger JC, Ajioka JW, Sibley LD. (2003). Recent expansion of *Toxoplasma* through enhanced oral transmission. *Science*. 299: 414-416. doi: 10.1126/science.1078035.

Su C, Khan A, Zhou P, Majumdar D, Ajzenberg D, Dardé ML, Zhu XQ, Ajioka JW, Rosenthal BM, Dubey JP, Sibley LD. (2012). Globally diverse *Toxoplasma gondii* isolates comprise six major clades originating from a small number of distinct ancestral lineages. Proc Natl Acad Sci U S A. 109(15): 5844-5849. doi: 10.1073/pnas.1203190109.

Su C, Zhang X, Dubey JP. (2006). Genotyping Of *Toxoplasma gondii* By multilocus PCR-RFLP markers: a high resolution and simple method for identification of parasites. *Int J Parasitol.* 36: 841–8. 10.1016/j.ijpara.2006.03.003

Swapna LS, Parkinson J. (2017). Genomics of apicomplexan parasites. Crit. Rev. Biochem. Mol. Biol. 52: 254–273. doi:10.1080/10409238.2017.1290043

Teixeira LE, Kanunfre KA, Shimokawa PT, Targa LS, Rodrigues JC, Domingues W, Yamamoto L, Okay TS. (2013). The performance of four molecular methods for the laboratory diagnosis of congenital toxoplasmosis in amniotic fluid samples. *Rev Soc Bras Med Trop*. 46:584-588.

Tenter AM, Barta JR, Beveridge I, Duszynski DW, Mehlhorn H, Morrison DA, Thompson RC, Conrad PA. (2002). The conceptual basis for a new classification of the coccidia. *Int J Parasitol*. 32: 595-616. doi: 10.1016/s0020-7519(02)00021-8.

Tenter AM, Heckeroth AR, Weiss LM. (2000). *Toxoplasma gondii*: from animals to humans. *Int J Parasitol*. 30: 1217-1258. doi: 10.1016/s0020-7519(00)00124-7.

Torgerson P R, Devleesschauwer B, Praet N, Speybroeck N, Willingham A L, Kasuga F, Rokni M B, Zhou X N, Fèvre E M, Sripa B, Gargouri N, Fürst T, Budke C M, Carabin H, Kirk M D, Angulo F J, Havelaar A, de Silva N. (2015). World Health Organization Estimates of the Global and Regional Disease Burden of 11 Foodborne Parasitic Diseases, 2010: A Data Synthesis. *PLoS medicine*. *12:* e1001920. doi: 10.1371/journal.pmed.1001920

Trees AJ, Williams DJL. (2005). Endogenous and exogenous transplacental infection in *Neospora caninum* and *Toxoplasma gondii*. *Trends Parasitol*. 21: 558–561. doi: 10.1016/j.pt..09.005

Uggla A, Sjöland L, Dubey JP. (1987). Immunohistochemical diagnosis of toxoplasmosis in fetuses and fetal membranes of sheep. *Am J Vet Res*. 48: 348-351.

Villard O, Breit L, Cimon B, Franck J, Fricker-Hidalgo H, Godineau N, Houze S, Paris L, Pelloux H, Villena I, Candolfi E; French National Reference Center for Toxoplasmosis Network. (2013). Comparison of four commercially available avidity tests for *Toxoplasma* gondiispecific IgG antibodies. *Clin Vaccine Immunol*. 20: 197-204. doi: 10.1128/CVI.00356-12.

Vismarra A, Barilli E, Miceli M, Mangia C, Bacci C, Brindani F and Kramer L. (2017). *Toxoplasma gondii* and pretreatment protocols for polymerase chain reaction analysis of

milk samples: a field trial in sheep from Southern Italy. *Ital. J. Food Saf*. 6, 6501. doi: 10.4081/ijfs.2017.6501.

Vismarra A, Mangia C, Barilli E, Brindani F, Bacci C, Kramer L. (2016). Meat Juice Serology for *Toxoplasma Gondii* Infection in Chickens. *Ital. J. Food Saf*. 5: 5586. doi: 10.4081/ijfs.2016.5586

Wainwright KE, Miller MA, Barr BC, Gardner IA, Melli AC, Essert T, Packham AE, Truong T, Lagunas-Solar M, Conrad PA.(2007). Chemical Inactivation of *Toxoplasma gondii* Oocysts in Water. *J. Parasitol*. 93: 925–931. doi: 10.1645/GE-1063R.1.

Wang Y, Wang G, Zhang D, Yin H, Wang M. (2013). Detection of acute toxoplasmosis in pigs using loop-mediated isothermal amplification and quantitative PCR. *Korean J Parasitol*. 51: 573–577. doi:10.3347/kjp.2013.51.5.573

Wang ZD, Wang SC, Liu HH, Ma HY, Li ZY, Wei F, Zhu XQ, Liu Q. (2017). Prevalence and burden of *Toxoplasma gondii* infection in HIV-infected people: a systematic review and meta-analysis. *Lancet HIV*. 4: e177-e188. doi: 10.1016/S2352-3018(17)30005-X

Webster JP (2007). The effect of *Toxoplasma gondii* on animal behavior: playing cat and mouse. *Schizophrenia bulletin*, 33(3): 752–756. doi: 10.1093/schbul/sbl073

Webster JP (1994). The effect of *Toxoplasma gondii* and other parasites on activity levels in wild and hybrid *Rattus norvegicus*. *Parasitology* 109: 583–589. doi: 10.1017/s0031182000076460.

Webster JP. (2001). Rats, cats, people and parasites: the impact of latent toxoplasmosis on behaviour. *Microbes Infect*. 3: 1–9. doi: 10.1016/s1286-4579(01)01459-9.

Webster JP, Brunton CFA, Macdonald DW. (1994). Effect of *Toxoplasma gondii* on neophobic behaviour in wild brown rats, *Rattus norvegicus*. *Parasitology*, 109: 37–43. doi: 10.1017/s003118200007774x.

Webster J P, Kaushik M, Bristow GC, McConkey GA. (2013). *Toxoplasma gondii* infection, from predation to schizophrenia: can animal behaviour help us understand human behaviour?. *J. Exp. Biol.* 216: 99–112. doi: 10.1242/jeb.074716

Wendte JM, Miller MA, Lambourn DM, Magargal SL, Jessup DA, Grigg ME. (2010). Self-mating in the definitive host potentiates clonal outbreaks of the apicomplexan parasites

Sarcocystis neurona and *Toxoplasma gondii*. *PLoS Genet*. 6: e1001261. doi: 10.1371/journal.pgen.1001261.

Xia J, Venkat A, Bainbridge RE, Reese ML, Le Roch KG, Ay F, Boyle JP. (2021). Thirdgeneration sequencing revises the molecular karyotype for *Toxoplasma gondii* and identifies emerging copy number variants in sexual recombinants. *Genome Res*.31: 834-851. doi: 10.1101/gr.262816.120.

Xiao J, Li Y, Gressitt K L, He H, Kannan G, Schultz T L, Svezhova N, Carruthers VB, Pletnikov M V, Yolken R H, Severance E G. (2016). Cerebral complement C1q activation in chronic *Toxoplasma* infection. *Brain Behav. Immun.* 58: 52–56. doi:10.1016/j.bbi.2016.04.009

Chapter 3

Spread of *Toxoplasma gondii* among animals and humans in Northern Italy: A retrospective analysis in a One Health framework

Dini FM, Morselli S, Marangoni A, Taddei R, Maioli G, Roncarati G, Balboni A, Dondi F, Lunetta F, Galuppi R. (2023). Spread of *Toxoplasma gondii* among animals and humans in Northern Italy: A retrospective analysis in a One-Health framework. *Food Waterborne Parasitol*. 8: 32:e00197. doi:10.1016/j.fawpar.2023.e00197.

Food and Waterborne Parasitology 32 (2023) e00197

Contents lists available at ScienceDirect

Food and Waterborne Parasitology

journal homepage: www.elsevier.com/locate/fawpar

Spread of *Toxoplasma gondii* among animals and humans in Northern Italy: A retrospective analysis in a One-Health framework

F.M. Dini [a,*,1], S. Morselli [b,1], A. Marangoni [b], R. Taddei [c], G. Maioli [c], G. Roncarati [d], A. Balboni [a], F. Dondi [a], F. Lunetta [a], R. Galuppi [a]

[a] *Department of Veterinary Medical Sciences, University of Bologna, Italy*
[b] *Department of Medical and Surgical Sciences, University of Bologna, Italy*
[c] *Istituto Zooprofilattico Sperimentale Della Lombardia e della Emilia-Romagna, Italy*
[d] *Microbiology Unit, IRCCS Azienda Ospedaliero-Universitaria di Bologna, 40138 Bologna, Italy*

ARTICLE INFO

Keywords:
Toxoplasmosis
Epidemiology
Humans
Animals
Emilia-Romagna region
Serology

ABSTRACT

Toxoplasmosis occurs worldwide and is considered one of the most important food-borne parasitic zoonoses. The consumption of undercooked meat containing viable tissue cysts and ingestion of environmental oocyst are the most important sources of infection. The aim of this retrospective study was to evaluate the spread of *Toxoplasma gondii* in the province of Bologna (Emilia-Romagna region) in northern Italy, with a One Health approach, comparing seropositivity rates in different animal species and in humans over the last 19 and 4 years respectively. Analyses were performed on serological data collected over different periods at three separate locations: Istituto Zooprofilattico Sperimentale della Lombardia e della Emilia-Romagna (IZSLER); Veterinary University Hospital Clinical Pathology Service, Department of Veterinary Medical Sciences, University of Bologna; and Unit of Microbiology, St. Orsola Hospital, Bologna. Most relevant seropositivity rates observed in animals were 15.5% (wild boar), 25% (roe deer), 18.7% (goat), 29.9% (sheep), 9.7% (pigs), 42.9% and 21.8% in cat and dog, respectively. A comprehensive screening was conducted on a population of 36,814 individuals, revealing a prevalence of 20.4%. Among pregnant women, a frequence of 0.39% for active toxoplasmosis was observed. Despite certain limitations, this study provided valuable insights into the extensive distribution of this parasitic infection among diverse animal species and human populations in the province of Bologna. These findings underscore the importance of implementing consistent and proactive toxoplasmosis screening protocols during pregnancy, while emphasizing the critical need for adopting a One Health approach for effective control of this parasitic disease.

1. Introduction

Toxoplasmosis is a zoonotic parasitic infection with a worldwide distribution caused by the apicomplexan protozoon *Toxoplasma*

* Corresponding author at: Department of Veterinary Medical Sciences, Alma Mater Studiorum University of Bologna, Via Tolara di Sopra 50, 40064 Ozzano Emilia, BO, Italy.
E-mail address: filippomaria.dini@unibo.it (F.M. Dini).
[1] Both authors contributed equally to this work.

https://doi.org/10.1016/j.fawpar.2023.e00197
Received 27 January 2023; Received in revised form 31 May 2023; Accepted 3 June 2023
Available online 8 June 2023

gondii. The sexual reproduction of the parasite occurs in felids, definitive hosts, that play an essential role in the contamination of the environment with oocysts, whereas a broad range of warm-blooded animals, including humans, act as intermediate hosts (Dubey et al., 2020a). Toxoplasmosis is recognized as one of the most significant food-borne parasitic zoonoses worldwide, and it is estimated that approximately one third of the global population is infected with *T. gondii*. (Montoya and Liesenfeld, 2004) In immunocompetent individuals, *Toxoplasma* infection is typically benign and self-limiting. However, it can cause life-threatening disease in fetuses and immunosuppressed individuals. Recent studies suggest that latent *Toxoplasma* infection may be associated with the development of specific neuropsychiatric conditions (Tyebji et al., 2019). The primary sources of infection are the consumption of undercooked meat containing viable tissue cysts and the ingestion of food and water contaminated with oocysts (Pereira et al., 2010, Guo et al., 2015). The development of a serological assay capable of differentiating oocyst-induced infection from bradyzoite-induced infection has facilitated the recognition of oocysts as the primary reservoir of infection during outbreaks in the United States (Hill et al., 2011; Boyer et al., 2011). Nonetheless, the widespread implementation of these diagnostically valuable epidemiological techniques remains limited, underscoring the necessity for a meticulous transdisciplinary approach to effectively prevent and control this parasite (Djurkovi'c-Djakovi'c et al., 2019).

In Southern Italy, a recent monitoring program has been implemented with the aim of enhancing the epidemiological knowledge regarding toxoplasmosis and identifying the risk factors associated with the infection in both animals and humans. This program adopts a multi-institutional approach to comprehensively investigate the subject (Pepe et al., 2021). In the Emilia-Romagna region of Italy, a three-year prospective observational study has shed light on the prevalence of toxoplasmosis among pregnant women. The study revealed that 22.3% of women tested positive for toxoplasmosis during early pregnancy. Notably, non-native women originating from Africa, Asia, Eastern Europe, and South America exhibited a higher likelihood of acquiring the infection during pregnancy compared to Italian women. Furthermore, the incidence rate of toxoplasmosis in this region was found to be higher than that reported in other European countries (Capretti et al., 2014). Serological analyses conducted during the same period in the Emilia-Romagna region also revealed a relatively high prevalence of toxoplasmosis in sheep flocks, reaching 41.9% (Parigi, 2014). Recently, a molecular investigation revealed a prevalence of 14% for parasite infections among wild water birds hunted in the aforementioned area (Dini et al., 2023).

To effectively implement appropriate control measures aimed at reducing the incidence of congenital toxoplasmosis, it is crucial to gain a comprehensive understanding of the extent of *T. gondii* circulation within the specific area of interest. Therefore, the objective of this study was to assess the prevalence of *T. gondii* infection in the province of Bologna, located within the Emilia-Romagna region of Italy, utilizing a One Health approach. The study aimed to compare the seroprevalence of *T. gondii* across various animal species with the seropositivity data observed in humans over the past years. By adopting this multidisciplinary approach, a more comprehensive and integrated understanding of the infection dynamics can be achieved, facilitating the development of targeted control strategies.

2. Materials and methods

The data utilized in this study were obtained through a retrospective analysis of serological investigations conducted at two distinct veterinary institutions and one human hospital.

Specifically, information pertaining to animal infections was extracted from the databases of the Istituto Zooprofilattico Sperimentale della Lombardia e della Emilia-Romagna (IZSLER) covering the period from 2002 to 2021. Similarly, data from the Veterinary University Hospital (VUH), specifically the Clinical Pathology Service of the Department of Veterinary Medical Sciences at the University of Bologna, were collected for the period from 2006 to 2021. This involved retrieving information from all samples that underwent serological testing for *T. gondii*.

IZLER is an Italian public health institute that is engaged in control and research initiatives, as well as offering services in the domains of animal health, food safety, and zoonoses. Within their scope of activities, IZLER conducts serological tests on diverse domestic and wild animal species for both routine institutional screening and diagnostic purposes upon request. During the specified period, various tests were employed at IZSLER: Latex Agglutination Test (LAT, Toxotest; Eiken Chemical, Tokyo, Japan), Enzyme-Linked Immunosorbent Assay (ELISA, ID Screen® Toxoplasmosis Indirect Multi-species; ID-Vet - Innovative Diagnostics, Grabels, France), and Immunofluorescence Antibody Test (IFAT, Toxo-Spot IF; bioMérieux, Marcy-l'Étoile, France). IFAT was performed using a commercial antigen (Toxo-Spot IF; bioMérieux) and, as conjugate, Anti-Dog and Anti- Cat IgG of IZSLER internal production were used. Antibody titer $\geq$1:40 was considered positive for IFAT, while antibody titer $\geq$1:32 was considered positive for LAT, as suggested by the manufacturers.

At the VUH, dogs and cats were tested for diagnostic purpose only by the means of IFAT (MegaFLUO TOXOPLASMA g, MegaCor Diagnostik, Hoerbranz, Austria) using Anti-Dog IgG-FITC antibody (Sigma-Aldrich, Saint Louis, MO, USA) and FITC IgG conjugate Anti-Cat (MegaCor Diagnostik, Hoerbranz, Austria); antibody titer $\geq$1:40 was considered positive.

In both laboratories, in addition to the detection of specific IgG antibodies, IFAT was also used for the detection of specific IgM antibodies. Concerning humans, this study encompassed all individuals who underwent immune status evaluation for *T. gondii* infection at the Unit of Microbiology in St. Orsola Hospital. This particular hospital serves the entire population in the metropolitan city of Bologna and its province, which consists of over 1 million inhabitants. The hospital's microbiology laboratory, equipped with an online database called DNLAB® (Dedalus), contains records of all tests conducted in the past four years (2018–2021). For serological analysis of human serum samples chemiluminescence immunoassays (Elecsys Toxo IgG and Elecsys Toxo IgM, Roche Diagnostics GmbH, Mannheim, Germany) were initially employed to detect IgM and IgG antibodies. Borderline or positive IgM screening results were subsequently confirmed using enzyme-linked fluorescent assays (ELFAs) (Vidas Toxo IgM, bioMerieux, Marcy l'Etoile, France), to exclude IgM residual. If IgM positivity was confirmed, and IgG Avidity test (Vidas Toxo IgG Avidity, bioMerieux) was performed.

Positivity in ELFAs and low Avidity Index were indicative of *T. gondii* active infection.

Data collected from animals were organized in databases. The two laboratories, IZSLER and VUH, provided different variables. For IZSLER, information included the animal species examined, the municipality of origin (Bologna province consists of 55 municipalities), and the date of sample submission. On the other hand, VUH provided additional data such as age, sex, breed, municipality of origin (based on owner address), the date of testing, and associated clinical information. All possible duplicate observations were removed with the first occurrence of the animal in the dataset retained.

Pearson's $\chi 2$ test was used to associate species and (when available) age, sex, and, for the cat, the origin (owned or unowned) with seroprevalence data. The level of statistical significance was 5% ($P < 0.05$).

For humans, data collected included prevalence and the number of active infections (positive IgM ELFA analyses and low avidity samples).

The Sample Size Calculator (https://www.surveysystem.com/sscalc.htm) was used to calculate 95% confidence intervals (CIs) for the observed prevalence values.

3. Results

Between 2002 and 2021, the Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna (IZSLER) conducted serological testing for *Toxoplasma gondii* infection on a total of 4263 sera derived from various animals, including those from farms, companion animals, and wild species (Table 1). Specifically, investigations were carried out on small ruminants, such as sheep and goats, in response to abortion outbreaks. Among the 64 goat samples examined, 12 samples (18.7%) tested positive. Notably, in the municipality of San Giovanni in Persiceto, clusters of positive cases were identified in three different years (2003, 2004, 2019). Out of the 117 sheep tested, 35 (29.9%) were found to be positive. Additionally, as part of a food safety research project, 31 pigs from the same municipality in the Bolognese Apennines were examined, and the observed frequency of positive cases was 9.7%. Concerning wild animals, of a total of 594 wild boar and 104 roe deer examined, 15.5% and 25% were seropositive, respectively, indicating infection was significantly more frequent in roe deer that in wild boar ($P = 0.025$). Very few wild carnivores were tested to allow for meaningful results, but it is noteworthy that one of the 5 wolves and both red foxes tested were seropositive. In contrast, none of the 34 tested hares was seropositive. Pigeons ($n = 105$), tested during a population control campaign, showed a seroprevalence of 3.8%. Among companion animals, the species most represented was the cat ($n = 3087$), which tested positive for total antibody (ELISA, LAT) or IgG in 1355 cases (43.9%). In most cases (3082) the sampling was carried out between 2002 and 2006. Evaluating stray cats from colonies versus owned cats, the former had a statistically higher seroprevalence (46.5% vs. 33.4%; $\chi^2 = 34.14$, $P < 0.001$). In dogs ($n = 114$), the frequency observed was lower (19.3%) with 22 positive dogs.

Concerning the results obtained from the VUH, data derived from 65 dogs and 208 cats of the province of Bologna: these were owned animals in which toxoplasmosis was a differential diagnosis based on their clinical and pathological findings. Out of 208 cats tested, 59 (28.4%) [C.I. 95%: 22.27–34.53] were positive. No significant difference has been detected in frequency of positivity between males and females (25.5% vs. 30.9%), although entire males appeared less frequently positive than neutered ones (13.6% vs 35.2%; $P = 0.02$). The seropositivity rate was 18.2% in subjects younger than one year of age, and 30.7% in cats $\geq$ one year, although the difference was not significant (Table 2).

Table 1
animal species tested at IZSLER, number of animals tested, number of municipalities of origin, frequency of seropositivity for *T. gondii* antibodies, confidence interval, number of municipalities of origin of positive animals and serological method used.

Animal Species	n. examined animals	n. municipalities of origin	Seropositive n (%) [CI 95%]	n. municipalities of origin of positive	Test Used
Goat	64	9	12 (18.7%) [12.45–24.95]	4	ELISA, LAT
Sheep	117	14	35 (29.9%) [21.6–38.2]	11	ELISA, LAT, IFAT
Pig	31	1	3 (9.7%) [0–20.12]	1	ELISA
Rabbit	6	2	0 (0%) [n.d.]	0	LAT
Roe Deer (*Capreolus capreolus*)	104	25	26 (25%) [16.68–33.32]	18	ELISA
Wild Boar (*Sus scrofa*)	594	19	92 (15.5%) [12.59–18.41]	14	ELISA
Hare (*Lepus europaeus*)	34	2	0 (0%) [n.d.]	0	IFAT
Pidgeon (*Columba livia*)	105	5	4 (3.8%) [0.14–7.46]	2	LAT
Wolf (*Canis lupus*)	5	5	1 (20%) [0–55.05]	1	ELISA
Red Fox (*Vulpes vulpes*)	2	2	2 (100%) [n.d.]	2	ELISA
Dog	114	21	22 (19.3%) [11.9–26.54]	11	ELISA, LAT, IFAT
Cat	3087	39	1355 (43.9%) [42.89–44.91]	30	ELISA, LAT, IFAT

Concerning dogs, the seropositivity rate observed was 26.2% (17/65) [C.I. 95%: 15.51–36.89]. There was no difference in prevalence according to sex (male or female, entire or neutered) or age (Table 2).

Fig. 1 illustrates the distribution of animals examined by IZSLER and VUH across various municipalities in the province of Bologna. The examination of wild species predominantly occurred in the southernmost region of the province, specifically in the Apennine area. On the other hand, dogs and cats were sampled from almost all municipalities throughout the entire province. The positive animals, indicating the presence of the infection, were found to be uniformly distributed across the different areas.

In the study period of four years, a comprehensive analysis was conducted on 122,377 serum samples at the Microbiology Unit of Sant'Orsola Hospital (Table 3). It should be noted that some individuals underwent multiple testing, resulting in a total of 36,814 patients (primarily adults) being evaluated (Table 3). Among these patients, the majority were women (88.2%), with an average of 3.61 samples assessed per woman or 1.16 per man. Notably, a significant proportion of women (87.9%) underwent testing during their pregnancy.

The observed prevalence of toxoplasmosis was 20.4% (95% CI: 20.8–20.0), ranging between 20.0% and 20.8% throughout the study period. There were no significant differences in prevalence based on year, season, or sex. During 2020, the number of tests conducted was markedly lower compared to other years, with a reduction of over 15%, which can likely be attributed to the impact of the COVID-19 pandemic.

IgM positivity confirmation by ELFA tests were performed on a total of 1915 sera. Among these, 504 sera (26.3%) tested positive for IgM. A diagnosis of acute *T. gondii* infection was established in 161 patients, determined by excluding cases of persistent IgM positivity and considering a low Avidity Index. This resulted in a frequency of 0.44% among the screened population. Most of the acute infection (113/161) were diagnosed in pregnant women. The frequencies of toxoplasmosis did not differ between men and women, or between pregnant and nonpregnant women.

Moreover, we divided the pregnant women into two groups: subjects attending the Family Advisory Health Centers of Bologna and its province for prenatal care and maternal screening and subjects attending the Maternal-Fetal Medicine Unit of Sant'Orsola Hospital, Bologna, for a second level toxoplasmosis diagnosis (women with clinical, echographic or laboratory suspicion of toxoplasmosis). In particular, in the former case, the frequence was 0.25% (69/27,513 subjects) and in the latter the value was 4.1% (44/1059), with $p = 0.0001$.

4. Discussion

In this study, a One Health approach was employed to analyse data concerning the presence of *Toxoplasma gondii* in the province of Bologna. The analysis focused on the key host species that play a significant role in the epidemiology of the parasite.

While the National Health Service has facilitated the collection of data on human health for the province through centralized analyses, the veterinary field presented a fragmented scenario due to the involvement of numerous private laboratories primarily focused on domestic animals and lacking a shared database. To overcome this limitation, data was obtained from two major institutional veterinary centres involved in diagnostic activities within the territory, resulting in a substantial number of samples from various animal species collected over the past 20 years. It is important to acknowledge that this approach has certain limitations. Firstly, the sampling methods varied across different categories, with some samples obtained through diagnostic processes and others through regional surveillance plans for toxoplasmosis in animals. Consequently, certain animal species may be underrepresented in terms of the number of specimens. Additionally, the available databases cover different time periods (2002 to 2021 for IZSLER, 2006 to 2021 for VUH, and 2018 to 2021 for St. Orsola Hospital), and the serological tests employed differ not only between different diagnostic centers but also within the same diagnostic unit at different time points. Nonetheless, despite these limitations, the collected data provides an overall understanding of the circulation of *T. gondii* in various animal categories and humans in the province of

Table 2
Signalment and serological results for *T. gondii* in dogs and cats examined at the VUH.

Cats			Positive n. (%)		Negative n. (%)		Chi-square test -P
Examined	208 [from 36 municipalities]		59 (28.4%) [from 23 municipalities]		149 (71.6%)		
Age	< 1 year		8 (18.2%)		36 (81.8%)		NS
(207 known)	≥ 1 year		50 (30.7%)		113 (69.3%)		
Sex	male	neutered	25 (25.5%)	19 (35.1%)	73 (74.5%)	35 (35.7%)	NS
		entire		6 (13.6%)		38 (38.7%)	4.84 P < 0.05
	female	neutered	34 (30.9%)	23 (30.3%)	76 (69.1%)	53 (69.7%)	NS
		entire		11 (32.4%)		23 (67.6%)	NS
Dogs examined	65 [from 25 municipalities]		17 (26.2%) [from 11 municipalities]		48 (73.8%)		
Age	< 1 year		1 (11.1%)		8 (88.9%)		NS
	≥ 1 year		16 (28.6%)		40 (71.4%)		
Sex	male	neutered	7 (28%)	0 (0%)	18 (72%)	2 (100%)	NS
		entire		7 (30.4%)		16 (69.6%)	NS
	female	neutered	10 (25%)	5 (35.7%)	30 (45%)	9 (64.3%)	NS
		unneutered		5 (19.2%)		21 (80.8%)	NS

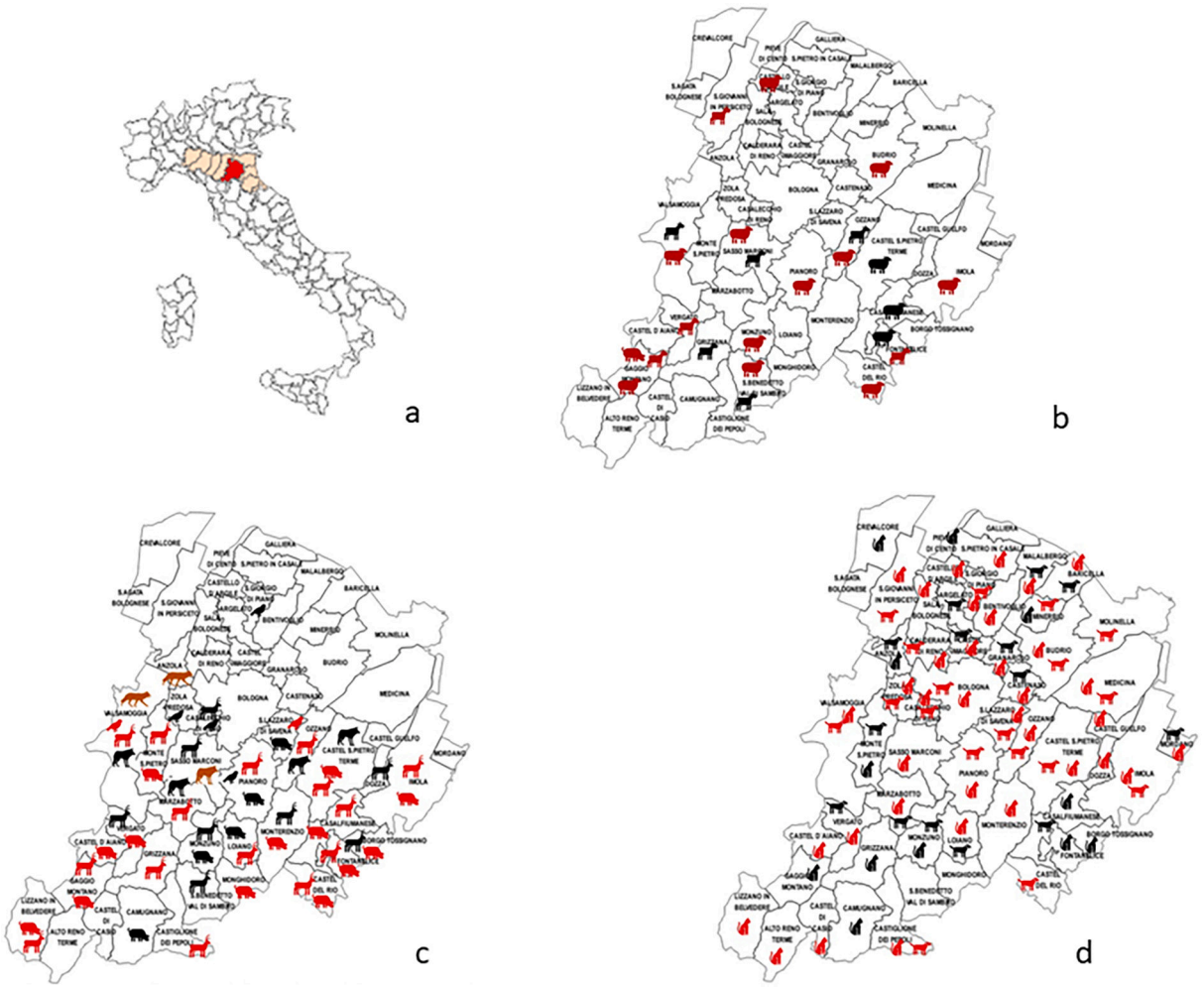


Fig. 1. Distribution of the animals examined by IZSLER and VUH: a) province of Bologna in Emilia Romagna region (Italy); b) Livestock species; c) Wild animals; d) dog and cat. The red figure means that at least one subject tested positive. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
Data concerning tests and serological results for *T. gondii* in humans. In the "acute infection" category, only active *T. gondii* infection cases, with positive IgM ELFA analyses and low-avidity samples, have been grouped.

	Total number	men (%)	women (%)	pregnant women (%)	non-pregnant women (%)
Screened subjects	36,814	4341 (11.8%)	32,473 (88.2%)	28,572 (87.9%)	3901 (12.1%)
Acute infection	161 (0.44%)	26	135	113	22
(frequence)		(0.60%)	(0.42%)	(0.39%)	(0.56%)

Bologna.

4.1. Livestock

In this retrospective study, diagnostic confirmation for *Toxoplasma gondii* was conducted in 181 small ruminants in the province of Bologna, specifically following abortion outbreaks, with seroprevalences of 29.9% in sheep and 18.7% in goats. The significance of toxoplasmosis in these animal species extends to both public health and economic aspects. They are considered a primary source of infection among certain ethnic groups that consume undercooked meat due to cultural reasons (Kijlstra and Jongert, 2008). Furthermore, *T. gondii* is recognized as one of the primary causes of abortion in sheep and goat farming (Dubey, 2022). Such losses can

be particularly devastating, especially for small family farms, which are prevalent in Mediterranean regions and have reported abortion rates as high as 75% (Edwards and Dubey, 2013). Although the seroprevalence values observed in our study were comparatively lower than those reported in other studies conducted in Northern Italy (Parigi, 2014, Gazzonis et al., 2015, 2020), it is crucial to consider the specific context of our data collection. The samples we analysed were obtained from farms in the area after abortion outbreaks, and it is possible that these outbreaks were not solely attributed to *T. gondii* infection. Previous studies have shown that goats generally exhibit lower positivity rates compared to sheep, consistent with the findings of the present survey. This variation can be attributed to the distinct feeding behaviors of the two species. Sheep, as grazers, are more vulnerable to the exposure of *T. gondii* oocysts and other soil-borne parasites, as indicated by prior research (Hoste et al., 2010). In contrast, goats, being browsers, have a relatively lower risk of contracting this parasitic infection.

4.2. Wild animals

The observed seroprevalences in wild boar (15.5%) and roe deer (25%), do not seem to reflect the increasing of the prevalence throughout the trophic chain previous described (Smith and Frenkel, 1995; Ferroglio et al., 2014) and are particularly intriguing: the higher exposure found in herbivores, suggests a relevant role of oocysts contamination in the considered territory. Oocyst environmental contamination could be linked to the presence of wild and domestic felids (Otranto et al., 2015). In recent years, the populations of wild boars and roe deer in Europe have shown significant expansion despite being among the most heavily hunted ungulate species (European Food Safety Authority (EFSA), 2014; Milner et al., 2006; Pittiglio et al., 2018). In recent decades, the roe deer population has undergone significant migration from northeastern regions to northwestern areas and the Apennines, establishing a relatively stable presence in our territories, especially in proximity to human-altered areas with a high abundance of free-roaming cats (Carnevali et al., 2009). The consumption of wild ungulate meat poses a potential risk of infection for other carnivorous hosts, including humans (Tenter et al., 2000). The meat of these ungulates is highly valued in certain regions of Italy, where culinary traditions may include the preparation of raw dishes.

Despite the limited sample size of wild carnivores in this survey, a notably high seroprevalence was observed, with 2 out of 2 red foxes and 1 out of 5 wolves testing positive for *T. gondii* antibodies. These findings are consistent with the results obtained in other surveys, where seroprevalence rates of up to 84.7% were reported in red foxes from north-eastern Europe (Kornacka-Strackonis, 2022), and *T. gondii* DNA was detected in 20% of grey wolves in Serbia (Uzelac et al., 2019). The higher susceptibility of these hosts to *T. gondii* infection, resulting from the consumption of both tissue cysts and environmentally-transmitted oocysts, positions them as valuable sentinels for monitoring the presence of the parasite within specific territories.

4.3. Dog and cat

Overall, in this study, cats demonstrated a seroprevalence of 42.9%, (1414 out of 3295 tested cats from both laboratories), in line with the estimate prevalence in Europe (43%) (Montazeri et al., 2020). Notably, colony cats exhibited significantly higher seroprevalence (46.5%) compared to owned cats (32.1%) ($P < 0.001$). Studies conducted in central Italy reported similar prevalence, such as 44% in colony cats from Florence province (Mancianti et al., 2010), and 62% in Rome (Macrì et al., 2009). However, it is important to consider the limitations of comparing these data due to variations in diagnostic techniques used over time, including in our laboratories, and the different cutoff titers.

Across the province of Bologna, seropositive cats were discovered in 36 out of 55 municipalities, indicating a widespread distribution of infected cats throughout the territory. This includes hilly and mountainous areas of the province, where positive cases were also found among wild ungulates. The detection of specific anti-*T. gondii* IgG in cats holds significant epidemiological implications. Cats that have developed protective IgG antibodies against *T. gondii* have likely shed oocysts in their living environment at some point, either in the distant or recent past, but are generally considered immune to further shedding of the parasite (Dubey, 1995).

Although not epidemiologically comparable to cats, dogs' exposure to *Toxoplasma* also has public health implications. Dogs can act as mechanical carriers of *T. gondii* oocysts, excreting them in their feces after ingestion from cat stool. Moreover, *T. gondii* oocysts can contaminate dog fur, potentially leading to human infection through contact with the dog's coat, mouth, and feet (Lindsay et al., 1997; Dubey et al., 2020b).

4.4. Humans

The seropositivity rate (20.4%) observed in this study is similar to the ones previously reported in other areas of Italy (Mosti et al., 2013; Fanigliulo et al., 2020).

The majority of available information on *Toxoplasma* seroprevalence in humans primarily focuses on women of reproductive age, utilizing prenatal screening data. Even in our retrospective study, over 95% of serological tests for *T. gondii* conducted between 2018 and 2021 were carried out on samples obtained from females, while only 4.1% were from males. This finding is not surprising, as serological testing during early pregnancy is strongly recommended (although not mandatory) according to the guidelines of the National Public Health Service. Moreover, *T. gondii* serology is provided free of charge to all pregnant women by the Italian Government (Italian Government, 2017). Consequently, pregnant women undergo testing for *T. gondii* during early pregnancy, and seronegative women are advised to undergo subsequent testing every 4–6 weeks until delivery. In addition to pregnant women, our study also included screenings conducted on transplant patients and individuals with clinical suspicion of toxoplasmosis.

In various high-income countries, a decline in *T. gondii* seroprevalence among the human population, specifically women of

childbearing age, has been observed since 2001 (Milne et al., 2023). This decrease has been attributed to shifts in dietary patterns, reduced prevalence in intensively farmed livestock, improved hygiene practices, and enhanced health education, collectively resulting in decreased exposure to the parasite (Pinto et al., 2012; Martini et al., 2020; Milne et al., 2023).

Although in many contexts, declining exposure to *T. gondii* commonly lead to a lower incidence of congenital toxoplasmosis due to fewer seroconversions in pregnancy, some studies have observed an unexpected increase in congenital toxoplasmosis incidence or IgM prevalence despite declining seroprevalence (Edelhofer and Prossinger, 2010; Mongua-Rodriguez et al., 2013). This phenomenon aligns with the epidemiological concept of "peak shift" dynamics, which suggests that as infection rates decrease, the risk of exposure is shifted to a higher age group (Woolhouse, 1998). Consequently, a naive population, including women of childbearing age, may become more susceptible to the infection due to increased exposure.

In the screened pregnant women population, the frequency of acute infections decreased to 0.25% (from the 0.39% of acute infection rate in the total population), which is consistent with findings from a previous study involving pregnant women in the Emilia-Romagna region (Billi et al., 2016). However, in the "second level *T. gondii* diagnosis group," the incidence was notably higher at 4.1%. Another study (Capretti et al., 2014) previously reported a significantly elevated incidence of toxoplasmosis among pregnant women who were non-native. A major limitation of our current survey is the inability to distinguish between native and non-native women, as well as the lack of clinical information and specific details regarding dietary habits.

Considering the seroprevalence in various animal species and humans observed in our specific study area, prenatal screening programs remain the mainstay of the prevention of congenital toxoplasmosis, allowing the early identification of maternal infection cases. An early detection of infection permits to start promptly the antenatal treatment to interrupt the vertical transmission, underlining the need to maintain an appropriate and active screening for toxoplasmosis during pregnancy.

5. Conclusions

The findings of this study provide compelling evidence for the wide distribution of the parasite in the study area. Specific antibodies were detected in a range of wildlife, livestock, domestic animals, and humans, indicating a constant presence of the parasite in diverse environments. The seropositivity rates observed in wildlife species like roe deer and wild boars underscore their significance in the parasite's epidemiology. They serve as indicators of environmental contamination in both wild and peri-urban settings, as well as potential sources of infection for humans. Furthermore, the presence of seropositivity in human populations, as well as in domestic and companion animals, highlights the occurrence of the parasite in anthropized environments. The interplay between anthropogenic and environmental factors shapes the epidemiology of this parasitic infection and influences its spread. Given the interdisciplinary nature of this issue, a One Health approach is crucial not only in prevalence surveys like this study but also in control, education, and prevention campaigns.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

Billi, P., Della Strada, M., Pierro, A., Semprini, S., Tommasini, N., Sambri, V., 2016. Three-year retrospective analysis of the incidence of *Toxoplasma gondii* infection in pregnant women living in the Greater Romagna Area (northeastern Italy). Clin. Microbiol. Infect. 22 https://doi.org/10.1016/j.cmi.2016.02.010, 572.e1–3. Epub 2016 Mar 9. PMID: 26970048.

Boyer, K., Hill, D., Mui, E., Wroblewski, K., Karrison, T., Dubey, J.P., Sautter, M., Noble, A.G., Withers, S., Swisher, C., Heydemann, P., Hosten, T., Babiarz, J., Lee, D., Meier, P., McLeod, R., Toxoplasmosis Study Group, 2011. Unrecognized ingestion of *Toxoplasma gondii* oocysts leads to congenital toxoplasmosis and causes epidemics in North America. Clin. Infect. Dis. 53, 1081–1089. https://doi.org/10.1093/cid/cir667.

Capretti, M.G., De Angelis, M., Tridapalli, E., Orlandi, A., Marangoni, A., Moroni, A., Guerra, B., Arcuri, S., Marsico, C., Faldella, G., 2014. Toxoplasmosis in pregnancy in an area with low seroprevalence: is prenatal screening still worthwhile? Pediatr. Infect. Dis. J. 33, 5–10. https://doi.org/10.1097/INF.0000000000000006. PMID: 24064557.

Carnevali, L., Pedrotti, L., Riga, F., Toso, S., 2009. Banca Dati Ungulati: Status, distribuzione, consistenza, gestione e prelievo venatorio delle popolazioni di Ungulati in Italia. Rapporto 2001–2005. Biol. Cons. Fauna 117, 1–168.

Dini, F.M., Graziosi, G., Lupini, C., Catelli, E., Galuppi, R., 2023. Migratory wild birds as potential long-distance transmitters of Toxoplasma gondii infection. Pathogens. 12, 478. https://doi.org/10.3390/pathogens12030478.

Djurkovi'c-Djakovi'c, O., Dupouy-Camet, J., Van Der Giessen, J., Dubey, J.P., 2019. Toxoplasmosis: overview from a One Health perspective. Food Waterborne Parasitol. 15, e00054 https://doi.org/10.1016/j.fawpar.2019.e00054.

Dubey, J.P., 1995. Duration of immunity to shedding of *Toxoplasma gondii* oocysts by cats. J. Parasitol. 81, 410–415. PMID: 7776126.

Dubey, J.P., 2022. Toxoplasmosis of Animals and Humans, second edition. CRC Press, Boca Raton, London New York.

Dubey, J.P., Cerqueira-Cézar, C.K., Murata, F.H.A., Kwok, O.C.H., Yang, Y.R., Su, C., 2020a. All about toxoplasmosis in cats: the last decade. Vet. Parasitol. 283, 109145 https://doi.org/10.1016/j.vetpar.2020.109145. Epub 2020 May 27. PMID: 32645556.

Dubey, J.P., Murata, F.H.A., Cerqueira-Cézar, C.K., Kwok, O.C.H., Yang, Y., Su, C., 2020b. *Toxoplasma gondii* infections in dogs: 2009–2020. Vet. Parasitol. 287, 109223 https://doi.org/10.1016/j.vetpar.2020.109223.

Edelhofer, R., Prossinger, H., 2010. Infection with *Toxoplasma gondii* during pregnancy: seroepidemiological studies in Austria. Zoonoses Public Health 57 (18–26), 79.

Edwards, J.F., Dubey, J.P., 2013. *Toxoplasma gondii* abortion storm in sheep on a Texas farm and isolation of mouse virulent atypical genotype *T. gondii* from an aborted lamb from a chronically infected ewe. Vet. Parasitol. 192, 129–136. https://doi.org/10.1016/j.vetpar.2012.09.037.

European Food Safety Authority (EFSA), 2014. Evaluation of possible mitigation measures to prevent introduction and spread of African swine fever virus through wild boar. EFSA J. 12, 3616. https://doi.org/10.2903/j.efsa.2014.3616.

Fanigliulo, D., Marchi, S., Montomoli, E., Trombetta, C.M., 2020. *Toxoplasma gondii* in women of childbearing age and during pregnancy: seroprevalence study in Central and Southern Italy from 2013 to 2017. Parasite. 27, 2. https://doi.org/10.1051/parasite/2019080.

Ferroglio, E., Bosio, F., Trisciuoglio, A., Zanet, S., 2014. *Toxoplasma gondii* in sympatric wild herbivores and carnivores: epidemiology of infection in the Western Alps. Parasit. Vectors 7, 196.

Gazzonis, A.L., Veronesi, F., Di Cerbo, A.R., Zanzani, S.A., Molineri, G., Moretta, I., Moretti, A., Piergili Fioretti, D., Invernizzi, A., Manfredi, M.T., 2015. *Toxoplasma gondii* in small ruminants in Northern Italy – prevalence and risk factors. Ann. Agric. Environ. Med. 22, 62–68.

Gazzonis, A.L., Zanzani, S.A., Villa, L., Manfredi, M.T., 2020. *Toxoplasma gondii* infection in meat-producing small ruminants: meat juice serology and genotyping. Parasitol. Int. 76, 102060.

Guo, M., Dubey, J.P., Hill, D., Buchanan, R.L., Ray Gamble, H., Jones, J.L., Pradhan, A.K., 2015. Prevalence and risk factors for *Toxoplasma gondii* infection in meat animals and meat products destined for human consumption. J. Food Prot. 78, 457–476. https://doi.org/10.4315/0362-028X.JFP-14-328.

Hill, D., Coss, C., Dubey, J.P., Wroblewski, K., Sautter, M., Hosten, T., Muñoz-Zanzi, C., Mui, E., Withers, S., Boyer, K., Hermes, G., Coyne, J., Jagdis, F., Burnett, A., McLeod, P., Morton, H., Robinson, D., McLeod, R., 2011. Identification of a sporozoite-specific antigen from *Toxoplasma gondii*. J. Parasitol. 97, 328–337. https://doi.org/10.1645/GE-2782.1.

Hoste, H., Sotiraki, S., Landau, S.Y., Jackson, F., Beveridge, I., 2010. Goat-nematode interactions: think differently. Trends Parasitol. 26, 376–381. https://doi.org/10.1016/j.pt.2010.04.007.

Italian Government, 2017. Decree of the president of ministers 12 January 2017. Off. J. Serie Generale n.65 del 18-03-2017. Ordinary Supplement n. 15, available at. https://www.gazzettaufficiale.it/eli/gu/2017/03/18/65/so/15/sg/pdf.

Kijlstra, A., Jongert, E., 2008. Control of the risk of human toxoplasmosis transmitted by meat. Int. J. Parasitol. 38, 1359–1370. https://doi.org/10.1016/j.ijpara.2008.06.002.

Kornacka-Strackonis, A., 2022. *Toxoplasma gondii* infection in wild omnivorous and carnivorous animals in Central Europe- a bried overview. Vet. Parasitol. 304, 109701.

Lindsay, D.S., Dubey, J.P., Butler, J.M., Blagburn, B.L., 1997. Mechanical transmission of *Toxoplasma gondii* oocysts by dogs. Vet. Parasitol. 77, 27–33.

Macrì, G., Sala, M., Linder, A.M., Pettirossi, N., Scarpulla, M., 2009. Comparison of indirect fluorescent antibody test and modified agglutination test for detecting *Toxoplasma gondii* immunoglobulin G antibodies in dog and cat. Parasitol. Res. 105, 35–40. https://doi.org/10.1007/s00436-009-1358-4.

Mancianti, F., Nardoni, S., Ariti, G., Parlanti, D., Giuliani, G., Papini, R.A., 2010. Cross-sectional survey of *Toxoplasma gondii* infection in colony cats from urban Florence (Italy). J. Feline Med. Surg. 12, 351–354.

Martini, A., Pietrafesa, E., Rondinone, B.M., Iavicoli, S., D'amelio, S., Cavallero, S., Bonafede, M., 2020. Toxoplasmosis and knowledge: what do the Italian women know about? Epidemiol. Infect. 148, 256. https://doi.org/10.1017/S0950268820002393.

Milne, G.C., Webster, J.P., Walker, M., 2023. Is the incidence of congenital toxoplasmosis declining? Trends Parasitol. 39, 26–37 https://doi.org/10.1016/j.pt.2022.10.003.

Milner, J.M., Bonenfant, C., Mysterud, A., Gaillard, J.-M., Csányi, S., Stenseth, N.C., 2006. Temporal and spatial development of red deer harvesting in Europe: biological and cultural factors. J. Appl. Ecol. 43, 721–734.

Mongua-Rodriguez, N., Díaz-Ortega, J.L., García-García, L., Piña-Pozas, M., Ferreira-Guerrero, E., Delgado-Sánchez, G., Ferreyra-Reyes, L., Cruz-Hervert, L.P., Baez-Saldaña, R., Campos-Montero, R., 2013. A systematic review of rubella vaccination strategies implemented in the Americas: impact on the incidence and seroprevalence rates of rubella and congenital rubella syndrome. Vaccine 31, 2145–2151 https://doi.org/10.1016/j.vaccine.2013.02.047.

Montazeri, M., Mikaeili Galeh, T., Moosazadeh, M., Sarvi, S., Dodangeh, S., Javidnia, J., Sharif, M., Daryani, A., 2020. The global serological prevalence of *Toxoplasma gondii* in felids during the last five decades (1967–2017): a systematic review and meta-analysis. Parasit. Vectors 13, 82. https://doi.org/10.1186/s13071-020-3954-1.

Montoya, J.G., Liesenfeld, O., 2004. Toxoplasmosis. Lancet 12, 1965–1976. https://doi.org/10.1016/S0140-6736(04)16412-X. PMID: 15194258.

Mosti, M., Pinto, B., Giromella, A., Fabiani, S., Cristofani, R., Panichi, M., Bruschi, F., 2013. A 4-year evaluation of toxoplasmosis seroprevalence in the general population and in women of reproductive age in central Italy. Epidemiol. Infect. 141, 2192–2195. https://doi.org/10.1017/S0950268812002841.

Otranto, D., Cantacessi, C., Pfeffer, M., Dantas-Torres, F., Brianti, E., Deplazes, P., Genchi, C., Guberti, V., Capelli, G., 2015. The role of wild canids and felids in spreading parasites to dogs and cats in Europe. Part I: Protozoa and tick-borne agents. Vet. Parasitol. 213, 12–23. https://doi.org/10.1016/j.vetpar.2015.04.022. Epub 2015 May 8. PMID: 26003669.

Parigi, M., 2014. *Toxoplasma gondii* in animals and the environment. PhD Thesis in Veterinary Sciences,. Alma Mater Studiorum University of Bologna. https://doi.org/10.6092/unibo/amsdottorato/6423.

Pepe, P., Bosco, A., Capuano, F., Baldi, L., Giordano, A., Mancusi, A., Buonanno, M., Morena, L., Pinto, R., Sarnelli, P., Cringoli, G., Rinaldi, L., 2021. Towards an integrated approach for monitoring toxoplasmosis in Southern Italy. Animals. 11, 1949. https://doi.org/10.3390/ani11071949.

Pereira, K.S., Franco, R.M., Leal, D.A., 2010. Transmission of toxoplasmosis (*Toxoplasma* gondii) by foods. Adv. Food Nutr. Res. 60, 1–19. https://doi.org/10.1016/S1043-4526(10)60001-0.

Pinto, B., Castagna, B., Mattei, R., Bruzzi, R., Chiumiento, L., Cristofani, R., Buffolano, W., Bruschi, F., 2012. Seroprevalence for toxoplasmosis in individuals living in north west Tuscany: access to Toxo-test in central Italy. Eur. J. Clin. Microbiol. Infect. Dis. 31, 1151–1156. https://doi.org/10.1007/s10096-011-1422-8.

Pittiglio, C., Khomenko, S., Beltran-Alcrudo, D., 2018. Wild boar mapping using population density statistics: from polygons to high resolution raster maps. PLoS One 13, e0193295. https://doi.org/10.1371/journal.pone.0193295.

Smith, D.D., Frenkel, J.K., 1995. Prevalence of antibodies to *Toxoplasma gondii* in wild mammals of Missouri and east central Kansas: biologic and ecologic considerations of transmission. J. Wildl. Dis. 31, 15–21. https://doi.org/10.7589/0090-3558-31.1.15.

Tenter, A.M., Heckeroth, A., Weiss, L.M.R., 2000. *Toxoplasma gondii*: from animals to humans. Int. J. Parasitol. 30, 1217–1258.

Tyebji, S., Seizova, S., Hannan, A.J., Tonkin, C.J., 2019. Toxoplasmosis: a pathway to neuropsychiatric disorders. Neurosci. Biobehav. Rev. 96, 72–92. https://doi.org/10.1016/j.neubiorev.2018.11.012.

Uzelac, A., Klun, I., Ćirović, D., Penezić, A., Ćirković, V., Djurković-Djaković, O., 2019. Detection and genotyping of *Toxoplasma gondii* in wild canids in Serbia. Parasitol. Int. 73, 101973 https://doi.org/10.1016/j.parint.2019.101973.

Woolhouse, M.E.J., 1998. Patterns in parasite epidemiology: the peak shift. Parasitol. Today 14, 428–434.

Chapter 4.1

Migratory Wild Birds as Potential Long-Distance Transmitters of *Toxoplasma gondii* Infection

Dini FM, Graziosi G, Lupini C, Catelli E, Galuppi R. (2023). Migratory wild birds as potential long-distance transmitters of *Toxoplasma gondii* infection. *Pathogens*. 12 (3): 478. doi:10.3390/pathogens12030478.

pathogens

MDPI

Communication

Migratory Wild Birds as Potential Long-Distance Transmitters of *Toxoplasma gondii* Infection

Filippo Maria Dini *,†, Giulia Graziosi †, Caterina Lupini, Elena Catelli and Roberta Galuppi

Department of Veterinary Medical Sciences, University of Bologna, Ozzano dell'Emilia, 40064 Bologna, Italy; roberta.galuppi@unibo.it (R.G.)

* Correspondence: filippomaria.dini@unibo.it

† These authors contributed equally to this work.

Abstract: *Toxoplasma gondii* is a worldwide distributed zoonotic protozoan capable of infecting a wide range of mammals (including humans) and birds as intermediate hosts. Migratory wild birds, through interconnecting countries along their flyways, can play a role in the spatial spread of *T. gondii* and could contribute to its sylvatic cycle. Additionally, hunted wild birds used for meat consumption could represent a further source of human infection. To determine the presence of *T. gondii* in wild birds, a total of 50 individuals belonging to the Anseriformes and Charadriiformes orders were sampled during the 2021–2022 hunting season in Northern Italy. Cardiac muscle samples of three Northern shovelers (*Anas clypeata)*, two wild mallards (*A. platyrhynchos*), one Eurasian teal (*A. crecca*), and one Northern lapwing (*Vanellus vanellus*) were positive for the molecular detection of *T. gondii* based on a targeted amplification of the B1 gene. A 14% (7/50) overall positivity was observed in the sampled population. Results from this study suggest a moderate exposure of wild aquatic birds to *T. gondii*, highlighting the importance of a further characterization of *T. gondii* in its wildlife hosts.

Keywords: *Toxoplasma gondii*; wild birds; One Health; PCR

Citation: Dini, F.M.; Graziosi, G.; Lupini, C.; Catelli, E.; Galuppi, R. Migratory Wild Birds as Potential Long-Distance Transmitters of *Toxoplasma gondii* Infection. *Pathogens* **2023**, *12*, 478. https://doi.org/10.3390/pathogens12030478

Academic Editor: Bernard Davoust

Received: 15 February 2023
Revised: 14 March 2023
Accepted: 17 March 2023
Published: 18 March 2023

1. Introduction

Toxoplasma gondii is a widespread zoonotic apicomplexan protozoan potentially able to infect all the warm-blooded animal species [1]. The life cycle of this parasite is complex, including definitive and intermediate hosts and several transmission pathways. Millions of unsporulated oocysts are shed in feces by felids, the definitive hosts of *T. gondii*. The parasite can therefore be found in various environmental matrices where, upon sporulation, oocysts become infectious and can remain viable up to several years [2]. This environmentally resistant stage is critical to the success of the parasite's life cycle, and it shapes the extensive range of its intermediate hosts. The oocysts can be further dispersed by wind, earthworms, arthropods, and water [3]; any warm-blooded animal can therefore become infected by the ingestion of oocysts-contaminated soil, water, or plant tissue [2]. For mammals, a vertical route of transmission is also expected [2]. When infecting an intermediate host, *T. gondii* forms life-long persistent cysts located prevalently in neural and muscular tissues, such as brain, retina, and skeletal and cardiac muscles [4]. Through predation between intermediate hosts, tissue cysts act as a reservoir of infection even in the absence of felids [5,6]. Nevertheless, sexual replication of *T. gondii* and the fecal excretion of its oocysts can only happen in the definitive hosts [2]. Environmental factors such as temperature and humidity can affect the life cycle of *T. gondii* by influencing the survival time of unsporulated oocysts; furthermore, seasonal fluctuation in precipitation rates influence the dispersion of sporulated oocysts [7].

Infections caused by *T. gondii* in wildlife and humans can determine heterogeneous clinical observations. Toxoplasmosis can indeed be fatal or chronic, with disease severity affected by host-dependent or parasite-dependent variables (e.g., individual immune

response, species-specific susceptibility, strain virulence, and infective dose) [2]. In avian populations, toxoplasmosis can be particularly concerning for endangered species [8–10].

Considering the terrestrial definitive host of *T. gondii*, oocysts are exclusively deposited on land and can therefore disperse in freshwater following heavy rainfalls, owing to the hydrophilic nature of their surface [3,11]; this sheds interest on surveys concerning the occurrence of *T. gondii* infections in wild aquatic species. In aquatic environments, intermediate avian hosts belonging to different feeding groups (herbivores, omnivores, carnivores, and insectivores) appear to be subjected to a similar infection probability [12]. In birds, several aquatic species are known hosts of relevant animal or zoonotic pathogens [13–17], including *T. gondii* [18,19]. Considering the ecology of waterfowl and the specialization as filter-feeders of some species, *T. gondii* infection in these animals suggests the presence of an oocysts-contaminated aquatic habitat. Furthermore, being huntable aquatic birds and a human food source, the consumption of raw or undercooked meat, especially derived from niche products, could represent an underappreciated source of *Toxoplasma gondii* infection [19,20]. Given the scarce available epidemiological data, the present study aimed to assess the occurrence of *T. gondii* in wild aquatic birds hunted in the wetlands of Northern Italy, where wintering migratory individuals from different breeding grounds congregate seasonally.

2. Materials and Methods

2.1. Population of Interest

A total of 50 hunted wild aquatic birds were included in this study, selected among 124 individuals sampled within the application of the National Avian Influenza (AI) Surveillance Plan 2021 (https://www.izsvenezie.it/documenti/temi/influenza-aviaria/piani-sorveglianza/piano-nazionale-influenza-aviaria-2021.pdf, accessed on 22 October 2021) and the Commission Delegated Regulation (EU) 2020/689. As an inclusion criterion, only individuals whose entire carcass was preserved were included in the molecular survey hereby presented. Sampling activities were conducted from October 2021 to January 2022 in two hunting grounds (Figure 1d) of the province of Bologna, Emilia-Romagna region, Northern Italy, in an area where wintering migratory birds congregate and intermingle with resident populations. Licensed hunters made available their hunting bags for AI surveillance purpose, and the samplings were performed on the behalf of the Local Health Authority A.U.S.L. of Imola (BO). Birds were hunted according to the National Hunting law 157/1992, without the necessity of any additional permits. Overall, 19 Northern shovelers (*Spatula clypeata*), 18 Eurasian teals (*Anas crecca*), 7 mallards (*A. plathyrhynchos*), 5 Northern lapwings (*Vanellus vanellus*), and 1 gadwall (*Mareca strepera*) were sampled. The sex and age class (juvenile of the year or adult) of each individual were recorded by trained ornithologists, as reported in Figure 1a and 1b, respectively.

2.2. Molecular Detection of Toxoplasma gondii

Heart tissue samples were collected and stored at −20 °C until processing. Genomic DNA was purified from 25 mg of tissue using the Pure Link® Genomic DNA Mini kit (Invitrogen by Thermo Fisher), according to the manufacturer's protocol. Nested PCR targeting the glycerol-3-phosphate dehydrogenase gene (B1) of *T. gondii* was performed with minor modifications of the protocol described by Jones et al. [21]. Briefly, the first round of amplification included a denaturation step at 96 °C for 2 min, followed by 40 cycles at 93 °C for 10 s, 57 °C for 10 s, and 72 °C for 30 s. The second round was as follows: denaturation step at 95 °C for 2 min, followed by 40 cycles at 93 °C for 10 s, 62.5 °C for 10 s, and 72 °C for 30 s. Amplifications were performed in a T-personal thermal cycler (Biometra, Goettingen, Germany). Amplicons from the first and second PCR rounds have an expected length of 193 bp and 96 bp, respectively. Water was used as a negative control, and *T. gondii* positive DNA was added as a positive control. PCR products were electrophoresed on 2% agarose gel stained with SYBR Safe DNA Gel Stain (Thermo Fisher Scientific, Carlsbad, CA, USA) in 0.5× TBE. For sequencing, the amplicons were excised and purified by Nucleo-Spin

Gel and PCR Clean-up (Macherey-Nagel, Düren, Germany) and sequenced with an ABI 3730 DNA analyzer (StarSEQ, Mainz, Germany). Consensus sequences were compared with published sequences by a BLAST search (https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 21 December 2022).

2.3. Statistical Analyses

Data analyses were performed using GraphPad Prism (version 8.0) software (GraphPad Software Inc., San Diego, CA, USA). Fisher's exact test (two sided) was used to determine the association between the molecular results and the species, age class, and sex of the birds tested. A p value below 0.05 ($p < 0.05$) was considered significant.

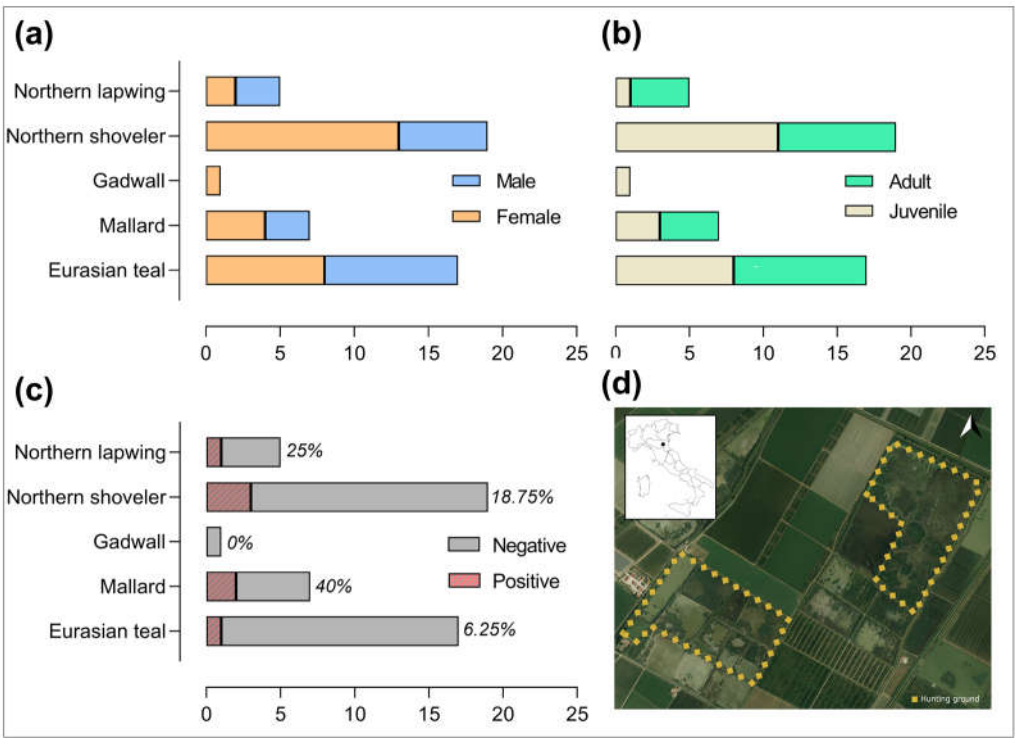


Figure 1. Population of interest, results, and study area. The number of sampled birds according to species and sex (**a**) and age class (**b**); the results of *T. gondii* molecular detection (**c**); and the hunting grounds (**d**), located in the Bologna province, Northern Italy. Map realized with QGIS software version 3.26 [22].

3. Results

The amplification of the B1 gene was successful in 7 out of the 50 heart specimens tested (14%) (Figure 2). BLAST searches on the obtained sequences gave a 100% identity with *T. gondii*. Sequence data were submitted to the NCBI GenBank database under the following accession numbers: OQ646717-23. As shown in Figure 1c, 3 Northern shovelers (1 juvenile female and 2 adult males), 1 Eurasian teal (adult male), 2 mallards (1 juvenile female and 1 adult male), and 1 Northern lapwing (juvenile male) tested positive. There was no association was observed between the molecular detection of *T. gondii* and species ($p > 0.1$), sex ($p = 0.43$), or age class ($p = 0.09$) of the birds tested.

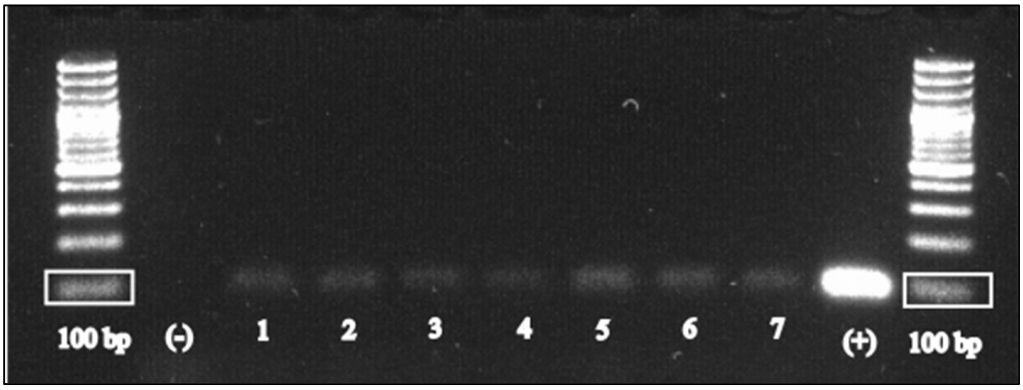


Figure 2. Agarose gel showing the 96 bp amplicons obtained from the individuals tested positive for *T. gondii*. The following order of samples is shown: negative control, 3 Northern shovelers (slots numbered 13), 1 Northern lapwing (slot 4), 1 Eurasian teal (slot 5), 2 mallards (slots 6 and 7), and *T. gondii* positive control.

4. Discussion

Considering *T. gondii* oocysts as widely distributed in the environment, especially in aquatic habitats [23,24], this study aimed to assess the molecular occurrence of *T. gondii* in hunted wild aquatic birds. The population tested included species with different habits and migratory strategies. As a result, parasitic DNA was found in 14% (7/50) of the heart samples tested, showing a moderate exposure of waterbirds to the infection.

Toxoplasma gondii molecular detection in individuals belonging to the Anseriformes order, namely, Northern shovelers, mallards, and Eurasian teals, confirmed previously published records for other geographic areas surveyed [16,25–28]. From an ecological point of view, the above-mentioned dabbling ducks are long-distance migrants along the Black Sea–Mediterranean flyway, which also encompasses the Italian wetlands. Mean estimates from ring recoveries data demonstrated that Eurasian teals and mallards can displace up to 326.5 km and 289.63 km per day during migration, respectively [29]. Epidemiological surveys carried out in wild and domestic animals sampled in North-Eastern Europe and Russia, where dabbling ducks' breeding grounds are located, reported serological or molecular detection of *T. gondii* in different intermediate hosts, suggesting a possible local infection [18,30,31]. Wild ducks could get infected since hatching and, through seasonal migratory movements between wintering grounds located in Italian wetlands and their breeding territories, could therefore play a role as long-distance transmitters in the *T. gondii* epidemiology.

For Northern lapwings, a species belonging to the Charadriiformes order commonly found in open lands and mudflats, *T. gondii* infection has already been reported by Nardoni et al. [26] in central Italy. Although considered migratory in other areas, lapwings are usually residents in southwestern Europe [32]; it is therefore likely that the adult individual hereby tested positive was locally exposed to the parasite, like the juvenile ducks (2 out of the 6 ducks tested). Wetlands in Northern Italy are represented by natural areas interspersed with anthropic environments used as water storage for cropland irrigation, hunting grounds, or wastewater plants. The environmental contamination of freshwater with *T. gondii* oocysts could be associated with inefficient sewage treatment, water discharge, and water runoff, as already demonstrated for marine environments [33]. Considering the earliest arrival of migratory ducks in Italian wintering grounds in August [34] and the *T. gondii* detection in the studied population since mid-October, local exposure to the parasite may also be likely for the adult ducks. In fact, an experimental study in chickens reported the detection of tissue cysts in brain, heart, liver, spleen, or lungs, starting from 7–15 days post-infection [35].

Molecular studies aimed at the detection of *T. gondii* in wild birds have been carried out testing different matrices [36]; in this survey, heart samples only were collected. These

are widely used for *T. gondii* PCR detection, even in wild aquatic birds [18,25,26], and can be associated with different techniques of DNA extraction developed to improve parasite detection and quantification [37,38]. Although the DNA extraction technique adopted in this study involved the use of a small amount of tissue, potentially resulting in less sensitivity than others such as magnetic-capture DNA extraction, higher overall molecular positivity (14%) has been hereby observed in comparison with previous records in wild aquatic birds [16,25,26,39–43]. For Italy, Mancianti et al. [25] and Nardoni et al. [26] performed serological investigations in waterbirds, and seropositive-only individuals were tested by PCR. As a result, 8.7% [25] and 8.1% [26] of the hunted waterfowls were seropositive and, among these, *T. gondii* was molecularly detected in 3 out of 9 and 8 out of 12 individuals, respectively. However, as reported by Opsteegh et al. [44] for *T. gondii* infection in cattle, also seronegative animals could harbor tissue cysts. The loss or absence of a detectable antibody titer in infected individuals has been observed in several bird species [45,46], while Bachand et al. [39] found discrepancies between serological and molecular results in both directions (e.g., seronegative animals with *T. gondii* positive tissues, and seropositive animals with tissues negative for parasite DNA). The molecular screening of all the individuals studied, as performed in this survey, could therefore reflect the actual percentage of birds that harbor tissue cysts. Furthermore, although no correlation with sex and age was hereby observed, these variables deserve attention in further investigations with a larger sample size to obtain a better understanding of *T. gondii* epidemiology in wild aquatic birds.

From a One Health point of view, the detection of *T. gondii* in heart samples may indicate the presence of tissue cysts in edible muscles, such as the pectoral ones, whose consumption could determine a meat-borne transmission of the parasite. In addition, it has been shown that handling wild or domestic animal carcasses without appropriate hygiene practices could lead to infection due to the accidental contamination of hand or other equipment from bradyzoites released from tissue cysts during cutting practices [47–50].

To conclude, results from this study highlight moderate exposure to *T. gondii* in wild aquatic birds from Northern Italy, suggesting their importance as biological indicators for *T. gondii* contamination of aquatic habitats and their potential contribution to the sylvatic cycle of the parasite. Furthermore, migratory species could act as *T. gondii* long-distance transmitters, considering their ecology and habits. An additional genetic characterization of the positive samples is needed to establish the role of migratory birds in linking countries where different genotypes circulate.

Author Contributions: Conceptualization, R.G., C.L. and E.C.; methodology, F.M.D. and G.G., software, G.G. and F.M.D.; validation, R.G., formal analysis, F.M.D. and G.G.; investigation, F.M.D.; resources, G.G., F.M.D. and R.G.; data curation, F.M.D. and G.G.; writing—original draft preparation, G.G. and F.M.D.; writing—review and editing, R.G.; visualization, R.G., C.L. and E.C.; supervision, R.G.; project administration, R.G. and E.C. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: The activities were carried out in accordance with the National Avian Influenza Surveillance Plan 2021 (https://www.izsvenezie.it/documenti/temi/influenza-aviaria/piani-sorveglianza/piano-nazionale-influenza-aviaria-2021.pdf, accessed on 23 October 2021) and the Commission Delegated Regulation (EU) 2020/689. Birds were hunted according to the National Hunting law 157/1992, without the necessity of any additional permits.

Informed Consent Statement: Not applicable.

Data Availability Statement: GenBank accession numbers: OQ646717-23.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Dubey, J.P. The history and life cycle of *Toxoplasma gondii*. In *Toxoplasma gondii*; Elsevier: Amsterdam, The Netherlands, 2020; pp. 1–19.
2. Dubey, J.P. *Toxoplasmosis of Animals and Humans*, 3rd ed.; CRC Press: Boca Raton, FL, USA, 2022.
3. Shapiro, K.; Bahia-Oliveira, L.; Dixon, B.; Dumetre, A.; de Wit, L.A.; VanWormer, E.; Villena, I. Environmental transmission of *Toxoplasma gondii*: Oocysts in water, soil and food. *Food Waterborne Parasitol.* **2019**, *15*, e00049. [CrossRef] [PubMed]
4. Dubey, J.P. Long term persistence of *Toxoplasma gondii* in tissues of pigs inoculated with *T. gondii* oocysts and effect of freezing on viability of tissue cysts in pork. *Am. J. Vet. Res.* **1988**, *49*, 910–913. [PubMed]
5. Akerstedt, J.; Lillehaug, A.; Larsen, I.-L.; Eide, N.E.; Arnemo, J.M.; Handeland, K. Serosurvey for canine distemper virus, canine adenovirus, *Leptospira interrogans*, and *Toxoplasma gondii* in free-ranging canids in Scandinavia and Svalbard. *J. Wildl. Dis.* **2010**, *46*, 474–480. [CrossRef] [PubMed]
6. Zanet, S.; Sposimo, P.; Trisciuoglio, A.; Giannini, F.; Strumia, F.; Ferroglio, E. Epidemiology of *Leishmania infantum*, *Toxoplasma gondii*, and *Neospora caninum* in *Rattus rattus* in absence of domestic reservoir and definitive hosts. *Vet. Parasitol.* **2014**, *199*, 247–249. [CrossRef] [PubMed]
7. Yan, C.; Liang, L.J.; Zheng, K.Y.; Zhu, X.Q. Impact of environmental factors on the emergence, transmission and distribution of *Toxoplasma gondii*. *Parasit Vectors* **2016**, *9*, 137. [CrossRef] [PubMed]
8. Frenkel, J.K.; Hassanein, K.M.; Hassanein, R.S.; Brown, E.; Thulliez, P.; Quintero-Nunez, R. Transmission of *Toxoplasma gondii* in Panama City, Panama: A Five-Year Prospective Cohort Study of Children, Cats, Rodents, Birds, and Soil. *Am. J. Trop. Med.* **1995**, *53*, 458–468. [CrossRef] [PubMed]
9. Dubey, J.P. A review of toxoplasmosis in wild birds. *Vet. Parasitol.* **2002**, *106*, 121–153. [CrossRef]
10. Wendte, J.M.; Gibson, A.K.; Grigg, M.E. Population genetics of *Toxoplasma gondii*: New perspectives from parasite genotypes in wildlife. *Vet. Parasitol.* **2011**, *182*, 96–111. [CrossRef]
11. Shapiro, K.; Largier, J.; Mazet, J.A.K.; Bernt, W.; Ell, J.R.; Melli, A.C.; Conrad, P.A. Surface properties of *Toxoplasma gondii* oocysts and surrogate microspheres. *Appl. Environ. Microbiol.* **2009**, *75*, 1185–1191. [CrossRef]
12. Wilson, A.G.; Lapen, D.R.; Mitchell, G.W.; Provencher, J.F.; Wilson, S. Interaction of diet and habitat predicts *Toxoplasma gondii* infection rates in wild birds at a global scale. *Glob. Ecol. Biogeogr.* **2020**, *29*, 1189–1198. [CrossRef]
13. Graziosi, G.; Catelli, E.; Fanelli, A.; Lupini, C. Infectious bursal disease virus in free-living wild birds: A systematic review and meta-analysis of its sero-viroprevalence on a global scale. *Transbound. Emerg. Dis.* **2021**, *69*, 2800–2815. [CrossRef] [PubMed]
14. Graziosi, G.; Lupini, C.; Catelli, E. Disentangling the role of wild birds in avian metapneumovirus (aMPV) epidemiology: A systematic review and meta-analysis. *Transbound. Emerg. Dis.* **2022**, *69*, 3285–3299. [CrossRef] [PubMed]
15. De Marco, M.A.; Delogu, M.; Facchini, M.; Di Trani, L.; Boni, A.; Cotti, C.; Graziosi, G.; Venturini, D.; Regazzi, D.; Ravaioli, V.; et al. Serologic Evidence of Occupational Exposure to Avian Influenza Viruses at the Wildfowl/Poultry/Human Interface. *Microorganisms* **2021**, *9*, 2153. [CrossRef] [PubMed]
16. Benskin, C.M.; Wilson, K.; Jones, K.; Hartley, I.R. Bacterial pathogens in wild birds: A review of the frequency and effects of infection. *Biol. Rev. Camb. Philos. Soc.* **2009**, *84*, 349–373. [CrossRef] [PubMed]
17. Ayala, A.J.; Yabsley, M.J.; Hernandez, S.M. A Review of Pathogen Transmission at the Backyard Chicken-Wild Bird Interface. *Front. Vet. Sci.* **2020**, *7*, 539925. [CrossRef]
18. Zhang, F.K.; Wang, H.J.; Qin, S.Y.; Wang, Z.D.; Lou, Z.L.; Zhu, X.Q.; Liu, Q. Molecular detection and genotypic characterization of *Toxoplasma gondii* in wild waterfowls in Jilin Province, Northeastern China. *Parasitol. Int.* **2015**, *64*, 576–578. [CrossRef]
19. Murao, T.; Omata, Y.; Kano, R.; Murata, S.; Okada, T.; Konnai, S.; Asakawa, M.; Ohashi, K.; Onuma, M. Serological Survey of *Toxoplasma gondii* in Wild Waterfowl in Chukotka, Kamchatka, Russia and Hokkaido, Japan. *J. Parasitol.* **2008**, *94*, 830–833. [CrossRef]
20. Guo, M.; Dubey, J.P.; Hill, D.; Buchanan, R.L.; Gamble, H.R.; Jones, J.L.; Pradhan, A.K. Prevalence and Risk Factors for *Toxoplasma gondii* Infection in Meat Animals and Meat Products Destined for Human Consumption. *J. Food Prot.* **2015**, *78*, 457–476. [CrossRef]
21. Jones, C.D.; Okhravi, N.; Adamson, P.; Tasker, S.; Lightman, S. Comparison of PCR Detection Methods for B1, P30, and 18S rDNA Genes of *T. gondii* in Aqueous Humor. *Investig. Ophtalmol. Vis. Sci.* **2000**, *41*, 634–644.
22. Team, Q.D.; QGIS Geographic Information System. Open Source Geospatial Foundation. 2016. Available online: http://qgis.org/ (accessed on 10 December 2022).
23. Adamska, M. Molecular detection of *Toxoplasma gondii* in natural surface water bodies in Poland. *J. Water Health* **2018**, *16*, 657–660. [CrossRef]
24. Aramini, J.J.; Stephen, C.; Dubey, J.P.; Engelstoft, C.; Schwantje, H.; Ribble, C.S. Potential contamination of drinking water with *Toxoplasma gondii* oocysts. *Epidemiol. Infect.* **1999**, *122*, 305–315. [CrossRef] [PubMed]
25. Mancianti, F.; Nardoni, S.; Mugnaini, L.; Poli, A. *Toxoplasma gondii* in waterfowl: The first detection of this parasite in *Anas crecca* and *Anas clypeata* from Italy. *J. Parasitol.* **2013**, *99*, 561–563. [CrossRef] [PubMed]
26. Nardoni, S.; Rocchigiani, G.; Varvaro, I.; Altomonte, I.; Ceccherelli, R.; Mancianti, F. Serological and Molecular Investigation on *Toxoplasma gondii* Infection in Wild Birds. *Pathogens* **2019**, *8*, 58. [CrossRef] [PubMed]
27. Puvanesuaran, V.R.; Noordin, R.; Balakrishnan, V. Isolation and genotyping of *Toxoplasma gondii* from free-range ducks in Malaysia. *Avian Dis.* **2013**, *57*, 128–132. [CrossRef]

28. Aubert, D.; Ajzenberg, D.; Richomme, C.; Gilot-Fromont, E.; Terrier, M.E.; de Gevigney, C.; Game, Y.; Maillard, D.; Gibert, P.; Dardé, M.L.; et al. Molecular and biological characteristics of *Toxoplasma gondii* isolates from wildlife in France. *Vet. Parasitol.* **2010**, *171*, 346–349. [CrossRef] [PubMed]
29. Waldenström, J.; van Toor, M.; Lewis, N.; Lopes, S.; Javakhishvili, Z.; Muzika, D.; Fouchier, R.A.M.; Brouwer, A. Active wild bird surveillance of avian influenza viruses, a report. *EFSA Support. Publ.* **2022**, *19*, 7791E. [CrossRef]
30. Fanelli, A.; Battisti, E.; Zanet, S.; Trisciuoglio, A.; Ferroglio, E. A systematic review and meta-analysis of *Toxoplasma gondii* in roe deer (*Capreolus capreolus*) and red deer (*Cervus elaphus*) in Europe. *Zoonoses Public Health* **2021**, *68*, 182–193. [CrossRef]
31. Damek, F.; Swart, A.; Waap, H.; Jokelainen, P.; Le Roux, D.; Deksne, G.; Deng, H.; Schares, G.; Lunden, A.; Alvarez-Garcia, G.; et al. Systematic Review and Modelling of Age-Dependent Prevalence of *Toxoplasma gondii* in Livestock, Wildlife and Felids in Europe. *Pathogens* **2023**, 12. [CrossRef]
32. Schüz, E.; Berthold, P.; Gwinner, E.; Oelke, H. *Grundriss der Vogelzugskunde*; Verlag: Berlin, Germany, 1971.
33. Dubey, J.P.; Murata, F.H.A.; Cerqueira-Cézar, C.K.; Kwok, O.C.H.; Grigg, M.E. Recent epidemiologic and clinical importance of *Toxoplasma gondii* infections in marine mammals: 2009–2020. *Vet. Parasitol.* **2020**, *288*, 109296. [CrossRef]
34. Spina, F.; Volponi, S. *Atlante Della Migrazione Degli Uccelli in Italia: Non-Passeriformi*; Ministero dell'Ambiente e della Tutela del Territorio e del Mare, Istituto Superiore per la Protezione e la Ricerca Ambientale (ISPRA): Roma, Italy, 2008.
35. Biancifiori, F.; Rondini, C.; Grelloni, V.; Frescura, T. Avian toxoplasmosis: Experimental infection of chicken and pigeon. *Comp. Immunol. Microbiol. Infect. Dis.* **1986**, *9*, 337–346. [CrossRef]
36. Dubey, J.P.; Murata, F.H.A.; Cerqueira-Cézar, C.K.; Kwok, O.C.H.; Su, C. Epidemiologic significance of *Toxoplasma gondii* infections in turkeys, ducks, ratites and other wild birds: 2009-2020. *Parasitology* **2021**, *148*, 1–30. [CrossRef]
37. Opsteegh, M.; Langelaar, M.; Sprong, H.; den Hartog, L.; De Craeye, S.; Bokken, G.; Ajzenberg, D.; Kijlstra, A.; van der Giessen, J. Direct detection and genotyping of *Toxoplasma gondii* in meat samples using magnetic capture and PCR. *Int. J. Food Microbiol.* **2010**, *139*, 193–201. [CrossRef] [PubMed]
38. Koethe, M.; Straubinger, R.K.; Pott, S.; Bangoura, B.; Geuthner, A.C.; Daugschies, A.; Ludewig, M. Quantitative detection of *Toxoplasma gondii* in tissues of experimentally infected turkeys and in retail turkey products by magnetic-capture PCR. *Food Microbiol.* **2015**, *52*, 11–17. [CrossRef] [PubMed]
39. Bachand, N.; Ravel, A.; Leighton, P.; Stephen, C.; Ndao, M.; Avard, E.; Jenkins, E. Serological and molecular detection of *Toxoplasma gondii* in terrestrial and marine wildlife harvested for food in Nunavik, Canada. *Parasites Vectors* **2019**, *12*, 155. [CrossRef] [PubMed]
40. Zou, Y.; Nie, L.B.; Zhang, N.Z.; Zou, F.C.; Zhu, X.Q.; Cong, W. First genetic characterization of *Toxoplasma gondii* infection in poultry meat intended for human consumption in eastern China. *Infect. Genet. Evol.* **2017**, *55*, 172–174. [CrossRef] [PubMed]
41. Work, T.M.; Verma, S.K.; Su, C.; Medeiros, J.; Kaiakapu, T.; Kwok, O.C.; Dubey, J.P. *Toxoplasma gondii* antibody prevalence and two new genotypes of the parasite in endangered hawaiian geese (Nene: *Branta sandvicensis*). *J. Wildl. Dis.* **2016**, *52*, 253–257, 255. [CrossRef] [PubMed]
42. Skorpikova, L.; Reslova, N.; Lorencova, A.; Plhal, R.; Drimaj, J.; Kamler, J.; Slany, M. Molecular detection of *Toxoplasma gondii* in feathered game intended for human consumption in the Czech Republic. *Int. J. Food Microbiol.* **2018**, *286*, 75–79. [CrossRef]
43. Karakavuk, M.; Aldemir, D.; Mercier, A.; Atalay Şahar, E.; Can, H.; Murat, J.B.; Döndüren, Ö.; Can, Ş.; Özdemir, H.G.; Değirmenci Döşkaya, A.; et al. Prevalence of toxoplasmosis and genetic characterization of *Toxoplasma gondii* strains isolated in wild birds of prey and their relation with previously isolated strains from Turkey. *PLoS ONE* **2018**, *13*, e0196159. [CrossRef]
44. Opsteegh, M.; Teunis, P.; Züchner, L.; Koets, A.; Langelaar, M.; van der Giessen, J. Low predictive value of seroprevalence of *Toxoplasma gondii* in cattle for detection of parasite DNA. *Int. J. Parasitol.* **2011**, *41*, 343–354. [CrossRef]
45. Sandström, C.A.; Buma, A.G.; Hoye, B.J.; Prop, J.; van der Jeugd, H.; Voslamber, B.; Madsen, J.; Loonen, M.J. Latitudinal variability in the seroprevalence of antibodies against *Toxoplasma gondii* in non-migrant and Arctic migratory geese. *Vet. Parasitol.* **2013**, *194*, 9–15, Epub 2012 Dec 20. [CrossRef] [PubMed]
46. Vitaliano, S.N.; Mineo, T.W.P.; André, M.R.; Machado, R.Z.; Mineo, J.R.; Werther, K.; Donato Castellane, P. Experimental infection of Crested Caracara (*Caracara plancus*) with *Toxoplasma gondii* simulating natural conditions. *Vet. Parasitol.* **2010**, *172*, 71–75. [CrossRef]
47. Ross, R.D.; Stec, L.A.; Werner, J.C.; Blumenkranz, M.S.; Glazer, L.; Williams, G.A. Presumed acquired ocular toxoplasmosis in deer hunters. *Retina* **2001**, *21*, 226–229. [CrossRef] [PubMed]
48. Conrady, C.D.; Besirli, C.G.; Baumal, C.R.; Kovach, J.L.; Etzel, J.D.; Tsui, J.C.; Elner, S.G.; Johnson, M.W. Ocular Toxoplasmosis after Exposure to Wild Game. *Ocul. Immunol. Inflamm.* **2022**, *30*, 527–532. [CrossRef] [PubMed]
49. Tenter, A.M.; Heckeroth, A.R.; Weiss, L.M. *Toxoplasma gondii*: From animals to humans. *Int. J. Parasitol.* **2000**, *30*, 1217–1258. [CrossRef] [PubMed]
50. Smith, J.L. Documented Outbreaks of Toxoplasmosis: Transmission of *Toxoplasma gondii* to Humans. *J. Food Prot.* **1993**, *56*, 630–639. [CrossRef]

Chapter 4.2

Sentinels in the Shadows: Exploring *Toxoplasma gondii* and other Sarcocystidae Parasites in Synanthropic Rodents and Their Public Health Implications

Dini FM, Caffara M, Cantori A, Luci V, Monno A, Galuppi R (2023). sentinels in the shadows: exploring *Toxoplasma gondii* and other Sarcocystidae parasites in synanthropic rodents and their public health implications. Submitted to *International Journal for Parasitology: Parasites and Wildlife*

Sentinels in the Shadows: Exploring *Toxoplasma gondii* and other Sarcocystidae Parasites in Synanthropic Rodents and Their Public Health Implications

Filippo Maria Dini[a#], Monica Caffara[a], Alessia Cantori[a], Valentina Luci[a], Antonio Monno[a], Roberta Galuppi[a]

[a]*Department of Veterinary Medical Sciences (DIMEVET), Alma Mater Studiorum University of Bologna, Via Tolara di Sopra 50, 40064 Ozzano Emilia (BO), Italy*

[#]Corresponding author Email address: filippomaria.dini@unibo.it

Abstract

Synanthropic rodents play a crucial role in maintaining the life cycle of *Toxoplasma gondii* in anthropized regions and can serve as indicators of environmental oocyst contamination. This investigation aimed to explore the occurrence of *T. gondii* infection within synanthropic rodent populations using a molecular diagnostic technique targeting the 18S rDNA gene, which is generic for Coccidia. We examined 97 brown rats (*Rattus norvegicus*), 67 black rats (*R. rattus*), 47 house mice (*Mus musculus*), and 1 common shrew (*Sorex araneus*). PCR tests were conducted on the brain, heart, and tongue tissues. PCR tested positive in at least one of the examined tissues in 26 *R. norvegicus* (26.8%), 13 *R. rattus* (19.4%), and 13 *M. musculus* (27.6%). Sequencing comparisons by BLAST allowed us to identify four different species of cyst-forming Apicomplexa. In particular, *T. gondii* was detected in 13 (6.1%) rodents, *Hammondia hammondi* (including *H. hammondi*-like organisms) in 36 (17%) subjects, *Besnoitia* sp. (in two cases identified as *B. besnoiti*) in 8 (3.7%), and *Sarcocystis gigantea* in two (0.94%). Rodents from peri-urban and urban environments can act as indicators of environmental contamination by oocysts of apicomplexan parasites with cats as definitive hosts, such as *T. gondii*, *H. hammondii*, and *S. gigantea*, the latter of which has never been previously recorded in rodents. Moreover, the presence of *B. besnoiti*, a parasite with an unidentified definitive host in Europe, sheds light on the potential role of these hosts as infection sentinels.

Keywords: Toxoplasmosis, zoonosis, Rodentia, Apicomplexa*, Hammondia hammondii*, *Besnoitia* sp, *Sarcocystis gigantea*

Introduction

Synanthropism, as defined by Klegarth (2016), encompasses the behaviour of wildlife (or flora) thriving within the shared ecosystems of humans. This behaviour, in turn, drives an increase in population density, reproduction rates, and survival advantages among these synanthropic species. Conversely, their territorial range diminishes due to their reliance on centralized anthropogenic resources (Gehrt et al., 2011; Hulme-Beaman et al., 2016).

From the time of the Neolithic Revolution, human activities have led to profound and enduring impacts on the natural environment. This process of settling down and adopting agricultural practices created a stable ecological niche that ensured sustenance over extended periods. Consequently, this environment began to attract initial rodent populations, as documented by Frynta et al. (2005) and Cucchi et al. (2020), which in turn drew the interest of subsequent cat populations (Krajcarz et al., 2022). Through the passing decades and centuries, these modest human settlements gradually expanded into villages and towns, forming the earliest instances of urban settings inhabited by synanthropic species (Baumann, 2023).

In this context, the establishment of a predator-prey relationship has given rise to the development of a peri-domestic life cycle of predation-associated parasites (Mendoza Roldan and Otranto, 2023). The process of predation stands out as one of the most effective mechanisms for facilitating the transmission of parasites, offering a direct pathway for the parasite to fulfil its life cycle within the trophic chain (Johnson et al., 2010; Médoc and Beisel, 2011). Parasites transmitted through trophic interactions have, in various instances, undergone adaptations to optimize predation through manipulation of their host preys (Seppälä et al., 2008). A pertinent illustration of this phenomenon can be observed in *Toxoplasma gondii* (Eucoccidiorida: Sarcocystidae), which relies on the predatory behaviour of felines (definitive hosts of the parasite), that include the consumption of small rodents and other prey species, to successfully conclude its life cycle (Vyas et al., 2007; Dubey et al., 2021). The reproductive fitness of *Toxoplasma* is intricately linked to the predation patterns exhibited by

54 felids. Disruption of the innate aversion mechanism, caused by the interaction of the parasite with the
55 SNC of the host, heighten predation rates, thereby increasing the reproductive fitness of the parasite.
56 This stance aligns with the 'behavioural manipulation' hypothesis, postulating that *T. gondii* can
57 induce alterations in host behaviour that directly contribute to the enhancement of their own
58 reproductive success, as proposed by Vyas et al. (2007) and Webster (2007).

59 Rodents play for this reason a crucial part in upholding the lifecycle of *T. gondii* and influencing
60 the spread of toxoplasmosis. This significance is particularly pronounced in species residing near
61 human settlements. The establishment of an infection transmission cycle via rodents (and other small
62 preys) results in the release of millions of unsporulated oocysts by cats, that can therefore be found
63 in various environmental matrices where, upon sporulation, become infectious and can remain viable
64 up to several years (Dubey, 2021). Consequently, this process heightens the risk of infection for all
65 hosts of the parasite in the ecosystem, most notably humans within these habitats (Mercier et al.,
66 2013).

67 Apart from their crucial role in maintaining the life cycle of *T. gondii* in anthropized regions,
68 synanthropic rodents, due to their feeding behaviour that predominantly facilitate oral transmission
69 of sporulated oocysts within the environment, can be regarded as indicative of environmental oocyst
70 contamination. Consequently, the finding of *T. gondii* in wild rodent populations might reflect the
71 dissemination of the parasitic environmental phase within a specific geographical area (Dubey et al.,
72 2021).

73 The primary objective of the present investigation is to explore the occurrence of *T. gondii*
74 infection within synanthropic rodent populations collected in a area of Italy that has previously
75 undergone retrospective scrutiny to determine seroprevalence rates in both animals and humans,
76 revealing a persistent presence of the parasite within the area (Dini et al., 2023). The central aim of
77 this study is to ascertain the potential environmental significance of the parasite within urbanized

settings. This is achieved by evaluating the occurrence of infections in rodents residing in the peri-domestic environment, utilizing molecular diagnostic techniques.

Materials and Methods

From June 2019 to March 2023, 212 carcasses of adults or subadults peridomestic rodent were collected and stored at -20 °C by professional rodent control services during pest control programs from urban and rural areas in the provinces of Ferrara, Forlì-Cesena, Ravenna, Bologna (Emilia Romagna Region) and Arezzo (Toscana Region) (Fig 1). In detail, 97 brown rats (*Rattus norvegicus*), 67 black rats (*Rattus rattus*), 47 house mouse (*Mus musculus*) and 1 common shrew *(Sorex araneus*) were sampled. The carcasses were identified morphologically according to CDC (2006); sex and weight of each rodents were recorded. Sampling was performed with sterile surgical instruments and, according to the state of the carcasses, 25-50 mg of tissue were collected from heart, 25-200 mg from brain and 25 mg from tongue muscle. Samples were placed in sterile 1.5 ml tubes and stored at -20 °C until DNA extraction. In total, 209 brains (due to poor condition of 3 *R. rattus*), 212 tongue, and 180 heart samples (poor condition of 19 *R norvegicus*, 10 *R. rattus*, and 3 *M. musculus*) were collected.

Genomic DNA was purified using Pure Link ® Genomic DNA Mini kit (Invitrogen by Thermo Fisher), according to the manufacturer's protocol. Initial end-point PCR targeting 18S rDNA gene of Coccidiawas performed on all the samples with the primers COC-1 and COC-2 as described by Ho et al. (1996) following some modification by Hornok et al. (2015). Briefly, a reaction volume of 25 µl, containing 12.5 µl 2x Dream Taq Hot Start Green PCR Master Mix (Thermo Scientific), 9.5 µl ddH2O, 0.25 µl (1 µM final concentration) of each primer, and 2.5 µl template DNA were used. For amplification, an initial denaturation step at 94 °C for 10 min was followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 54 °C for 30 s and extension at 72 °C for 30 s. Final

102 extension was performed at 72 °C for 10 min. In all the PCRs, sterile water was included as negative
103 control. Amplifications were performed in a T-personal thermal cycler (Biometra, Goettingen,
104 Germany). The PCR products were electrophoresed on 1.5% agarose gel stained with SYBR Safe
105 DNA Gel Stain (Thermo Fisher Scientific, Carlsbad, CA, USA) in 0.5× TBE. For sequencing, the
106 amplicons were excised and purified by Nucleo-Spin Gel and PCR Clean-up (Mackerey-Nagel,
107 Düren, Germa-ny), and sequenced with an ABI 3730 DNA analyzer (StarSEQ, Mainz, Germany).

108 The trace files were assembled with Contig Express (VectorNTI Advance 11 software, Invitrogen,
109 Carlsbad, CA, USA), and the consensus sequences were compared with published data by BLAST
110 tools (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequence alignments were carried out by BioEdit
111 7.2.5 (Hall, 1999), while p-distance and maximum-likelihood (ML) tree (GTR+G+I substitution
112 model for both genes and bootstrap of 1,000 replicate) were calculated by MEGA 7 (Kumar et al.,
113 2016). The sequences obtained in this study were deposited in GenBank under accession numbers
114 XXX.

115 Student t-test was used to compare the average weight of male and female; Pearson's $\chi 2$ test was
116 used to associate sex with prevalence data. Differences were considered significant when $P \leq 0.05$.
117 The Sample Size Calculator (https://www.surveysystem.com/sscalc.htm) was used to calculate 95%
118 confidence intervals (CIs) for the observed prevalence values.

119 **Results**

120 Among the 212 rodents collected, 133 were males while 78 were females as detailed in table 1
121 together with the species sex and weight. In each species no significant weight differences between
122 sexes were detected by Student's t-test. The single shrew collected, was a male weighing 4 g.

123 Overall, 53 rodents (25%) were PCR positive in at least one of the examined tissues; splitting
124 the results: *R. norvegicus* 26 out of 97 (26.8%, CI 95%=7.99-35.61), *R. rattus* 13 out of 67 (19.4%,
125 CI 95%=9.93-28.87) while *M. musculus* 13 out of 47 (27.6%, CI 95%=14.87-40.45). In table 1 the

PCR results are also reported in relation to sex. Notably, in *R. norvegicus*, females showed a significantly higher positivity rate (42.4%) than males (18.7%) (Yates-corrected chi-square: 5.7; p = 0.0243), while no significant sex differences were observed for *R. rattus* and *M. musculus*.

Sequences comparison by BLAST allows to identify four different species of cyst-forming Apicomplexa (Sarcocystidae): *Toxoplasma gondii, Hammondia hammondi* (including *H. hammondi*-like organism), *Besnoitia* sp. and *Sarcocystis gigantea*.

In particular *T. gondii* was detected in 13 (6.1%) rodents, (similarities 99.7%-100%), *H. hammondi* (and *H. hammondi*-like organism) in 36 (17%) subject (similarities 99.3%-100%), *Besnoitia* sp. was found in 8 rodents (3.7%), with low sequence similarity (94%-95%). Notably, only two samples showed 100% similarity with *B. besnoiti* from cattle. Finally, *S. gigantea* was detected in two heart samples of *R. norvegicus*, constituting 0.94% of the total rodent population and 2% of the *R. norvegicus* samples. These samples displayed 100% sequence similarity with sequences of *S. gigantea* available in GenBank.

In the context of host species differentiation, within *R. norvegicus*, the most frequently encountered cyst-forming coccidia was *H. hammondi* (and *H. hammondi*-like) with a prevalence of 17.5%, followed by *Besnoitia* sp. at 7.2%. Among the latter only one sequence showed 100% similarity with *B. besnoiti*. Trailing behind in terms of prevalence were *T. gondii* at 4% and *Sarcocystis gigantea* at 2%. In *R. rattus*, only two Apicomplexan species were molecularly identified in the analysed tissues: *H. hammondi*/*H. hammondi-like* (11.9%), and *T. gondii* (9%). Concerning *M. musculus*, the most prevalent parasite was *H. hammondi/H. hammondi*-like (23.4%), followed by *T. gondii* (4.2%). Intriguingly, in one instance, *B. besnoiti* was detected in a heart sample with 100% sequence similarity.

148 Regarding the distribution of positive matrices in relation to parasites, *H. hammondi/H.*
149 *hammondi*-like was more frequently observed in CNS and heart samples; *T. gondii* was equally
150 distributed among all matrices, while *B. besnoiti* occurred only in heart samples.

151 Co-infections were only observed in *Rattus* spp. and *M. musculus*. Among *Rattus* species, one
152 *R. norvegicus* and one *R. rattus* exhibited co-infection by *T. gondii/H. hammondi* + *H. hammondi*-
153 like. Additionally, two *R. norvegicus* showed co-infection by *H. hammondi*-like and *Besnoitia* sp.,
154 while one *R. norvegicus* exhibited co-infection by *H. hammondi*-like and *Sarcocystis gigantea*. In the
155 case of *Mus musculus*, co-infection by *T. gondii* and *H. hammondi*-like was detected. (Tables 2-4).
156 The sole analyzed *S. araneus* was found positive for *T. gondii* (100% sequence similarity) in heart
157 tissue.

158 The alignment of all *T. gondii* obtained in the present study (plus the *T. gondii* type I, RH
159 reference strain) with *H. hammondi/H. hammondi*-like showed the presence of two transitions C/T
160 and A/G as the only differences between the two species over 297 bp of the 18S rDNA. Moreover,
161 the A/G transition further separate the "true" *H. hammondi* from the *H. hammondi*-like group. The
162 p-distance observed between *T. gondii* and the group of *H. hammondi* is very low ranging from 0%
163 to 0.1%, due to the low genetic resolution of the 18S rDNA.

164 The ML tree obtained (tree not reported), despite with a low bootstrap support, showed 3
165 separated clusters, one composed by our *T. gondii* together with the reference strain, the second by
166 the "true" *H. hammondi* (having 100% identity with AH008381) and the latter by *H. hammondi*-like
167 group.

168

169 **Discussion**

170 *Rattus* spp. and *M. musculus* exemplify species capable of coexisting within anthropogenically
171 influenced environments. This coexistence raises concerns for potential human health risks owing to

the close proximity between these species and human habitats. Rodents constitute a notably significant group of mammals, particularly in terms of serving as reservoirs for various pathogens, some of which are of zoonotic concern (Han et al., 2015). Their biological attributes, characterized by elevated reproductive rates, opportunistic behaviours, adaptability, and worldwide distribution, position them strategically, thereby enhancing the likelihood of disease transmission among wildlife, domestic animals, and human populations (Han et al., 2015; Luis et al., 2013)

Within the scope of this research, we examined the occurrence of Sarcocystidae infections in synanthropic rodents using a broad-spectrum PCR assay targeting the 18S rRNA of Coccidia. This molecular method, involving a single PCR assay followed by Sanger sequencing, enabled us to reveal the presence of various protozoan species, namely *T. gondii*, *H. hammondi/H. hammondi*-like, *Besnoitia* sp., and *S. gigantea*, within the studied host species (*R. norvegicus*, *R. rattus*, *M. musculus*, and *S. araneus*).

In the current study, *T. gondii* DNA was detected in all the rodent host species, with an overall prevalence rate of 6.1%. Notably, among these species, *R. rattus* exhibited the highest infection rate, with 9% prevalence. The worldwide seroprevalence is approximately 6%, with the highest rates recorded in Africa (24%) and South America (18%), and the lowest in Europe (1%) (Galeh et al., 2020). It's important to note that serological tests cannot definitively predict the presence/absence of the parasite. This limitation has been previously observed (Dubey, 2022), as viable *T. gondii* has been isolated from rodents that tested seronegative (Araújo et al., 2010). Hence, molecular studies appear to offer more epidemiological reliability.

Rodents play a pivotal role in the perpetuation of the *T. gondii* life cycle and the epidemiology of toxoplasmosis. They are recognized as reservoirs and carriers of the disease, serving as the primary source of infection for cats and their related species (Dabritz et al., 2008). This role becomes particularly significant in species inhabiting close proximity to human habitats due to the profound implications for both the environment and human health. The establishment of the infection

197 transmission cycle through rodents results in the release of oocysts from infected felids, leading their
198 dissemination into environment. Consequently, this amplifies the infection risk for various hosts
199 including humans (Mercier et al., 2013). The importance of rodents in maintaining the lifecycle of *T.*
200 *gondii* has been further strengthened and highlighted following studies involving the neuroanatomical
201 interaction of chronical established CNS cysts in the behavioural pattern of these hosts. Numerous
202 studies indicate that *T. gondii* alters rodent behaviour, making them more susceptible to predation by
203 cats (Webster, 2007). These alterations include increased activity, reduced neophobia (fear of
204 novelty), and decreased predator vigilance (Webster, 1994, 2001, 2007; Webster et al., 1994; Berdoy
205 et al., 1995; Lamberton et al., 2008). These changes likely facilitate parasite transmission from the
206 intermediate host to the feline host. (Berdoy et al., 2000; Vyas et al., 2007, Kaushik et al., 2014).

207 *Hammondia hammondi* (including *H. hammondi*-like), found in the 17% of the analysed rodents,
208 is a non-zoonotic coccidian parasite that bears a close resemblance to *T. gondii* (Frenkel and Dubey,
209 1975). The life cycles of these two parasites also share similarities like in the definitive hosts (Felidae)
210 but differently from *T. gondii*, *H. hammondi* follows an obligatory two-host lifecycle, consequently,
211 only sporulated oocysts are infective to rodents, and solely bradyzoite cysts are infective to cats
212 (Frenkel and Dubey, 2000). Intermediate hosts include mice (*Mus musculus*), rats (*Rattus*
213 *norvegicus*), and other rodents but also, rabbits, hamsters, goats, dogs, and pigs (Shimura et al., 1987).
214 The prevalence of *H. hammondi* remains undisclosed in both definitive and intermediate hosts. Only
215 few research reported the sporadically presence of *H. hammondi* in cat and dog faeces (Schares et al.,
216 2005; Schares et al., 2008, Dubey et al., 2013) and in intermediate host tissues (Cabral et al., 2021).

217 In our study the consistent presence of *H. hammondi/H. hammondi*-like in the rodent hosts are
218 not comparable with other data, since no previous report are, to the best of our knowledge, present in
219 available literature.

220 The distinction between *T. gondii* and *H. hammondi* is of paramount importance, particularly
221 because the latter lacks zoonotic relevance, despite its close genetic relationship. In our investigations,

the 18S rDNA despite being highly conserved, emerged as a sensitive genetic marker within the Apicomplexa subphylum, able to distinguish between the two parasites. Notably, there are only a few nucleotide variations between the complete 18S rDNA sequences of *T. gondii* and *H. hammondi* (Jenkins et al., 1998). Within our sequences, a single nucleotide variation, represented by C/T and A/G transition, effectively separated *T. gondii* from *H. hammondi* samples, leading to the formation of two distinct clusters among our positive sample set. Moreover, the latter transition (A/G) further delineated these samples into two sub-clusters, "*H. hammondi*" and "*H. hammondi*-like organism." In light of the limited knowledge regarding the global population structure of *H. hammondi*, it remains conceivable that yet undiscovered lineages of *H. hammondi* may exist, displaying potential genetic diversity (Shares et al., 2021).

Within our study, 3.7% of the rodents exhibited DNA sequences associated with *Besnoitia* sp. However, it's noteworthy that only two sequences matched with 100% identity to *B. besnoiti*. The other specimens exhibited a sequence similarity of 94-95% with sequences of *Besnoitia* spp. available in GenBank, and lower similarity with other Sarcocystidae parasites. This outcome renders the accurate assignment of a species identification for these positive samples challenging, thereby impeding our ability to make informed epidemiological inferences.

This parasite is responsible for bovine Besnoitiosis, a chronic and debilitating disease that has been causing significant economic losses in cattle and has been considered endemic in Italy since 2011 (Gentile et al., 2012). In Europe, no definitive hosts for this parasite have been identified (Basso et al., 2011). The presence of parasite cysts in the skin suggests that the primary way of transmission is likely mechanical, with hematophagous flies serving as the carriers of the parasite (Bigalke, 1968). The intriguing aspect is the molecular identification of this parasite in the internal organs of rodent hosts, particularly in the heart, which raises questions about the epidemiology and potential host range of this Sarcocystidae. Notably the two subjects in which B. besnoiti was detected, were collected from two areas where a consistent population of domestic ruminant is present.This discovery hints

the possibility that rodent hosts could serve as competent hosts in the life cycle of *Besnoitia*. It's worth noting that other *Besnoitia* species, such as *B. jellisoni* and *B. wallacei*, have been previously described in rodents but have not been documented in Europe until now (Alvarez-García et al., 2013).

Finally, our research has unveiled the occurrence of *S. gigantea* infection in the heart muscles of two *R. norvegicus*. The genus *Sarcocystis* comprises apicomplexan protozoa forming cysts, with a life cycle obligatorily entailing two hosts (Dubey et al., 2015). These cysts are typically located in the striated muscles of herbivorous or omnivorous intermediate hosts, while carnivores serve as the definitive hosts. Remarkably, more than 40 *Sarcocystis* species have been identified to use rodents as their intermediate hosts, including *S. microti* (Votýpka et al., 1998; Mugridge et al., 1999), *S. muris* (Mugridge et al., 1999; Gajadhar et al., 1991), *S. myodes* (Rudaitytė-Lukošienė et al., 2022), and *S. ratti* (Zeng et al., 2020; Prakas et al., 2029). An apparent gap in research exists concerning the global prevalence of *Sarcocystis* spp. in small mammals. Researchers have proposed that infection rates of various *Sarcocystis* species are contingent upon factors such as the specific parasite species, intermediate host species, geographical region, as well as the presence and abundance of definitive hosts within the studied area (Rudaitytė-Lukošienė et al., 2022; Hu et al., 2022). *Sarcocystis gigantea* infection is considered to be mildly pathogenic yet relatively common in sheep, with the cat as the definitive host (Dubey et al., 2015). While *S. gigantea* has not been documented in rodent hosts to date, recent findings have indicated its capacity to cause infections in horses, which serve as non-specific intermediate hosts (Veronesi et al., 2020). This observation suggests that in peri-urban settings where definitive hosts, such as cats, are abundant and the parasite is disseminated within the environment, non-specific intermediate hosts, such as rodents, may potentially develop bradyzoite cysts within their muscle tissue. Our findings support the hypothesis that certain *Sarcocystis* spp. may possess a broader range of intermediate hosts than was previously recognized (Veronesi et al., 2020).

Conclusions

In conclusion, the findings of this study highlight that synanthropic rodents sampled in urban and peri-urban environments serve as valuable indicators of environmental contamination by oocysts of apicomplexan parasites with cat as definitive host. This applies not only to parasites like *T. gondii* and *H. hammondi*, which are closely related apicomplexans with distinct epidemiological implications but both having cat-rodent cycle as a robust framework. This pattern is also applicable for *S. gigantea*, recovered in the hearts of two *R. norvegicus*, and recognizing cats as the definitive host. This species has never been recorded previously in rodents. Lastly, the presence of *B. besnoiti* in *R. norvegicus* and *M. musculus*, parasite with an unidentified definitive host in Europe, sheds light on the potential role of these hosts as infection sentinels.

Acknowledgements

Authors would like to thank social cooperative For.B and dr. Guglielmo Pampiglione for kindly providing rodent carcasses from the provinces of Ferrara, Forlì-Cesena and Ravenna of Emilia-Romagna Region.

Authors' contributions

Filippo Maria Dini wrote the main manuscript text. Filippo Maria Dini, Valentina Luci, Alessia Cantori and Antonio Monno performed sampling. Filippo Maria Dini, Valentina Luci, Alessia Cantori and Monica Caffara carried out the molecular analyses. Roberta Galuppi analized the data and performed the statistical analysis. Monica Caffara and Roberta Galuppi revised the manuscript. All authors reviewed the manuscript and approved the final manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

AraùJo, J. B., A. V. Da Silva, R. C. Rosa, R. J. Mattei, R. C. Da Silva, V. B. Richini-Pereira, And H. Langoni. 2010. Isolation And Multilocus Genotyping Of *Toxoplasma Gondii* In Seronegative Rodents In Brazil. Vet. Parasitol. 174: 328–331

Basso, W., Schares, G., Gollnick, N.S., Rütten, M., Deplazes, P. 2011. Exploring the life cycle of *Besnoitia besnoiti* - experimental infection of putative definitive and intermediate host species. Vet Parasitol. 178:223–34.

Baumann C., 2023. The paleo synanthropic niche: a first attempt to define animal's adaptation to a human made micro environment in the Late Pleistocene. Archaeol. Anthropol. Sci.15:63 https://doi.org/10.1007/s12520-023-01764-x

Berdoy, M., Webster, J.P., Macdonald, D.W. 2000. Fatal attraction in *Toxoplasma*-infected rats: a case of parasite manipulation of its mammalian host. Proc R Soc B. 267:1591–1594

Berdoy, M., Webster J.P., Macdonald D.W. 1995: Parasite altered behaviour: is the effect of Toxoplasma gondii on Rattus norvegicus specific? Parasitology 111: 403–409

Bigalke, RD. 1968. New concepts on the epidemiological features of bovine besnoitiosis as determined by laboratory and feld investigations. Onder- stepoort J Vet Res. 35:3–137.

Cabral, A. D., Su, C., Soares, R. M., Gennari, S. M., Sperança, M. A., da Rosa, A. R., & Pena, H. F. J. 2021. Occurrence and diversity of Sarcocystidae protozoa in muscle and brain tissues of bats from São Paulo state, Brazil. Int J Parasitol Parasites Wildl. 14, 91–96. https://doi.org/10.1016/j.ijppaw.2021.01.003

Centers for Disease Control and Prevention and U.S. Department of Housing and Urban Development (2006). Healthy housing reference manual. Atlanta: US Department of Health and Human

316 Services. https://www.cdc.gov/nceh/publications/books/housing/
317 housing_ref_manual_2012.pdf.

318 Cucchi, T., Vigne. J.D., Aufray, J.C. 2005. First occurrence of the house mouse (*Mus musculus*
319 *domesticus* Schwarz & Schwarz, 1943) in the Western Mediterranean: a zooarchaeological
320 revision of subfossil occurrences. Biol J Lin Soc. 84(3):429–445

321 Dabritz, H.A., Miller, M.A., Gardner, I.A., Packham, A.E., Atwill, E.R., Conrad, P.A. 2008. Risk
322 factors for *Toxoplasma gondii* infection in wild rodents from central coastal California and a
323 review of T. gondii prevalence in rodents. J Parasitol. 94:675–84. doi: 10.1645/GE-1342.1

324 Dubey, J.P., Calero-Bernal R., Rosenthal, B.M., Speer, C.A., Fayer, R. 2015. Sarcocystosis of
325 Animals and Humans. 2nd ed. CRC Press.

326 Dubey, J. P., Murata, F. H. A., Cerqueira-Cézar, C. K., Kwok, O. C. H., & Su, C. 2021.
327 Epidemiological Significance of *Toxoplasma gondii* Infections in Wild Rodents: 2009-2020. J.
328 Parasitol. 107(2), 182–204. https://doi-org.ezproxy.unibo.it/10.1645/20-121.

329 Dubey, J. P., Tilahun, G., Boyle, J. P., Schares, G., Verma, S. K., Ferreira, L. R., Oliveira, S., Tiao,
330 N., Darrington, C., & Gebreyes, W. A. 2013. Molecular and biological characterization of first
331 isolates of *Hammondia hammondi* from cats from Ethiopia. J. Parasitol. 99(4), 614–618.
332 https://doi.org/10.1645/12-51.1

333 Dubey, J.P. 2022. Toxoplasmosis of Animals and Humans, 3rd ed.; CRC Press: Boca Raton, FL,
334 USA.

335 Frenkel, J. K., & Dubey, J. P. 1975. *Hammondia hammondi* gen. nov., sp.nov., from domestic cats, a
336 new coccidian related to *Toxoplasma* and *Sarcocystis*. Zeitschrift fur Parasitenkunde (Berlin,
337 Germany), 46(1), 3–12. https://doi.org/10.1007/BF00383662

338 Frenkel, J. K., & Dubey, J. P. 2000. The taxonomic importance of obligate heteroxeny: distinction of
339 *Hammondia hammondi* from *Toxoplasma gondii*--another opinion. Parasitol. Res. 86(10), 783–
340 786. https://doi.org/10.1007/s004360000261

341 Frynta, D., Slabova, M., Vachova, H., Volfova, R., Munclinger, P. 2005. Aggression and
342 commensalism in house mouse: a comparative study across Europe and the Near East. Behav
343 Aggress. 31(3):283–293

344 Gajadhar, A.A.; Marquardt, W.C.; Hall, R.; Gunderson, J.; Ariztia-Carmona, E.V.; Sogin, M.L. 1991.
345 Ribosomal RNA sequences of *Sarcocystis muris*, *Theileria annulata* and *Crypthecodinium*
346 *cohnii* reveal evolutionary relationships among apicomplexans, dinoflagellates, and ciliates.
347 Mol. Biochem. Parasitol. 45, 147–154.

348 Galeh, T. M., Sarvi, S., Montazeri, M., Moosazadeh, M., Nakhaei, M., Shariatzadeh, S. A., &
349 Daryani, A. 2020. Global Status of *Toxoplasma gondii* Seroprevalence in Rodents: A
350 Systematic Review and Meta-Analysis. Front. Vet. Sci. 7, 461.
351 https://doi.org/10.3389/fvets.2020.00461

352 Gehrt, SD, Brown JL, Anchor, C. 2011. Is the urban coyote a misanthropic synanthrope? The case
353 from Chicago. Cities and the Environment (CATE) 4(1): article 3

354 Gentile, A., Militerno, G., Schares, G., Nanni, A., Testoni, S., Bassi, P., & Gollnick, N. S. 2012.
355 Evidence for bovine besnoitiosis being endemic in Italy--first in vitro isolation of Besnoitia
356 besnoiti from cattle born in Italy. Vet. Parasitol. 184(2-4), 108–115.
357 https://doi.org/10.1016/j.vetpar.2011.09.014

358 Hall, T.A. 1999. BioEdit: A user-friendly biological sequence alignment editor and analysis program
359 for Windows 95/98/NT. Nucleic Acids Symp. Ser. 41, 95-98

360 Han, B. A., Schmidt, J. P., Bowden, S. E., & Drake, J. M. 2015. Rodent reservoirs of future zoonotic
361 diseases. Proceedings of the National Academy of Sciences of the United States of America,
362 112(22), 7039–7044. https://doi.org/10.1073/pnas.1501598112

363 Ho, M. S., Barr, B. C., Marsh, A. E., Anderson, M. L., Rowe, J. D., Tarantal, A. F., Hendrickx, A.
364 G., Sverlow, K., Dubey, J. P., & Conrad, P. A. 1996. Identification of bovine *Neospora* parasites
365 by PCR amplification and specific small-subunit rRNA sequence probe hybridization. J. Clin.
366 Microbiol. 34(5), 1203–1208. https://doi.org/10.1128/jcm.34.5.1203-1208.1996

367 Hornok, S.; Mester, A.; Takács, N.; Baska, F.; Majoros, G.; Fok, É.; Biksi, I.; Német, Z.; Hornyák,
368 Á.; Jánosi, S.; Farkas, R. 2015. *Sarcocystis*-infection of cattle in Hungary. Parasit. Vectors. 4–
369 9, doi:10.1186/s13071-015-0685-9.

370 Hu, J., Sun, J., Guo, Y., Zeng, H., Zhang, Y., Tao, J. 2022. Infection of the Asian gray shrew
371 *Crocidura attenuata* (Insectivora: Soricidae) with *Sarcocystis attenuati* n. sp. (Apicomplexa:
372 Sarcocystidae) in China. Parasite. Vector. 15:13. doi: 10.1186/s13071-021-05136-z.

373 Hulme-Beaman, A., Dobney, K., Cucchi, T., Searle, J.B. 2016. An ecological and evolutionary
374 framework for commensalism in anthropogenic environments. Trends Ecol Evol 31(8):633–
375 645. https://doi.org/10.1016/j.tree.2016.05.001

376 Johnson, P. T., Dobson, A., Lafferty, K. D., Marcogliese, D. J., Memmott, J., Orlofske, S. A., Poulin,
377 R., & Thieltges, D. W. 2010. When parasites become prey: ecological and epidemiological
378 significance of eating parasites. Trends Ecol Evol. 25(6), 362–371. https://doi-
379 org.ezproxy.unibo.it/10.1016/j.tree.2010.01.005.

380 Kaushik, M., Knowles, S. C., & Webster, J. P. 2014. What makes a feline fatal in Toxoplasma gondii's
381 fatal feline attraction? Infected rats choose wild cats. Integr. Comp. Biol. 54(2), 118–128.
382 https://doi.org/10.1093/icb/icu060

383 Klegarth, A.R. 2016. Synanthropy. The International Encyclopedia of Primatology: 1–5.
384 https://doi.org/10.1002/9781119179313. wbprim0448

385 Krajcarz, M., Krajcarz, M.T., Baca, M., Golubiński, M., Bielichová, Z., Bulatović, J., Csippán, P.,
386 Dimitrijević, V., Kyselý, R., Makowiecki, D. 2022. The history of the domestic cat in Central
387 Europe. Antiq: 1–6. https://doi.org/10.15184/aqy.2022.128

388 Kumar, S., Stecher, G., Li, M., Knyaz, C., Tamura, K. 2018. MEGA X: molecular evolutionary
389 genetics analysis across computing platforms. Mol Biol Evol 35:1547–1549.
390 https://doi.org/10.1093/ molbev/msy096

391 Kumar, S.; Stecher, G.; Tamura, K. 2016. MEGA7: Molecular Evolutionary Genetics Analysis
392 version 7 for bigger datasets. Mol. Biol. Evol. 33, 1870-1874.
393 https://doi.org/10.1093/molbev/msw054

394 Lamberton, P.H.L., Donnelly, C.A., Webster, J.P. 2008: Specificity of the *Toxoplasma gondii*-altered
395 behaviour to definitive versus non-definitive host predation risk. Parasitology 135: 1143–1150.

396 Luis, A.D., Hayman, D.T.S., O'Shea, T.J., Cryan, P.M., Gilbert, A.T., Mills, J.N., Timonin, M.E.,
397 Willis, C.K.R., Cunningham, A.A., Rupprecht, C.E., Wood, J.L.N., Webb, C.T., 2013. A
398 comparison of bats and rodents as reservoirs of zoonotic viruses: are bats special? Proceed.
399 Royal Soc. Biol. Sci. 280, 1–9.

400 Médoc, V., Beisel, J.N. 2011. When trophically-transmitted parasites combine predation
401 enhancement with predation suppression to optimize their transmission. Oikos.120:1452–8.

402 Mendoza Roldan, J. A., & Otranto, D. 2023. Zoonotic parasites associated with predation by dogs
403 and cats. Parasit. Vectors. 16(1), 55. https://doi-org.ezproxy.unibo.it/10.1186/s13071-023-
404 05670-y

405 Mercier, A., Garba, M., Bonnabau, H., Kane, M., Rossi, J. P., Dardé, M. L., & Dobigny, G. 2013.
406 Toxoplasmosis seroprevalence in urban rodents: a survey in Niamey, Niger. Memorias do
407 Instituto Oswaldo Cruz, 108(4), 399–407. https://doi.org/10.1590/S0074-0276108042013002

408 Mugridge, N.B.; Morrison, D.A.; Johnson, A.M.; Luton, K.; Dubey, J.P.; Votýpka, J.; Tenter, A.M.
409 1999. Phylogenetic relationships of the genus *Frenkelia*: A review of its history and new
410 knowledge gained from comparison of large subunit ribosomal ribonucleic acid gene
411 sequences. Int. J. Parasitol. 29, 957–972

412 Prakas, P., Kirillova, V., Gavarāne, I., Grāvele, E., Butkauskas, D., Rudaitytė-Lukošienė, E.,
413 Kirjušina, M. 2019. Morphological and molecular description of *Sarcocystis ratti* n. sp. from
414 the black rat (*Rattus rattus*) in Latvia. Parasitol. Res. 118:2689–2694. doi: 10.1007/s00436-
415 019-06393-9

416 Prakas, P.; Kirillova, V.; Gavarāne, I.; Grāvele, E.; Butkauskas, D.; Rudaitytė-Lukošienė, E.;
417 Kirjušina, M. 2019. Morphological and molecular description of *Sarcocystis ratti* n. sp. from
418 the black rat (*Rattus rattus*) in Latvia. Parasitol. Res. 118, 2689–2694

419 Rudaitytė-Lukošienė, E.; Jasiulionis, M.; Balčiauskas, L.; Prakas, P.; Stirkė, V.; Butkauskas, D. 2022.
420 Morphological and Molecular Description of *Sarcocystis myodes* n. sp. from the Bank Vole
421 (*Clethrionomys glareolus*) in Lithuania. Biology. 1, 512

422 Schares, G., Pantchev, N., Barutzki, D., Heydorn, A. O., Bauer, C., & Conraths, F. J. 2005. Oocysts
423 of *Neospora caninum*, *Hammondia heydorni*, *Toxoplasma gondii* and *Hammondia hammondi*
424 in faeces collected from dogs in Germany. Int. J. Parasitol. 35(14), 1525–1537.
425 https://doi.org/10.1016/j.ijpara.2005.08.008

426 Schares, G., Vrhovec, M. G., Pantchev, N., Herrmann, D. C., & Conraths, F. J. 2008. Occurrence of
427 *Toxoplasma gondii* and *Hammondia hammondi* oocysts in the faeces of cats from Germany and

428 other European countries. Vet. Parasitol. 152(1-2), 34–45.
429 https://doi.org/10.1016/j.vetpar.2007.12.004

430 Seppälä, O., Valtonen, E.T., Benesh, D.P. 2008. Host manipulation by parasites in the world of dead-
431 end predators: adaptation to enhance transmission? Proc Royal Soc B. 275:1611–5.

432 Shimura, K., & Ito, S. 1987. Goats as natural intermediate hosts of *Hammondia hammondi*.
433 Zentralblatt fur Bakteriologie, Mikrobiologie, und Hygiene. Series A, Medical microbiology,
434 infectious diseases, virology, parasitology, 264(3-4), 348–352. https://doi.org/10.1016/s0176-
435 6724(87)80055-x

436 Veronesi, F., Di Palma, S., Gabrielli, S., Morganti, G., Milardi, G. L., Middleton, B., & Lepri, E.
437 2020. *Sarcocystis gigantea* infection associated with granulomatous eosinophilic myositis in a
438 horse. J. Vet: Diagn. 32(4), 611–615. https://doi.org/10.1177/1040638720935847

439 Votýpka, J.; Hypsa, V.; Jirků, M.; Flegr, J.; Vávra, J.; Lukes, J. 1998. Molecular phylogenetic
440 relatedness of *Frenkelia* spp. (Protozoa, Apicomplexa) to *Sarcocystis falcatula* Stiles 1893: Is
441 the genus *Sarcocystis* paraphyletic? J. Eukaryot. Microbiol. 45, 137–141.

442 Vyas, A., Kim, S.K., Giacomini, N., Boothroyd, J.C., Sapolsky, R.M. 2007. Behavioral changes
443 induced by *Toxoplasma* infection of rodents are highly specific to aversion of cat odors. Proc
444 Natl Acad Sci U S A. 2007;104:6442–6447.

445 Webster, J. P. 2007. The effect of *Toxoplasma gondii* on animal behavior: playing cat and mouse.
446 Schizophr. Bull. 33(3), 752–756. https://doi.org/10.1093/schbul/sbl073

447 Webster, J.P. 1994: The effect of *Toxoplasma gondii* and other parasites on activity levels in wild and
448 hybrid *Rattus norvegicus*. Parasitology 109: 583–589.

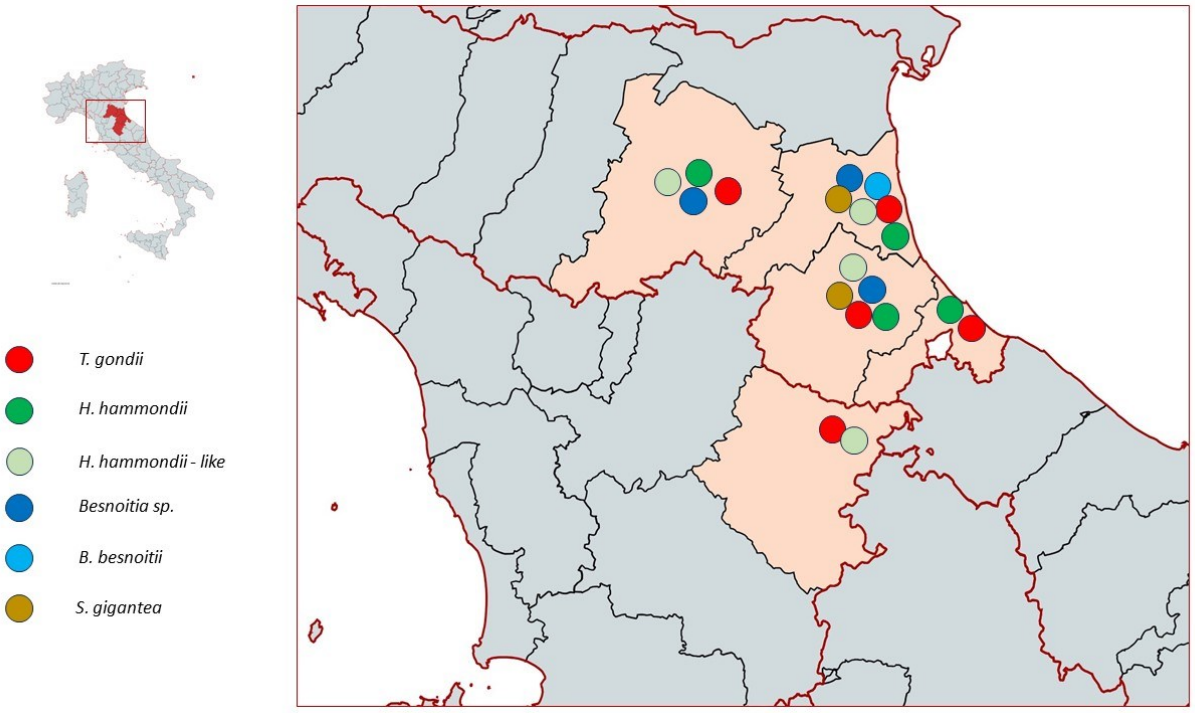
449 Webster, J.P. 2001: Rats, cats, people and parasites: the impact of latent toxoplasmosis on behaviour.
450 Microbes Infect. 3: 1–9.

451 Webster, J.P., Brunton, C.F.A. 1994. Macdonald DW. Effect of *Toxoplasma gondii* on neophobic
452 behaviour in wild brown rats, *Rattus norvegicus*. Parasitology. 109:37–43.

453 Zeng, H.; Guo, Y.; Ma, C.; Deng, S.; Hu, J.; Zhang, Y. 2020. Redescription and molecular
454 characterizationof *Sarcocystis cymruensis* from Norway rats (*Rattus norvegicus*) and
455 *Sarcocystis ratti* from black rats (*R. rattus*) in China. Parasitol. Res. 2020, 119, 3785–3791.

456

457 Figure 1: Geographical distribution of positive rodents.



458

459

460

461 Table 1: Descriptive statistics and PCR results

Species	n.	Sex	n. (%)	Weight (g)			PCR positive (%)	CI 95%
				min	max	median		
Rattus norvegicus	97	M	64 (66%)	28.5	490	228.5	12 (18.7%)	[9.19-28.31]
		F	33 (34%)	45	440	188	14 (42.4%)	[25.56-59.28]
Rattus rattus	67	M	41 (61.2%)	6	165	90	8 (19.5%)	[7.38-31.64]
		F	26 (38.81%)	5.2	195	106	5 (19.2%)	[4.08-34.4]
Mus musculus	47	M	28 (59.6%)	4.6	70	14.6	9 (32.1%)	[14.84-49.44]
		F	19 (40.4%)	4.9	25	16	4 (21)	[2.72-39.38]

462

463

464 Table 2: List of the 26 *R. norvegicus* positive at 18s PCR and result of sequencing.

ID number	Sex	Weight	Brain	Tongue	Heart
20	F	390	x	x	*Besnoitia sp.*
82	M	40	*H. hammondi*-like	x	x
83	F	320	x	*H. hammondi*-like	x
84	M	155	x	x	*Besnoitia* sp.
87	F	320	*T. gondii*	x	x
135	M	74,5	x	*Besnoitia* sp.	
136	F	240	*H. hammondi*	x	
137	F	370	x	*Besnoitia* sp.	x
140	M	265	*H. hammondi* -like	x	x
144	M	470	x	*Besnoitia besnoiti*	x
216	M	191	*H. hammondi*	*H. hammondi*-like	*H. hammondi*-like
217	F	156	x	x	*S. gigantea*
219	F	185,7	*H. hammondi*-like	x	x
220	F	86,19	*H. hammondi*-like	x	*H. hammondi*-like
221	M	36,6	x	*H. hammondi*-like	*H. hammondi*-like
3	F	180	x	*H. hammondi*-like	*H. hammondi*-like
4	F	314	*H. hammondi*-like	*H. hammondi*-like	*H. hammondi*-like
19	M	96	*T. gondii*	x	x
22	F	45	*H. hammondi*	x	*T. gondii*
25	F	80	*H. hammondi*-like	x	*Besnoitia* sp.
26	M	294,5	*H. hammondi*-like	x	*S. gigantea*
69	M	48	*H. hammondi*-like	x	x
71	F	220	*Besnoitia* sp.	x	*H. hammondi*-like
78	F	94,7	*H. hammondi*-like	x	*H. hammondi*
79	M	82	x	x	*H. hammondi*-like
81	M	132	x	x	*T. gondii*

465

466

Table 3: List of the 13 *R. rattus* positive at 18s PCR and result of sequencing.

ID number	**Sex**	**weight**	**Brain**	**Tongue**	**Heart**
76	F	5,2	x	*T. gondii*	x
77	F	6	x	x	*H. hammondi*-like
148	M	91	x	*T. gondii*	x
215	F	82	*H. hammondi*	*T. gondii*	*H. hammondi*
218	F	97	*H. hammondi*-like	x	x
1	M	104	*H. hammondi*-like	*H. hammondi*-like	*H. hammondi*-like
2	M	90	*H. hammondi*-like	*H. hammondi*-like	*H. hammondi*-like
21	F	139,22	*T. gondii*	x	x
30	M	162,95	x	*T. gondii*	x
31	M	141,3	*H. hammondi*-like	x	*H. hammondi*-like
38	M	59,6	x	x	*H. hammondii*
51	M	140	*H. hammondi*-like	*H. hammondi*-like	*H. hammondi*-like
75	M	45	x	x	*T. gondii*

472

473 Table 4 list of the 13 *M. musculus* positive at 18s PCR and result of sequencing

ID number	Sex	Weight	Brain	Tongue	Heart
101	F	17	x	x	*T. gondii*
109	F	25	x	*H. hammondi*-like	x
111	M	17	x	x	*Besnoitia besnoiti*
112	F	22	x	x	*H. hammondi*-like
113	F	15	*H. hammondi*-like	x	x
115	M	14	*H. hammondi*-like	x	*H. hammondi*-like
117	M	23	*H. hammondi*-like	x	x
119	M	7,9	x	*H. hammondi*-like	*H. hammondi*-like
120	M	7	*H. hammondi*	x	*H. hammondi*-like
122	M	14,7	*H. hammondi*-like	x	x
7	M	21	x	x	*H. hammondi*-like
32	M	9,7	x	*H. hammondi*-like	
80	M	18,5	*T. gondii*	*H. hammondi*-like	x

474

475

476

477

478

Chapter 4.3

Sero-epidemiological investigation on *Toxoplasma gondii* infection in Apennine wolf (*Canis lupus italicus*) and wild boar (*Sus scrofa*) in Italy.

Dini FM, Musto C, De Nigris VM, Bellinello E, Sampieri M, Merialdi G, Barca L, Delogu M, Galuppi R (2024). Sero-epidemiological investigation on *Toxoplasma gondii* infection in Apennine wolf (*Canis lupus italicus*) and wild boar (*Sus scrofa*) in Italy. BMC Veterinary Sciences, Accepted.

Sero-epidemiological investigation on *Toxoplasma gondii* infection in Apennine wolf (*Canis lupus italicus*) and wild boar (*Sus scrofa*) in Italy.

Filippo Maria Dini[a*], Carmela Musto[a], Vincenzo Maria De Nigris[b], Enrica Bellinello[c], Maria Sampieri[d], Giuseppe Merialdi[d], Lorella Barca[e], Mauro Delogu[a], Roberta Galuppi[a]

[a] *Department of Veterinary Medical Sciences, University of Bologna, 40064 Ozzano Emilia, Bologna, Italy*

[b]*AUSL Bologna dipartimento di Sanità Pubblica Veterinaria- UO Veterinaria B, Via del Seminario, 1 San Lazzaro di Savena, Bologna, Italy.*

[c]*AUSL Modena, dipartimento di Sanità Pubblica Veterinaria, via Suore di San Giuseppe Benedetto Cottolengo, 5 41026 Pavullo nel Frignano, Modena, Italy*

[d]*Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia-Romagna Bruno Ubertini, Brescia, Italy*

[e]*Istituto Zooprofilattico Sperimentale del Mezzogiorno, Portici, Napoli, Italy*

*Correspondence: Filippo Maria Dini

E-mail: filippomaria.dini@unibo.it

Abstract

- **Background:** The wild boar (*Sus scrofa*) and the Apennine wolf (*Canis lupus italicus*) are two wild species that have both increased their presence in the Italian territory, albeit in varying numbers. They can be occasionally found in peri-urban areas as well. Both of these species can serve as intermediate hosts for *Toxoplasma gondii*, as they can become infected either through the consumption of oocysts found in water, soil, or on vegetables, or through the ingestion of meat containing bradyzoites. Consequently, these animals can be regarded as key indicators of *Toxoplasma* presence in the wild or peri-urban environment. In our study, we examined a total of 174 wild boar meat juice samples and 128 wolf sera samples from Italy for the detection of *T. gondii* IgG using the indirect fluorescent antibody test (IFAT).
- **Results:** The results showed that 40 (22.6%) of the wild boar meat juice and 34 (26.6%) of the wolf serum samples tested positive. Interestingly, there were no significant differences in seropositivity with respect to gender, age group, or the region of origin in both species.
- **Conclusions:** Overall the results indicate a moderate exposure in both the species under investigation, highlighting the spread of *T. gondii* in sylvatic and periurban environments. The prevalence of *T. gondii* in wild boar is consistent with findings from other studies conducted in Europe. Our study, with a considerably larger sample size, compared to the available research in European context, provides valuable data on the seroprevalence of *T. gondii* in wolves.

Key-Words: Toxoplasmosis, Wild boar, Wolf, Serology, IFAT

Background

Toxoplasma gondii is a globally distributed apicomplexan protozoan. Its widespread epidemiological success can be attributed to its ability to infect both definitive and intermediate hosts through various modes [1].

Definitive hosts, primarily members of the Felidae family, facilitate the parasite's sexual reproduction in their intestinal tract, potentially leading to the excretion of millions of oocysts into the environment. In our regions wild and domestic cats play a crucial role in perpetuating this parasite [2]. In Italy, the native European wildcat (*Felis silvestris silvestris*) maintains a relatively small population size in the wild, despite beingclassified as Least Concern in the IUCN Red List of Threatened Species [3]. However, free-roaming domestic cats are prevalent in rural and peri-urban regions [4].

All warm-blooded vertebrates, including humans, can serve as intermediate hosts in which cysts housing long-lasting bradyzoites develop. These hosts become infected by ingesting sporulated oocysts, although the parasite may persist through predation among them, even in the absence of a definitive host [1].

Omnivorous wild boars (*Sus scrofa*) are susceptible to infection through two plausible routes: ingestion of highly resistant oocysts present in water and vegetation, and consumption of remains of infected intermediate hosts [5]. Additionally, wild boars represent a potential risk to human health through the consumption of raw or undercooked game meat [6]. The wolf (*Canis lupus*) can also act as an intermediate host of *T gondii*. Despite the wolf's primarily carnivorous diet, which includes predation on live animals, including wild boar, it has been established that they also frequently consume fruits (Rosaceae), other plant matter, and insects [7]. Consequently, both modess of infection are viable in these animals, positioning them at the apex of receptive intermediate hosts range.

The IUCN Red List of Threatened Species has classified the European assessment of *Canis lupus* as "Least Concern" [8]. In Italy, a subspecies of the grey wolf known as the Apennine wolf (*Canis lupus italicus*) has seen a population expansion throughout the Italian peninsula in recent years [9], with the

exception of the islands. Over the past few decades, both the number and distribution of wolf populations in Italy have increased. Wolves have been progressively reclaiming their historic habitats, moving from the Apennines to the western areas of the Italian Alps [10, 11].In the past decade, they have also expanded into the eastern Alps [12]. While wolves tend to prefer locations at a considerable distance from human settlements, they have been observed in close proximity to urban areas in densely populated regions [13]."

Despite being among the most heavily hunted ungulate species, wild boars have undergone a population expansion throughout Europe. In Italy, the density of wild boars has been estimated to range from 0.01 to 0.05 animals per square kilometer, increasing to as high as 2.32 to 10.5 animals per square kilometre across the entire Italian peninsula [14]. The simultaneous expansion of human-inhabited areas and the wild boar populations has facilitated the intrusion of this species into various European urban areas, including Rome [15].

In the present study, we conducted a serological survey on wolves and wild boars from different regions of Italy. The objective was to gather data on their exposure to *T. gondii* infection, serving as indicators of *Toxoplasma* presence within the wild or peri-urban environment.

Results

The wild boars displayed nearly equal representation across sex and age groups, with a notable portion originating from the Tuscany region (as reported in Table 1). Among the 177 meat juice samples, 40 (22.6%) tested positive for *Toxoplasma* IgG at IFAT. No statistically significant differences of seropositivity were observed in relation to sex, age groups and region of origin and between wolves and wild boar.

Table 1
Descriptive statistics and serological tests result in wild boar examined.

	Category	n. wild boar tested	Relative distribution %	IFAT positive	Seroprevalence %	95%CI
Total		177		40	22.6	[16.44 – 28.76]
Gender	Male	83	53.2	14	16.9	[8.84 – 24.96]
	Female	73	46.8	21	28.8	[18.41 – 39.19]
Age groups	Young	92	52.3	25	27.2	[18.11 – 36.29]
	Elderly	84	47.7	14	16.7	[8.72 – 24.68]
Region of origin	Tuscany	76	42.9	15	19.7	[10.76 – 28.64]
	Emilia Romagna	51	28.8	9	17.6	[7.15 – 28.05]
	Abruzzo	48	27.2	16	33.3	[20 – 46.63]
	Molise	2	1.1	0	0	[]

Note: in 21 cases, the sex of the subjects could not be ascertained, and in one case, the age was unknown, due to incomplete filling of the animal's identification form.

The region of origin of wolves and their cause of death are summarized in Table 2). Thirty-four (26.6%) out of 128 serum analysed, were positive at IFAT, with antibody titres ranging from 1:20 to 1:160. It is

noteworthy that no statistically significant differences were observed to seropositivity in relation to sex, age group, geographic origin or cause of death.

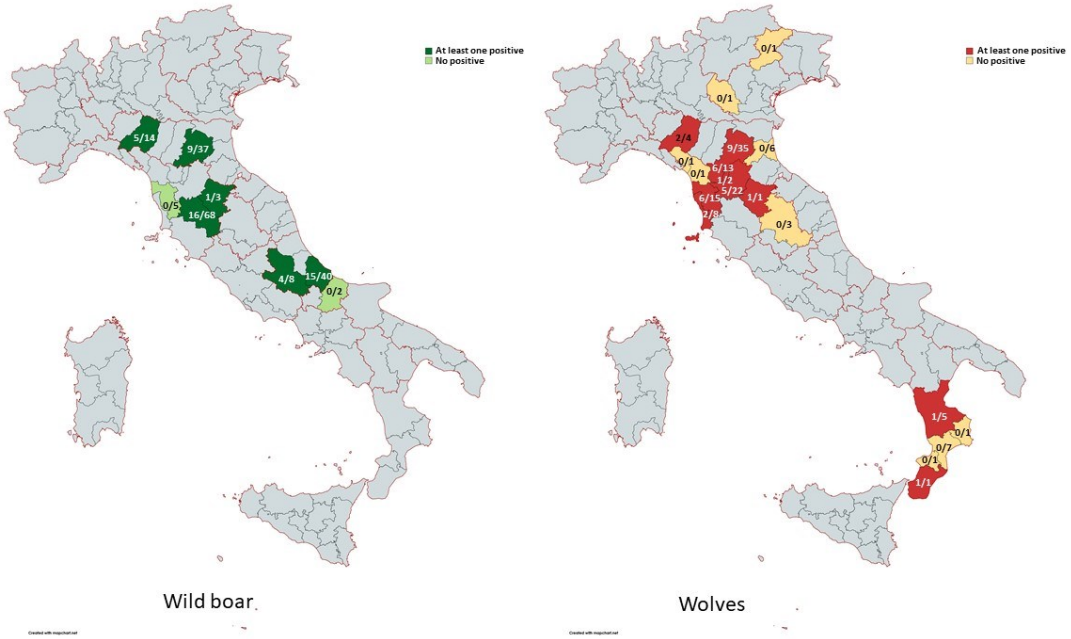
Table. 2
Descriptive statistics and serological test results in wolf examined.

	Category	n. wolf tested	Relative distribution %	IFAT positive	Seroprevalence %	95%CI
Total		128		34	26.6	[18.95 - 34.25]
Sex	Male	79	62.2	26	32.9	[22.54 - 43.26]
	Female	48	37.8	8	16.7	[6.15 – 27.25]
Age class	1: <12 months	42	33.1	10	23.8	[10.92 - 36.68]
	2: 1-2 years	31	24.4	5	16.1	[3.16 – 29.04]
	3: > 2 years	54	42.18	19	35.19	[24.87 – 45.51]
Region of origin	Tuscany	63	49.2	21	33.3	[21.66 – 44.94]
	Emilia-Romagna	45	35.2	11	24.4	[11.85 – 36.95]
	Calabria	15	11.7	2	13.3	[0 – 31.1]
	Umbria	3	2.3	0	0	[]
	Veneto	2	1.6	0	0	[]
Cause of death	Car crash	75	60.5	16	21.3	[12.3 – 30.57]
	Other cause	49	39.5	16	32.6	[19.4 – 45.8]

Note: in one subject it was not possibly to know the gender and in one the age due to the poor condition of the carcasses. In four subjects (two positive and two negative) the cause of death was undetermined.

.

Figure 1: distribution of wild boar and wolves examined; number positive/number examined



In figure 1 the distribution of wolves and wild boar examined are illustrated, with the number of seropositive/number of examined samples in the different Italian provinces.

In the geographical areas where there was an overlap in the sampling of wild boars and wolves (Emilia-Romagna and Tuscany regions), the seroprevalences were 18.9% and 29.6%, respectively, even though the differences were not significant.

Discussion

In this study, we evaluated the seroprevalence of *T. gondii* in two species, wild boar and wolves. Despite their role as intermediate hosts, these species could play a significant role in mantaining the effective continuity of the parasite's life cycle in the wild. Both these animals can become infected through the ingestion of robust, environmentally enduring oocysts, as well as via the consumption of prey or carrion. Consequently, they serve as valuable indicators to detect the presence of *T. gondii* contamination within specific ecological contexts [16, 17].

During this study, we utilized two different matrices: serum samples from wolves and meat juice from wild boar. The choice of these two matrices was driven by practical considerations. In the case of wolves, which were found deceased, we were able to conduct a comprehensive necropsy, including the collection of clotted blood from the heart cavity and subsequent extraction of serum. On the other hand, for wild boars, a different approach was necessary. These animals were hunted and eviscerated before slaughtering, making it impossible to collect blood directly. Therefore, we chose meat juice as a more appropriate and easily accessible matrix in this situation.

This matrix has been used in previous studies for the detection of antibodies against *T. gondii* [18] as well as other zoonotic pathogens such as *Trichinella* sp. [19, 20], *Salmonella* sp., and Hepatitis E virus [21]. The use of meat juice as a matrix is particularly advantageous, as it can be easily obtained from wildlife carcasses, often found deceased, thereby providing valuable serological data that would otherwise be challenging to collect. However, it's important to note that meat juice has been perceived as a matrix with lower sensitivity in comparison to serum, primarily due to the lower antibody concentration it contains [22]. While serological data derived from either sera or meat juice samples offer insights into an animal's exposure to the parasite, they do not provide information concerning the presence of tissue cysts within organs, which directly relates to the risk for consumers [23, 24].

In the present study, an overall seroprevalence rate of 22.6% was observed in wild boars (ranging from 0 to 33.3% across the different regions), and no statistically significant differences were observed among the variables considered, including age, in line with the findings of some authors [25-27]. Recent meta-analyses have shown that the global pooled seroprevalence of *T. gondii* in wild boars from 1995 to 2017 was 23%, which aligns closely with our findings [17]. However, various seroprevalence rates have been documented on wild boars in different geographical settings. For instance, in Europe, seroprevalence values ranging from 8% to 38% have been reported [17, 28- 30]. Specifically, surveys conducted in central and southern regions of Italy, reported values ranging from 12.2% [31] to 14% [32, 21], while recent surveys in Northern Italy have identified seroprevalences spanning from 15.5% [33] to as high as 53.1% [27]. These seroprevalence differences could be related to specific local epidemiological conditions, such as variations in environmental factors, wildlife populations, or human activities, highlighting the importance of considering local risk factors in understanding the epidemiology of Toxoplasmosis.

In wolves, a seroprevalence rate of 26.6% was observed in this study. When comparing seroprevalences between wolves and wild boars, despite wolves occupying higher trophic levels and

exhibiting a higher prevalence of T. gondii, no statistically significant differences were observed between these two populations. This finding aligns with the results of Dakraub et al.. [4]. Reliable *T. gondii* seroprevalence data for wolves in European countries, including Italy, are notably scarce. Recent reports from Italy have indeed documented seropositivity in wolves, albeit with relatively small sample sizes: Dini et al. [33] identified one positive wolf out of 5 samples, while Dakraub et al. [4] reported 4 positives out of 14. In other European countries, such as Spain, a seroprevalence rate of 46.9% was observed (n=32 wolves sampled) [34]. Due to the considerable higher sample size, the present study offers a comprehensive assessment of *T. gondii* seroprevalence in wolves, thereby contributing valuable data on a European scale.

No significant differences in seroprevalence were observed among sex, age class, region of origin, or cause of death. While an age-related increase in parasite prevalence could be a plausible hypothesis, our recent data on dogs also indicate that age categories do not significantly impact toxoplasmosis seroprevalence [35].

In addition to its epidemiological significance, seropositivity in wolves has been associated with ecological implications, particularly in the United States. Recent research [36] demonstrated that the overlap of wolf territories with regions characterized by a high cougar population density serves as a significant predictor of *T. gondii* infection in wolves. Furthermore, wolves that tested positive through serological analysis were found to be more inclined to make high-risk decisions, such as dispersing and assuming leadership roles within packs [36]. These decisions have a pivotal impact on individual fitness and the broader dynamics of wolf. In the current study, we did not observe a positive correlation between seropositivity and the cause of death being a car crash. Instead, even when considering seropositivity as a factor contributing to increased wolf dispersion, it does not appear to be linked to car collision as cause of death in our sample set.

Conclusion

This study provides an update on the spread of *T. gondii* in sylvatic and peri urban settings, highlining a moderate exposure in both the species under investigation. Additional research endeavours should be undertaken to explore the correlation between *T. gondii* seropositivity in wolves and factors like dispersal rates, causes of death, and spatial overlap with other species, including humans. These studies will be able to contribute to a more comprehensive understanding of the significance of *T. gondii* seroprevalence, including its ecological implications.

Methods

Approximately 25 g of diaphragm tissue from wild boars were systematically collected at a specialized game meat processing facility located in the Bologna province (Emilia-Romagna region). This facility routinely receives eviscerated carcasses of hunted wild boars from various regions of Italy, encompassing Emilia-Romagna, Tuscany, and Abruzzo. Sex, and age class were determined, the latter assessed by the evaluation of the dental table. The diagnostic matrix employed in this study was the meat juice, as carcasses have already been bled and eviscerated. To extract the meat juice from the diaphragm tissue, the samples were placed in hermetically sealed plastic container, and frozen at -20 °C. Following this step, the meat samples were thawed over-night, at a controlled temperature of 4 °C. The resulting meat juice was then transferred into sterile tubes, preserved at -20 °C until use [37].

The examined wolves came mainly from Toscana and Emilia-Romagna region (Central Italy), in less extent they were collected from Calabria (south), Umbria (centre), and Veneto (north) regions. The wolves were found dead and delivered to authorized centers in order to proceed with the necropsy. Necropsy examinations on wolf carcasses were carried out at the Experimental Zooprophylactic

Institute of Lombardy and Emilia-Romagna, the Wildlife and Exotic Service of the University of Bologna and at the Experimental Zooprophylactic Institute of Southern Italy. At the arrival of each carcass, a first form containing the following information was filled: subject's identification data with the attribution of a unique ID code, the discovery location (reported as GPS coordinates), sex, weight (in kg) and nutritional status. The age of the animal was determined by assessing dental development and wear [38, 39], as well as considering body size and weight. Here, all individuals were aged using 3 categories as follows: class 1: ≤12 months; class 2: 1–2 years; class 3: > 2 years. The age determination of class 1 (based on months of life) was defined in relation to the reproductive cycle of the wolf [40]. Besides the biometrics information, phenotypic characteristics and anatomopathological activities were carried out to investigate the cause of death [41]. During necropsy the entire heart was collected, and the heart blood clot was extracted and centrifuged at 980g for 20 minutes. The haemolytic serum was then collected in a 2 ml tube and stored at -20 °C until use.

A total of 177 meat juices of wild boars and 128 wolf sera were analysed for *T. gondii* IgG by indirect fluorescent antibody test (IFAT) following the manufacturer's instructions (MegaFLUO TOXO-PLASMA g, MegaCor Diagnostik, Hoerbranz, Austria). As conjugated, anti-dog IgG antibody diluted in PBS at concentration of 1:64 (Anti-Dog IgG-FITC antibody, Sigma-Aldrich, Saint Louis, MO) and anti-pig. IgG antibody diluted in PBS at concentration of 1:32 (Anti-pig IgG-FITC antibody, Sigma-Aldrich, Saint Louis, MO) were used. Meat juice from wild boars with an antibody titre ≥ 1:4 were considered positive (due to the scarce concentration of antibody in this matrix) [22], while wolf serum samples with antibody titre ≥ 1:20 were considered positive (due to the haemolytic characteristics of the sera) [42].

Pearson's $\chi 2$ test was used to correlate sex, age group, region of origin (and cause of death in wolf) with seroprevalence. Statistical significance was set at $P \leq 0.05$. The Sample Size Calculator (https://www.surveysystem.com/sscalc.htm) was used to calculate 95% confidence intervals (CIs) for the observed prevalence values.

Declarations

- **Ethics approval and consent to participate**
  All samples were gathered in compliance with local regulations. Specifically, wild boar samples were collected by an official veterinarian as part of the official *Trichinella* sampling for game meat. As for wolves, the local authority collected all animals, and necropsies were conducted at specialized centers (such as IZS or University institutions) dedicated to wildlife disease control. All the methods were performed in accordance with relevant guidelines and regulations, no specific permission was required to perform the sampling.

- **Animal Ethics declaration**
  Not applicable

- **Consent for publication**
  Not applicable

- **Availability of data and materials**
  The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request

- **Competing interests**
  The authors declare that they have no competing interests

- **Funding**

The co-author Carmela Musto was partially supported by a research grant funded by the Vienna Science and Technology Fund (WWTF) [10.47379/ESR20009].

- **Authors' contributions**
  F.M.D. and R.G. designed the study and analysed data, F.M.D., C.M., V.M.D.N., E.B., M.S., G.M., L.B., M.D. contributed to collecting the samples. F.M.D. carried out the analysis. F.M.D. and R.G. assembled data, RG. was the supervisor, F.M.D, R.G., E.B., C.M. contributed to writing, reviewing and editing the final manuscript. All authors read and approved the final manuscript.

- **Acknowledgements**
  The authors are grateful to Dr. Bruno Marasco from AUSL of Bologna, for his availability in organizing the sampling of wild boar, and Prof. Monica Caffara for the English revision.

References

1. Tenter AM, Heckeroth AR, Weiss LM. *Toxoplasma gondii*: from animals to humans. Int J Parasitol. 2000 Nov;30(12-13):1217-58. doi: 10.1016/s0020-7519(00)00124-7.

2. Montazeri M, Mikaeili Galeh T, Moosazadeh M, Sarvi S, Dodangeh S, Javidnia J, Sharif M, Daryani A. The global serological prevalence of Toxoplasma gondii in felids during the last five decades (1967-2017): a systematic review and meta-analysis. Parasit Vectors. 2020 Feb 17;13(1):82. doi: 10.1186/s13071-020-3954-1.

3. Gerngross P, Ambarli H, Angelici FM, Anile S, Campbell R, Ferreras de Andres P, Gil-Sanchez JM, Götz, M., Jerosch S, Mengüllüoglu D, Monterosso P, Zlatanova D, *Felis silvestris*. 2022. *The IUCN Red List of Threatened Species*: e.T181049859A181050999. https://dx.doi.org/10.2305/IUCN.UK.2022-1.RLTS.T181049859A181050999.en. Accessed on 17 October 2023.

4. Dakroub H, Sgroi G, D'Alessio N, Russo D, Serra F, Veneziano V, Rea S, Pucciarelli A, Lucibelli MG, De Carlo E, Fusco G, Amoroso MG. Molecular Survey of *Toxoplasma gondii* in Wild Mammals of Southern Italy. Pathogens. 2023 Mar 16;12(3):471. doi: 10.3390/pathogens12030471.

5. Guardone L, Armani A, Mancianti F, Ferroglio E. A Review on *Alaria alata*, *Toxoplasma gondii* and *Sarcocystis* spp. in Mammalian Game Meat Consumed in Europe: Epidemiology, Risk Management and Future Directions. Animals (Basel). 2022 Jan 21;12(3):263. doi: 10.3390/ani12030263. PMID: 35158587.

6. Kornacka-Stackonis A. Toxoplasma gondii infection in wild omnivorous and carnivorous animals in Central Europe - A brief overview. Vet Parasitol. 2022 Apr;304:109701. doi: 10.1016/j.vetpar.2022.109701

7. Meriggi A, Rosa P, Brangi A, Matteucci C.. Habitat Use And Diet Of The Wolf In Northern Italy. Acta Theriol. 1991 36: 141 – 151.

8. IUCN, Lista Rossa dei vertebrati italiani 2022. https://www.iucn.it/pdf/Lista-Rossa-vertebratiitaliani-2022.pdf

9. La Morgia V, Marucco F, Aragno P, Salvatori V, Gervasi V, De Angelis D, Fabbri E, Caniglia R, Velli E, Avanzinelli E, Boiani MV, Genovesi P, Stima della distribuzione e consistenza del lupo a scala nazionale 2020/2021. Relazione tecnica realizzata nell'ambito della convenzione ISPRA-Ministero della Transizione Ecologica "Attività di monitoraggio nazionale nell'ambito del Piano

di Azione del lupo". 2022. https://www.isprambiente.gov.it/it/attivita/biodiversita/monitoraggio-nazionale-del-lupo/file-monitoraggio/report-nazionale-lupo-20_21.pdf

10. Scandura M, Apollonio M, Mattioli L. Recent recovery of the Italian wolf population: a genetic investigation using microsatellites. Mamm Biol. 2001, 66: 321–31.

11. Fabbri E, Miquel C, Lucchini V, Santini A, Caniglia R, Duchamp C, Weber JM, Lequette B, Marucco F, Boitani L, Fumagalli L, Taberlet P, Randi E. From the Apennines to the Alps: colonization genetics of the naturally expanding Italian wolf (*Canis lupus*) population. Mol Ecol. 2007 Apr;16(8):1661-71. doi: 10.1111/j.1365-294X.2007.03262.x.

12. Marucco F., Avanzinelli E, Boiani MV, Menzano A, Perrone S, Dupont P, Bischof R, Milleret C, von Hardenberg A, Pilgrim K, Friard O, Bisi F, Bombieri G, Calderola S, Carolfi S, Chioso C, Fattori U, Ferrari P, Pedrotti L, Righetti D, Tomasella M, Truc F, Aragno P, La Morgia V, Genovesi P. La popolazione di lupo nelle regioni alpine Italiane 2020-2021. Relazione tecnica dell'Attività di monitoraggio nazionale nell'ambito del Piano di Azione del lupo ai sensi della Convenzione ISPRA-MITE e nell'ambito del Progetto LIFE 18 NAT/IT/000972 WOLFALPS EU. 2022.

13. Zanni M, Brogi R, Merli E, Apollonio M. The wolf and the city: insights on wolves conservation in the anthropocene. Anim Conserv. 2023 doi:10.1111/acv.12858. 1-15.

14. Pittiglio C, Khomenko S, Beltran-Alcrudo D. Wild boar mapping using population-density statistics: From polygons to high resolution raster maps. PLoS One. 2018 May 16;13(5):e0193295. doi: 10.1371/journal.pone.0193295.

15. Amendoli S, Lombardini M, Pierucci P, Meriggi A. Seasonal spatial ecology of the wild boar in a peri-urban area. Mammal Res 2019 64:387–96. https://doi.org/10.1007/s13364-019-00422-9

16. Beral M, Rossi S, Aubert D, Gasqui P, Terrier ME, Klein F, Villena I, Abrial D, Gilot-Fromont E, Richomme C, Hars J, Jourdain E. Environmental factors associated with the seroprevalence of *Toxoplasma gondii* in Wild Boars (Sus scrofa), France. Ecohealth. 2012 Sep;9(3):303-9. doi: 10.1007/s10393-012-0786-2.

17. Rostami A, Riahi SM, Fakhri Y, Saber V, Hanifehpour H, Valizadeh S, Gholizadeh M, Pouya RH, Gamble HR. The global seroprevalence of *Toxoplasma gondii* among wild boars: A systematic review and meta-analysis. Vet Parasitol. 2017 Sep 15;244:12-20. doi: 10.1016/j.vetpar.2017.07.013.

18. Opsteegh, M., Swart, A., Fonville, M., Dekkers, L., van der Giessen, J., 2011. Age-related *Toxoplasma gondii* seroprevalence in Dutch wild boar inconsistent with lifelong persistence of antibodies. PloS one, 6(1), e16240. https://doi.org/10.1371/journal.pone.0016240.

19. Calero-Bernal R, Pérez-Martín JE, Reina D, Serrano FJ, Frontera E, Fuentes I, Dubey JP. Detection of Zoonotic Protozoa *Toxoplasma gondii* and *Sarcocystis suihominis* in Wild Boars from Spain. Zoonoses Public Health. 2016 Aug;63(5):346-50. doi: 10.1111/zph.12243.

20. Villa L, Allievi C, Gazzonis AL, Ventura G, Gradassi M, Zanzani SA, Manfredi MT. Serological Prevalence of *Toxoplasma gondii*, *Neospora caninum*, and *Sarcoptes scabiei* var. *suis* in Wild Boars (*Sus scrofa*) Hunted in a Highly Anthropized Area in Italy. Animals (Basel). 2023 May 23;13(11):1730. doi: 10.3390/ani13111730.

21. Kijlstra A, Jongert E. Control of the risk of human toxoplasmosis transmitted by meat. Int J Parasitol. 2008 Oct;38(12):1359-70. doi: 10.1016/j.ijpara.2008.06.002.

22. Puchalska M, Pyziel AM, Wiśniewski J, Steiner-Bogdaszewska Ż, Klich D, Anusz K. Prevalence of *Toxoplasma gondii* antibodies in wild boar (*Sus scrofa*) from Strzałowo Forest Division, Warmia and Mazury Region, Poland. Ann Agric Environ Med. 2021 Jun 14;28(2):237-42. doi: 10.26444/aaem/118883.

23. Olsen A, Berg R, Tagel M, Must K, Deksne G, Enemark HL, Alban L, Johansen MV, Nielsen HV, Sandberg M, Lundén A, Stensvold CR, Pires SM, Jokelainen P. Seroprevalence of *Toxoplasma gondii* in domestic pigs, sheep, cattle, wild boars, and moose in the Nordic-Baltic region: A systematic review and meta-analysis. Parasite Epidemiol Control. 2019 Mar 4;5:e00100. doi: 10.1016/j.parepi.2019.e00100

24. Papini RA, Vannucci S, Rocchigiani G, Nardoni S, Mancianti F. Prevalence of *Toxoplasma gondii* and potentially zoonotic helminths in wild boars (*Sus scrofa*) hunted in central Italy. Mac Vet Rev. 2018 41: 83–93.

25. Ranucci D, Veronesi F, Moretti A, Branciari R, Miraglia D, Manfredi MT, Piergili Fioretti D. Seroprevalence of *Toxoplasma gondii* in wild boars (*Sus scrofa*) from Central Italy. Parasite. 2013;20:48. doi: 10.1051/parasite/2013048.

26. Sarno E, Costanzo N, Quaranta V, Santoro AML, Stephan R,. Prevalence of IgG against hepatitis E virus, *Salmonella* spp., and *Toxoplasma gondii* in meat juice samples from wild boars hunted in Southern Italy. J. Food Safety Food Qual. 2014 65: 141–4.

27. Dini FM, Morselli S, Marangoni A, Taddei R, Maioli G, Roncarati G, Balboni A, Dondi F, Lunetta F, Galuppi R. Spread of *Toxoplasma gondii* among animals and humans in Northern Italy: A retrospective analysis in a One-Health framework. Food Waterborne Parasitol. 2023 Jun 8;32:e00197. doi: 10.1016/j.fawpar.2023.e00197.

28. Halos L, Thébault A, Aubert D, Thomas M, Perret C, Geers R, Alliot A, Escotte-Binet S, Ajzenberg D, Dardé ML, Durand B, Boireau P, Villena I. An innovative survey underlining the significant level of contamination by Toxoplasma gondii of ovine meat consumed in France. Int J Parasitol. 2010 Feb;40(2):193-200. doi: 10.1016/j.ijpara.2009.06.009.

29. Nöckler K, Serrano FJ, Boireau P, Kapel CM, Pozio E. Experimental studies in pigs on Trichinella detection in different diagnostic matrices. Vet Parasitol. 2005 Sep 5;132(1-2):85-90. doi: 10.1016/j.vetpar.2005.05.033.

30. Frey CF, Schuppers ME, Nöckler K, Marinculić A, Pozio E, Kihm U, Gottstein B. Validation of a Western Blot for the detection of anti-*Trichinella* spp. antibodies in domestic pigs. Parasitol Res. 2009 Jun;104(6):1269-77. doi: 10.1007/s00436-008-1321-9.

31. Carella E, Caruso C, Moreno A, Di Blasio A, Oberto F, Vitale N, Masoero L. Meat Juice and Oral Fluid as Alternatives to Serum for Aujeszky Disease Monitoring in Pigs. Microorganisms. 2023 Sep 27;11(10):2418. doi: 10.3390/microorganisms11102418.

32. Pinto P, Bozzo G, Novello L, Terio V. Detection of *Toxoplasma gondii* cysts from wild boar muscles: Does it represent a risk for ready to eat food? IJFS. 2010 Oct. 27,1(8):31-34. https://www.pagepressjournals.org/index.php/ijfs/article/view/ijfs.2010.8.31.

33. Gazzonis AL, Gjerde B, Villa L, Minazzi S, Zanzani SA, Riccaboni P, Sironi G, Manfredi MT. Prevalence and molecular characterisation of *Sarcocystis miescheriana* and *Sarcocystis suihominis* in wild boars (*Sus scrofa*) in Italy. Parasitol Res. 2019 Apr;118(4):1271-87. doi: 10.1007/s00436-019-06249-2.

34. Sobrino R, Cabezón O, Millán J, Pabón M, Arnal MC, Luco DF, Gortázar C, Dubey JP, Almeria S. Seroprevalence of Toxoplasma gondii antibodies in wild carnivores from Spain. Vet Parasitol. 2007 Sep 30;148(3-4):187-92. doi: 10.1016/j.vetpar.2007.06.038.

35. Dini FM, Stancampiano L, Poglayen G, Galuppi R. Risk factor for *Toxoplasma gondii* infection in dogs: a serological survey. 2023 Under revision in Acta Veterinaria Scandinavica.

36. Meyer CJ, Cassidy KA, Stahler EE, Brandell EE, Anton CB, Stahler DR, Smith DW. Parasitic infection increases risk-taking in a social, intermediate host carnivore. Commun Biol. 2022 Nov 24;5(1):1180. doi: 10.1038/s42003-022-04122-0.

37. Gipson PS, Ballard WB, Nowak RM, Mech LD. Accuracy and precision of estimating age of gray wolves by tooth wear. J Wildl Manag. 2000 64 (3): 752-8.

38. Brasington TJ, Hadley JM, Stahler DR, Stahler EE, Cassidy KA 2023. A Visual guide to wolf dentition and age determination. Wildlife Biology, https://www.researchgate.net/publication/374921532_A_Visual_Guide_to_Wolf_Dentition_and_Age_Determination_For_Researchers_and_Wildlife_Professionals

39. Boitani L. Lupo *Canis lupus*. In: Pavan M. (Eds), Distribuzione e biologia di 22 specie di Mammiferi in Italia Rome (IT) IBS; 1981. [61–67] https://www.ibs.it/distribuzionebiologia-di-22-specie-libri-vintage-vari/e/2560038844628

40. Musto C, Cerri J, Galaverni M, Caniglia R, Fabbri E, Apollonio M, Mucci N, Bonilauri P, Maioli G, Fontana MC, Gelmini L, Prosperi A, Rossi A, Garbarino C, Fiorentini L, Ciuti F, Berzi D, Merialdi G, Delogu M. Men and wolves: Anthropogenic causes are an important driver of wolf mortality in human-dominated landscapes in Italy. Glob Ecol Conserv. 2021 e01892, https://doi.org/10.1016/j.gecco.2021.e01892.

41. Meemken D, Tangemann AH, Meermeier D, Gundlach S, Mischok D, Greiner M, Klein G, Blaha T. Establishment of serological herd profiles for zoonoses and production diseases in pigs by "meat juice multi-serology". Prev Vet Med. 2014 Mar 1;113(4):589-98. doi: 10.1016/j.prevetmed.2013.12.006.

42. Nardoni S, Rocchigiani G, Varvaro I, Altomonte I, Ceccherelli R, Mancianti F. Serological and Molecular Investigation on *Toxoplasma gondii* Infection in Wild Birds. Pathogens. 2019 Apr 29;8(2):58. doi: 10.3390/pathogens8020058.

Chapter 5.1

Risk factor for *Toxoplasma gondii* infection in dogs: a serological survey

Dini FM, Stancampiano L, Poglayen G, Galuppi R. (2023). Risk factor for *Toxoplasma gondii* infection in dogs: a serological survey. *Acta Veterinaria Scandinavica* (under revision).

Risk factor for *Toxoplasma gondii* infection in dogs: a serological survey

Filippo Maria Dini[1]*, Laura Stancampiano [1], Giovanni Poglayen [1], Roberta Galuppi[1]

[1]Department of Veterinary Medical Sciences, Alma Mater Studiorum - University of Bologna;

*Correspondence: filippomaria.dini@unibo.it;

Abstract

Background

Dogs, as well as a wide variety of other warm-blooded animals, act as intermediate host of *Toxoplasma gondii*. In this species, most cases of toxoplasmosis are subclinical, although clinical disease has been sporadically reported.

Beyond its role in diagnostic pathways, seropositivity also functions as a reflection of the parasite's spread within the dog's living environment. The aim of the present study was to evaluate the possible risk factor associated with seropositivity to *T.gondii* in dogs in Central-Northen Italy, analysing 120 dogs sera for the presence of IgG antibodies by indirect fluorescence antibody test (IFAT).

Results

The population examined were composed of 54.17% hunting dogs, 24.17% companion dogs, 14.17% truffle dogs and 7.5% watchdogs. Thirty-four (29.17%) dogs tested positive for *T. gondii* IgG, with titers ranging from 1:40 to 1:1280. Seroprevalence and antibodies titers were not related to dog gender, age and function. The logistic regression and ordered logistic regression results indicate that seroprevalence, and antibody titers are significantly higher in dogs cohabiting with cats, exhibiting coprophagy habits, and living constantly outdoors. Notably, the lifestyle factor showed the highest odds-ratios in the study: dogs living constantly outdoors were found to be at approximately 5 times

greater risk of testing positive and having higher antibody titers compared to dogs living both indoors and outdoors.

Conclusion

The consistency between logistic and ordered logistic regression results supports the key role of living with cats, engaging in coprophagy behaviors, and maintaining an outdoor lifestyle in increasing the risk of *T. gondii* infection in dogs. These identified risk factors collectively suggest that both ingesting oocysts, as observed through cat cohabitation and coprophagy, and engaging in predatory behaviors, as possible for outdoor living dogs, are likely sources of *T. gondii* infection in this host species.

Keywords: *Toxoplasma gondii*, Serology, Dog, Risk factors.

1. Background

Toxoplasma gondii is a worldwide Apicomplexan protozoan that infects virtually all the warm-blooded species including humans, livestock, birds, and pets [1]. It has been estimated that approximately one third of the world population is infected with *T. gondii*, with prevalences varying greatly depending on the geographical area [2,3]. Domestic and wild felids are definitive host, harbouring in their small intestine the sexual stages of the parasite, which resolves in the release of environmentally resistant oocysts. In all the other hosts, after the infection, asexual reproduction occurs, lasting in bradyzoites cysts formation in several tissues. However, *T. gondii* can also undergo asexual reproduction in felids, that can therefore act also as intermediate host [4].

In many animal species, infection is typically subclinical, although toxoplasmosis can be lethal in several host, including pets. *Toxoplasma* infection in dogs is often associated with low morbidity and mortality rates; indeed, infrequently is observed a primary clinical toxoplasmosis in dogs, which is instead usually linked to former immunosuppression [5]. The clinical aspects of canine toxoplasmosis range from nonspecific symptoms such as fever, lymphadenopathy, dyspnoea and

gastrointestinal signs, to neurological syndromes characterized by epilepsy, cranial nerve deficits, tremors, ataxia, paresis, and paralysis. Other clinical features described are noise sensitivity, myositis, ocular diseases, and cutaneous signs associated with immunosuppressant therapies [5, 6-10]

Seropositivity for *Toxoplasma* in dogs is not only an aid in the differential diagnosis of clinical cases, but has also epidemiological significance, reflecting the circulation of the parasite in the environment [1]. The presence of antibodies merely indicates previous contact with the parasite, either by ingestion of bradyzoite cysts from meat or oocysts from the environment. The contact with oocysts may have other consequences besides infection of the dog. Indeed, it has been shown that dogs can act as mechanical transporters of *T. gondii* oocysts. They can excrete infective oocyst after ingestion of infected cat faeces, suggesting that coprophagy, with a subsequent intestinal passage by dogs, plays a role in the dissemination of *T. gondii*. [11]. Additionally, dogs can vehicle oocysts on the fur after rolling over cat stool [12,13]. As a result, mechanical transmission of *T. gondii* oocysts to humans can occur from dogs via their body surface, mouth, and feet [1].

Toxoplasma gondii infection has a cosmopolitan distribution, and seroprevalence in dogs depends on geographical region, living environment, and lifestyle of the dog. In general, according to the data reported in literature, the risk of infection with *T. gondii* increases throughout life, due to an increasing cumulative risk of exposure, and the seroprevalence is higher in rural than in urban areas [1,14,15]. In addition, it has been observed in several studies that dogs living outdoors have a higher risk of infection than indoors dogs [16-20].

As in humans, dogs can become infected with *T. gondii* through a variety of sources, including ingestion of water containing sporulated oocysts, ingestion of raw or inadequately cooked meat containing cysts with bradyzoites, or transplacental infection [4]. Depending on the living environment of the dog, seropositivity may have different epidemiological implications. On the one hand, dogs living in anthropogenic areas have been shown to mirror seropositivity in humans, probably due to similar exposure to contaminated water and the environment [21]. On the other hand,

74 stray or hunting dogs, whose *Toxoplasma* exposure is also related to the consumption of small wild
75 preys, may be an indicator of the spread of the parasite in a wild area [16,22].

76 The aim of the present work is to evaluate the risk factors for *T. gondii* infection in dogs with
77 different uses in an area of Italy where *Toxoplasma* infection have already been detected in these
78 species [23], but without analysing the risk factors, and where positivity findings for the parasite have
79 been detected in hunted wild animals [23-24].

80

81 **2. Methods**

82 The study was based on convenience sampling involving the use of sera from 120 dogs collected for
83 other research/diagnostic purpose from 20 municipalities in three provinces (Bologna, Rimini and
84 Pesaro-Urbino) in 2018-2019. Blood sampling was carried out by venipuncture. Sera were obtained
85 by centrifugation for 10 min at 2000 rpm and stored at -20 °C until use. Inclusion criteria to enrolment
86 included: regular outdoor access; no treatment for internal worms (included *Dirofilaria immitis*
87 prophylaxis) in the month before the study, six months of age or older, and signed informed consent
88 of the owner. A questionnaire was submitted to the owners in order to obtain information about age,
89 gender, main use or function, housing (hosted or not in house during the night), lifestyle (living
90 exclusively outdoor or hosted in house/boxes when not in activity), cohabitation with cats and
91 coprophagy habits.

92 *T. gondii* indirect fluorescent antibody test (IFAT) for IgG (MegaFLUO TOXOPLASMA g,
93 MegaCor Diagnostik, Hoerbranz, Austria) was performed on serum samples, following the
94 manufacturer's instructions. Briefly, slides coated with *T. gondii* infected cells were probed with
95 20µL of serum diluted in phosphate-buffered saline (PBS) with a starting dilution of 1:40. Slides were
96 incubated for 30 min at 37 °C and washed two times with PBS. Internal canine positive and negative
97 sera controls were included on each slide. The slides were therefore probed with 20µL of fluorescein

isothiocyanate (FITC) conjugated anti-dog IgG antibody diluted in PBS at a concentration of 1:32 (Anti-Dog IgG-FITC antibody, Sigma-Aldrich, Saint Louis, MO) and incubated for 30 min at 37 °C. After two further washing steps with PBS, they were examined under a fluorescent microscope. The highest dilution showing fluorescence was the final antibody titre. Serum samples with antibody titre ≥1:40 were assessed positive, as 1:40 is the cut-off adopted for diagnostic purpose in different diagnostic facilities in the same area [23] (Dini et al., 2023a).

Statistical analysis was conducted using STATA 12.1. Prior to the analysis, the age of the dogs was grouped into three categories: ≤3 years, >3 years and ≤7 years, >7 years in order to obtain a uniform distribution of dogs in three age groups. The relationship between the prevalence of toxoplasmosis and various dog-related factors (such as age categories, gender, function, housing, lifestyle, cohabitation with cats, and coprophagy habits) was assessed using multivariable logistic regression. This approach allowed us to estimate the odds-ratio while holding all other factors in the model constant; odds-ratio is a common approximation of the relative risk in cross-sectional surveys, indicating the likelihood of testing positive for toxoplasmosis in relation to each factor. To evaluate the relationship between antibody titres and the same dog-related factors considered for the prevalence analysis, multivariable ordered logistic regression was employed. Before this analysis, positive titres were log-transformed as $\log_2(\text{titre}/10)$ to create a more manageable scale for calculations. The transformation did not alter the significance of the model, but it facilitated result interpretation. The dependent variable of the model, the transformed titre, represents an ordinal scale reflecting an underlying continuous measure, i.e., the concentration of antibodies. By using this model, we were able to estimate the odds ratio for each tested factor, considering the influence of each factor on increasing or decreasing antibody titres while keeping all other factors in the model constant.

3. Results

In table 1, the distribution of the dog examined in the different risk categories and serological results were described. The dogs were uniformly distributed in gender and age categories. Concerning their function, hunting dogs made up the largest group (51.67%). All the dogs had regular outdoor access, as inclusion criteria, and most of them (71.67%) were not hosted in house during the night. Nevertheless, only a small part of dogs lived exclusively outdoors (19.17%). The main function of the dogs influenced the housing and the lifestyle: all the hunting dogs (100%) were hosted outside (in kennel boxes) during the night, significantly differing from the other categories (Chi-square test: $p<0.01$). In fact, most pet dogs (96.55%), and some truffle dogs (20%) and watchdogs (22.22%) were housed inside the owners' homes during the night. On the other hand, considering lifestyle, hunting dog lived in kennel when not actively engaged in hunting activities, but only 12.73% of them had only outdoor lifestyle (not differing significantly from pets: 3.44%). The predominant lifestyle for watchdogs was to remain outdoor (88.89%), differing from the other categories (Chi-square test: $p<0.05$).

Approximately 39.17% of the dogs included in the study were reported to cohabit with cats, irrespective of their function. Interestingly, the habit of coprophagy, i.e., consuming faeces, was primarily observed in truffle dogs, with 65% of them exhibiting this behaviour. Companion dogs ranked second, with 50% of them having records of coprophagy according to the owner report.

Concerning the serological analysis, 35 out of 120 sera samples examined tested positive for *T. gondii* antibodies, resulting in a seroprevalence of 29.17% (95% CI = 21.09%-37.25%).

The logistic regression results (Table 2) indicate that the seroprevalence, representing the probability of having been infected, is significantly higher in dogs cohabiting with cats, exhibiting coprophagy habits, and living constantly outdoors. This finding is consistent with the results from the ordered logistic regression (Table 3), where the antibody titers were significantly higher in dogs living with cats, having coprophagy habits, and constant outdoor living. Notably, the lifestyle factor showed the highest odds-ratios in the study. Dogs living outdoors constantly were found to be at approximately 5 times greater risk of testing positive and having higher antibody titers compared to

dogs living both indoors and outdoors. In positive subjects, there appears to be a tendency for the antibody titers to increase with the age cathegory; however, the differences observed are not statistically significant (Graph 1).

4. Discussion

In the current investigation, a comprehensive spectrum of factors encompassing age categories, gender, function, housing arrangements, lifestyle, cohabitation with cats, and coprophagy habits has been systematically scrutinized to discern and assess the risk factors intrinsically associated with *T. gondii* infection in dogs.

Recent data available about *T. gondii* seroprevalence in dogs in different countries of the world are quite divergent, even in the context of the same country: in Brazil it varies from 7.95% to 48.8% [25,26], in China prevalences range from 4.4% to 40.3% [27,28]. In the same Asian continent, the 7.9% of owened dogs of Bangok (Thailand) were seen to be *T. gondii* positive in 2021 [20]. Regarding Europe, the prevalences reported (from Spain and Poland) are about 30% [16,29], showing a similarity with our results. The seroprevalence observed in the present study, quantified at 29.17%, aligns harmoniously with seroprevalence data recently elucidated in Northern Italy by Dini et al. [23] pertaining to owned dogs, albeit bereft of concurrent risk factor analysis. It is noteworthy that the seroprevalence figures available in Italy are marked by notable variability. In the Campania Region, a survey involving a canine cohort of 398 hunting dogs unveiled a prevalence of 24% [30], in accordance with our findings. In contradistinction, findings presented by Macrì et al. [31] in Rome, encompassing both public kennel occupants and privately-owned dogs, disclosed a prevalence of 64%. The conspicuous divergence in these infection indexes is attributed, in part, to the utilization of disparate cut-off titres for seropositivity determination—1:50 and 1:20, respectively, for the aforementioned studies. This variance in cut-off titres unquestionably imparts a substantial influence on the ascertained prevalence figures. The overarching challenge arising from these dissimilarities is

the absence of standardized serological techniques and universally accepted initial cut-offs for diagnosing dog toxoplasmosis. Information available in scientific literature shows that the cut-off values employed for serological diagnosis of *T. gondii* in dogs using IFAT vary between 1:16 and 1:64 [1]. The absence of a standardized approach compromises the comparability of epidemiological data across studies, thereby precluding a comprehensive analysis of the actual epidemiological landscape prevalent within a given region.

The outcomes of the logistic regression analysis offer notable insights into the factors associated with *T. gondii* infection in the canine population under study.

Firstly, it is noteworthy that the seroprevalence, exhibited a noticeable increase in dogs cohabiting with cats. This observation aligns with the findings of the ordered logistic regression analysis, where higher antibody titres were consistently observed in dogs sharing a living environment with cats. This correspondence across both regression analyses reinforces the notion that feline cohabitation serves as a significant predictor of heightened *T. gondii* infection risk. Following the excretion of the parasite in the feces of infected felids, *T. gondii* oocysts have the potential to contaminate soil [32]. Given the restricted spectrum of definitive host species for *T. gondii*, limited exclusively to felids, the distribution of oocysts within the soil does not occur randomly. Instead, there is a discernible propensity for oocysts to aggregate in proximity to or within sites of cat defecation [33,34]. These factors imply that living alongside cats increases the probability of being exposed to an environment contaminated with *Toxoplasma* oocysts, consequently increasing the potential for infection in the dogs that share the living space with felids.

Secondly, the coprophagy habits exhibited a similar pattern of association. Dogs displaying this behaviour showed an increased likelihood of seropositivity, as substantiated by their increased antibody titres. The inclination to coprophagy, predominantly observed in this study among truffle dogs, followed by pet dogs, seems to be less prevalent among hunting dogs based on the data analysis. However, the unique housing conditions associated with this dog category might lead to an underestimation of this variable, as these animals frequently remain out of the owner's direct

observation, potentially resulting in a lack of documentation for this behaviour. Coprophagy is a common behaviour among dogs. Dogs may consume their own faeces, faeces of other dogs and/or faeces of other species [35], including cats. Given that cats can shed millions of oocysts through their faeces during the course of sexual reproduction of *T. gondii* [4], the consistent habit of coprophagy, where dogs consume feline stool, places them at a significantly heightened risk of infection through oocysts.

Thirdly, the consistent outdoor residency of dogs emerged as a particularly prominent risk factor. It is noteworthy that this is true regardless of the dog's function.

Actually, it might be expected that hunting dogs, that can more easily engage in predatory behaviour and are more likely exposed to game meat, would have been at higher risk of infection [36] Our results, thanks to multivariable analysis that evaluates different covariates avoiding possible confounding effects among them, do not support this assumption disentangling the importance of function and lifestyle as risk factors.

The consistency of the association between toxoplasmosis and "living outdoor" underscores the significance of the outdoor environment as a risk factor; it implies that the dog is subjected to prolonged exposure to potential sources of infection, including environmental oocysts, feline faeces and potentially infected small mammals or avian prey, independently to their function.

5. Conclusions

In essence, both the logistic and the ordered logistic regression findings substantiates the pivotal role of cohabitation with cats, coprophagy behaviours, and perpetual outdoor habitation in amplifying the risk of *T. gondii* infection among dogs. This comprehensive understanding of the interplay between these factors and infection likelihood contributes to the broader comprehension of the epidemiological landscape and underscores the necessity for targeted preventive strategies, particularly for dogs exhibiting these risk-associated behaviours and conditions. Furthermore, the

results of our study indicate that gender, age category, and function do not have a significant influence on toxoplasmosis seroprevalence. Instead, the findings suggest that habits play a more substantial role as risk factors for this zoonotic agent, compared to the individual's function or receptivity.

Authors' contributions

Filippo Maria Dini and Roberta Galuppi wrote the main manuscript text, Filippo Maria Dini, Roberta Galuppi and Giovanni Poglayen performed sampling. Filippo Maria Dini carried out the serological analyses. Laura Stancampianoo performed the statistical analysis. Filippo Maria Dini, Laura Stancampiano and Roberta Galuppi revised the manuscript. All authors reviewed the manuscript and approved the final manuscript.

Funding

This study received no external funding

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Availability of data and materials

All data are included in this published article.

247

248 **References**:

249 1. Dubey JP, Murata FHA, Cerqueira-Cézar CK, Kwok OCH, Yang Y, Su C. *Toxoplasma*
250 *gondii* infections in dogs: 2009-2020. Vet Parasitol. 2020; 31;287:109223. doi:
251 10.1016/j.vetpar.2020.109223. Epub ahead of print. PMID: 33160144.
252 2. Montoya JG, Liesenfeld O. Toxoplasmosis. Lancet. 2004;363:1965-76. doi:
253 10.1016/S0140-6736(04)16412-X. PMID: 15194258.
254 3. Dubey JP, Lago EG, Gennari SM, Su C, Jones JL. Toxoplasmosis in humans and animals
255 in Brazil: high prevalence, high burden of disease, and epidemiology. Parasitol.
256 2012;139:1375–424.
257 4. Dubey JP. Toxoplasmosis of Animals and Humans, third edition, CRC Press, Boca Raton,
258 London New York. 2022.
259 5. Calero-Bernal R, Gennari SM. Clinical Toxoplasmosis in Dogs and Cats: An Update.
260 Front Vet Sci. 2019;26;6:54. doi: 10.3389/fvets.2019.00054. PMID: 30863754; PMCID:
261 PMC6399377.
262 6. Wolfer J, Grahn B. Diagnostic ophtalmology. case report of anterior uveitis and
263 endophtalmitis. Can Vet J. 1996; 37:506–7.
264 7. Patitucci AN, Alley MR, Jones BR, Charleston WAG. Protozoal encephalomyelitis of
265 dogs involving *Neospora caninum* and *Toxoplasma gondii* in New Zealand. N Z Vet J.
266 1997;45:231–5. doi: 10.1080/00480169.1997.36035
267 8. Papini R, Mancianti F, Saccardi E. Noise sensitivity in a dog with toxoplasmosis. Vet Rec.
268 2009;165:62. doi: 10.1136/vetrec.165.2.62-b
269 9. Swinger RL, Schmidt KA Jr , Dubielzig RR. Keratoconjunctivitis associated with
270 *Toxoplasma gondii* in a dog. Vet Ophthalmol. 2009;12:56–60. doi: 10.1111/j.1463-
271 5224.2009.00675.x 12.

10. Migliore S, La Marca S, Stabile C, Di Marco Lo Presti V, Vitale M. A rare case of acute toxoplasmosis in a stray dog due to infection of *T. gondii* clonal type I: public health concern in urban settings with stray animals? BMC Vet Res. 2017; 13:249. doi: 10.1186/s12917-017-1176-3

11. Schares G, Pantchev N, Barutzki D, Heydorn AO, Bauer C, Conraths FJ. Oocysts of *Neospora caninum*, *Hammondia heydorni*, *Toxoplasma gondii* and *Hammondia hammondi* in faeces collected from dogs in Germany. Int J Parasitol. 2005;35:1525-37. doi: 10.1016/j.ijpara.2005.08.008. Epub 2005 Sep 15. PMID: 16197949.

12. Lindsay DS, Dubey JP, Butler JM, Blagburn BL. Mechanical transmission of *Toxoplasma gondii* oocysts by dogs. Vet Parasitol. 1997;73:27-33. doi: 10.1016/s0304-4017(97)00048-4. PMID: 9477489.

13. Frenkel JK, Lindsay DS, Parker BB, Dobesh M. Dogs as possible mechanical carriers of *Toxoplasma*, and their fur as a source of infection of young children. Int J Infect Dis. 2003;7:292-3. doi: 10.1016/s1201-9712(03)90112-3. PMID: 14656424.

14. Cañón-Franco WA, Bergamaschi DP, Labruna MB, Camargo LM, Silva JC, Pinter A et al. Occurrence of anti-*Toxoplasma gondii* antibodies in dogs in the urban area of Monte Negro, Rondônia, Brazil. Vet Res Commun. 2004;28:113-8. doi:10.1023/b:verc.0000012114.71235.73. PMID: 14992241

15. Azevedo SS, Batista CS, Vasconcellos SA, Aguiar DM, Ragozo AM, Rodrigues AA, Alves CJ, Gennari SM. Seroepidemiology of *Toxoplasma gondii* and *Neospora caninum* in dogs from the state of Paraíba, Northeast region of Brazil. Res Vet Sci. 2005; 79:51-6. doi: 10.1016/j.rvsc.2004.10.001. Epub 2004 Dec 15. PMID: 15894024.

16. Cano-Terriza D, Puig-Ribas M, Jiménez-Ruiz S, Cabezón Ó, Almería S, Galán-Relaño Á et al. Risk factors of *Toxoplasma gondii* infection in hunting, pet and watchdogs from southern Spain and northern Africa. Parasitol Int. 2016;65:363-366. doi:

17. Ding H, Gao YM, Deng Y, Lamberton PHL and Lu DB A systematic review and meta-analysis of the seroprevalence of *Toxoplasma gondii* in cats in mainland China. Parasit Vectors. 2017;10: 2–9. doi: 10.1186/ s13071-017-1970-6

18. 18. Wang S, Zhou YH, Xie Q, Zheng B, Zhang HZ, Yao ZJ, et al. Prevalence of *Toxoplasma gondii* antibodies in dogs in central China. Isr J Vet Med. 2017;2:11–15.

19. Zarra-Nezhad F, Borujeni MP, Mosallanejad B, Hamidinejat H. A seroepidemiological survey of *Toxoplasma gondii* infection in referred dogs to Veterinary Hospital of Ahvaz, Iran. Int J Vet Sci Med. 2017;5(2):148-151. doi: 10.1016/j.ijvsm.2017.08.006. PMID: 30255064; PMCID: PMC6137845.

20. Huertas-López A, Sukhumavasi W, Álvarez-García G, Martínez-Subiela S, Cano-Terriza D, Almería S, Dubey JP, García-Bocanegra I, Cerón JJ, Martínez-Carrasco C. Seroprevalence of *Toxoplasma gondii* in outdoor dogs and cats in Bangkok, Thailand. Parasitology. 2021;148:843-849. doi: 10.1017/S0031182021000421. Epub 2021 Mar 10. PMID: 33750492.

21. Mortari APG, Tagarra LG, de Souza ML, Roman IJ, Ratzlaff FR, Braunig P, de Andrade CM, Cargnelutti JF, Sangioni LA, Vogel FSF. Increased seroprevalence of anti-*Toxoplasma gondii* antibodies in dogs in southern Brazil after an outbreak of human toxoplasmosis. Parasitol Res. 2023;122:1009-1014. doi: 10.1007/s00436-023-07808-4. Epub 2023 Mar 7. PMID: 36881160.

22. Cabezón O, Millán J, Gomis M, Dubey JP, Ferroglio E, Almería S. Kennel dogs as sentinels of *Leishmania infantum*, *Toxoplasma gondii*, and *Neospora caninum* in Majorca Island, Spain. Parasitol Res. 2010;107:1505-8. doi: 10.1007/s00436-010-2015-7. Epub 2010 Aug 6. PMID: 20689966.

23. Dini FM, Morselli S, Marangoni A, Taddei R, Maioli G, Roncarati G, Balboni A, Dondi F, Lunetta F, Galuppi R. Spread of *Toxoplasma gondii* among animals and humans in

Northern Italy: A retrospective analysis in a One-Health framework. Food Waterborne Parasitol. 2023a;8;32:e00197. doi: 10.1016/j.fawpar.2023.e00197. PMID: 37333686; PMCID: PMC10273278.

24. Dini FM, Graziosi G, Lupini C, Catelli E, Galuppi R. Migratory Wild Birds as Potential Long-Distance Transmitters of *Toxoplasma gondii* Infection. Pathogens. 2023;18;12(3):478. doi: 10.3390/pathogens12030478. PMID: 36986400; PMCID: PMC10051421.

25. Da Cunha GR; Pellizzaro M; Martins CM; Rocha SM; Yamakawa AC; Da Silva EC et al. Spatial serosurvey of anti-*Toxoplasma gondii* antibodies in individuals with animal hoarding disorder and their dogs in Southern Brazil. PLoS ONE 2020, 15, e0233305.

26. Magalhães FJ; Ribeiro-Andrade M; Souza FM; Filho CDL; Biondo AW; Vidotto O et al. Seroprevalence and spatial distribution of *Toxoplasma gondii* infection in cats, dogs, pigs and equines of the Fernando de Noronha Island, Brazil. Parasitol. Int. 2017, 66, 43–46.

27. Zhu N, Yang L, Xin S, Huang W, Jiang Y, Yang Y. Low Prevalence of *Toxoplasma gondii* in Dogs From Central China. Front Cell Infect Microbiol. 2022 Apr 28;12:885348. doi: 10.3389/fcimb.2022.885348. PMID: 35573782; PMCID: PMC9097580.

28. Yan C, Fu LL, Yue CL, Tang RX, Liu YS, Lv L et al. Stray dogs as indicators of *Toxoplasma gondii* distributed in the environment: the first report across an urban-rural gradient in China. Parasit Vectors. 2012 5:5. doi: 10.1186/1756-3305-5-5. PMID: 22217112; PMCID: PMC3271967.

29. Turlewicz-Podbielska H, Ruszkowski JJ, Gogulski M, Pomorska-Mól M. Seroprevalence of *Toxoplasma gondii* in domestic cats, dogs and rabbits from Poland. Vet Res Commun. 2023 doi: 10.1007/s11259-022-10055-0. Epub ahead of print. PMID: 36645566.

30. Machacova T, Bartova E, Sedlak K, Slezakova R, Budikova M, Piantedosi D, et al. Seroprevalence and risk factors of infections with *Neospora caninum* and *Toxoplasma*

gondii in hunting dogs from Campania region, southern Italy. Folia Parasitol. 2016;6;63:2016.012. doi: 10.14411/fp.2016.012. PMID: 27189127.

31. Macrì G, Sala M, Linder AM, Pettirossi N, Scarpulla M. Comparison of indirect fluorescent antibody test and modified agglutination test for detecting *Toxoplasma gondii* immunoglobulin G antibodies in dog and cat. Parasitol Res. 2009;105:35-40. doi: 10.1007/s00436-009-1358-4. Epub 2009 Feb 17. PMID: 19221795

32. Gilot-Fromont E, Lélu M, Dardé ML, Richomme C, Aubert D, Afonso E, et al. The life cycle of *Toxoplasma gondii* in the natural environment. In: Djaković, O.D. (Ed.), Toxoplasmosis. IntechOpen, London, UK. 2012.

33. Shapiro K, Bahia-Oliveira L, Dixon B, Dumètre A, de Wit LA, VanWormer E, et al. Environmental transmission of *Toxoplasma gondii*: Oocysts in water, soil and food. Food Waterborne Parasitol. 2019;15:e00049. doi: 10.1016/j.fawpar.2019.e00049. PMID: 32095620; PMCID: PMC7033973.

34. Afonso E, Thulliez P, Pontier D, Gilot-Fromont E. Toxoplasmosis in prey species and consequences for prevalence in feral cats: not all prey species are equal. Parasitology. 2007;134:1963-1971. doi:10.1017/S0031182007003320

35. Nijsse R, Mughini-Gras L, Wagenaar JA, Ploeger HW. Coprophagy in dogs interferes in the diagnosis of parasitic infections by faecal examination. Vet Parasitol. 2014;204:304-9. doi: 10.1016/j.vetpar.2014.05.019. Epub 2014 May 20. PMID: 24880647.

36. Mendoza Roldan JA, Otranto D. Zoonotic parasites associated with predation by dogs and cats. Parasit Vectors. 2023;16:55. doi: 10.1186/s13071-023-05670-y. PMID: 36747243; PMCID: PMC9901148

.

	Category	n. dog tested	Relative distribution %	n. positive at IFAT	Seroprevalence %	95% CI
Gender	Male	67	58.83	22	32.84	21.09-44.59
	Female	53	44.17	13	24.53	11.2-37.86
Age groups	6 m – 3y	37	30.83	8	21.62	8.38-34.86
	>3 – 7 years	45	37.50	16	35.55	21.6-49.5
	> 7 years	38	31.67	11	28.95	14.56-43.34
Use	Pet dog	29	24.17	6	20.69	5.97-35.41
	Watchdog	9	7.50	4	44.44	11.99-76.89
	Hunting dog	62	51.67	19	30.64	19.2-42.08
	Truffle dog	20	16.68	6	30.00	9.4-50.6
Housing	House	34	28.33	8	23.53	9,3-37.76
	Outside	86	71.67	27	31.39	21.62-41.16
Lifestyle	Indoor/outdoor	97	80.83	22	22.68	14.39-30.97
	Outdoor	23	19.17	13	56.52	35.97-76.47
Cohabitation with cats	No	73	60.83	18	24.66	14.81-34.51
	Yes	47	39.17	17	36.17	22.46-49.88
Coprophagy	No	87	72.50	16	18.39	10.28-26.5
	Yes	33	27.50	19	57.57	40.73-74.41

371 Table 1 - Descriptive statistics and serological test results. CI= confidence interval.

372

373

374

375

376

377

		O.R.	95% C.I.	p-value
Gender	Male	ref		
	Female	0.457	0.178 -1.177	0.105
Age group	6 m – 3years	ref		
	>3 – 7 years	1.975	0.650-6.003	0.230
	> 7 years	1.603	0.492-5.220	0.433
Use	Pet dog	ref		
	Watchdog	0.630	0.077-5.133	0.666
	Hunting dog	1.958	0.608-5.133	0.260
	Truffle dog	0.570	0.110-2.941	0.502
Lifestyle	Indoor/outdoor	ref		
	outdoor	5.289	1.319-21.209	0.019
Cohabitation with cats	No	ref		
	Yes	2.783	1.058-9.645	0.038
Coprophagy	No	ref		
	Yes	3.250	1.095-9.645	0.034

378 Table 2 Result of the logistic regression model having seropositivity as dependent variable. The term
379 ref refers to the reference category of the covariates. O.R= odds-ratio; C.I.=confidence interval.

380

381

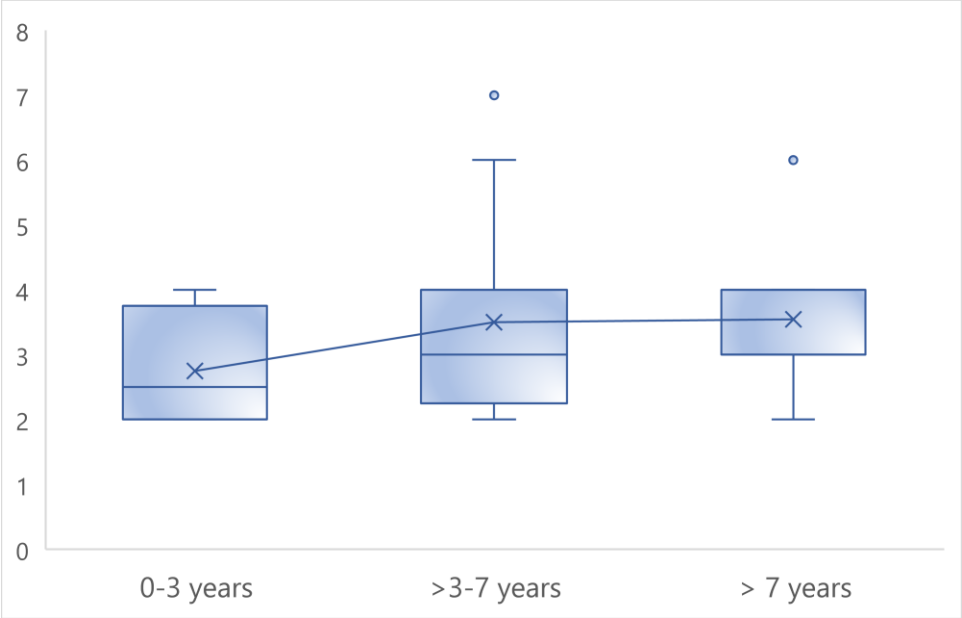
382

383

Category		O.R.	95% C.I.	p-value
Gender	male	ref		
	female	0.430	0.173-1.070	0.070
Age group	6 m – 3years	ref		
	>3 – 7 years	2.515	0.837-7.556	0.100
	> 7 years	1.960	0.616-6.240	0.255
Use	Pet dog	ref		
	Watchdog	0.629	0.092-4.308	0.637
	Hunting dog	1.976	0.605-6.454	0.259
	Truffle dog	0.486	0.010-2.368	0.372
Lifestyle	Inside/outside	ref		
	Outside	5.370	1.607-17.945	0.006
Cohabitation with cat	No	ref		
	Yes	3.068	1.224-7.694	0.017
Coprophagy	No	ref		
	Yes	4.051	1.443-11.370	0.008

Table 3: result of Ordered logistic regression model having the log-transformed titre as dependent variable. The term ref refers to the reference category of the covariates. O.R= odds-ratio; C.I.=confidence interval.

388 Figure 2:



389

390

391 Caption Fig.2: Box and whisker plot showing age-dependent kinetics of IgG Anti-*Toxoplasma*
392 antibodies in positive subjects across different age groups: log-transformed antibody titres
393 ($\log_2$(titre/10)) were analyzed in three age categories: 0-3 years, 3-7 years, and >7 years. The × is the
394 arithmetic mean. While an observable trend of increasing antibody titres was observed across the
395 considered age ranges, statistical analysis revealed that this upward trend did not reach significance.

396

397

Chapter 5.2

Seropositivity to *Toxoplasma gondii* as a potential risk factor for anxiety in companion dog (*Canis lupus familiaris*)

Dini FM, Marliani G, Amadei E, Tosco S, Cavallini D, Accorsi PA, Galuppi R. (2023). Seropositivity to *Toxoplasma gondii* as a potential risk factor for anxiety in companion dog (*Canis lupus familiaris*). Submitted to Applied Animal Behaviour Science

Seropositivity to *Toxoplasma gondii* as a potential Risk Factor for Anxiety in Companion Dogs (*Canis lupus familiaris*)

Authors

F. M. Dini[*], G. Marliani [1*], E. Amadei [2], S. Tosco [1], D. Cavallini [1], P. A. Accorsi [1], R. Galuppi [1]

[1] *Department of Veterinary Medical Sciences, University of Bologna, Via Tolara di sopra, 50, 40064, Ozzano dell' Emilia (BO), Italy*

[2]*Indipendent researcher*

[*] These authors contributed equally to this work

*Corresponding author: Tel: + 39 051 2097056

E-mail address: filippomaria.dini@unibo.it

Abstract

Toxoplasma gondii is a widespread apicomplexan protozoan parasite that can infect a variety of warm-blooded species, exploiting numerous transmission pathways, including the ingestion of oocysts or tissue cysts. Recent research has revealed that neural localization of bradyzoite cysts in intermediate hosts potentially can lead to behavioural modifications. This double-blinded study explores the correlation between serological evidence of exposure to *T. gondii* and anxiety disorders in companion dogs. Furthermore, we evaluated the risk related to the consumption of raw meat and exposure to *T. gondii*. A veterinary surgeon, expert in animal behavior, used positive and negative activation (PANAS) scale and the Lincoln Canine Anxiety Scale to classify 124 dogs as affected or unaffected by anxiety disorders. During the interview, the veterinarian asked information about the consumption or not of raw meat. Additionally, *T. gondii* indirect fluorescent antibody test (IFAT) for IgG was performed on serum samples of each subject. The results indicated that dogs regularly consuming raw meat were more likely to test positive for *T. gondii* antibodies, with a significant ($p=0.05$) risk of 2.60. Furthermore, through a ROC curve analysis, the population was divided based on dog size, with small-sized dogs (weighing less than 15 kg) and large/medium-sized dogs (weighing over 15 kg). The study found that smaller dogs exhibited a 2.34 times higher risk ($p=0.01$) of developing anxiety compared to large/medium sized dogs, regardless of *T. gondii* exposure. Conversiley, larger dogs were more likely (3.41 times; $p =0.07$) to develop anxiety when testing positive for *T. gondii*. These findings suggest a potential link between *T. gondii* exposure and the development of anxiety disorders in dogs, with the consumption of raw meat acting as a contributing risk factor. However, it is essential to recognize that the onset of anxiety is influenced by multiple factors.

Key-Words: Serology, chronic toxplasmosis, dogs, behaviour

1. Introduction

Toxoplasma gondii, a highly successful apicomplexan protozoan, exhibits remarkable worldwide presence, infecting warm-blooded species, often at elevated prevalence levels. Felidae members, stand as the sole definitive hosts, enabling the parasite to complete full gametogenesis within the small intestine, ultimately yielding oocysts housing sporozoites that are shed in feline feces (Attias et al., 2020). Intermediate hosts can contract the infection by ingesting oocysts (via contaminated soil, water, or food) or tissue cysts (via consumption of raw or undercooked infected meat, predation or cannibalism), and in some cases, congenital transmission has been documented (Tenter et al., 2000). Inside intermediate hosts, the parasite embarks on asexual reproduction, characterized by rapidly dividing tachyzoites and the more slowly dividing bradyzoites. Bradyzoites encyst in various tissues such as the brain and heart, where they can persist for the host's lifetime (Dubey, 2022). Transmission to the feline definitive host occurs when an immunologically naive cat consumes an infected intermediate host through predation or the ingestion of contaminated meat. Given that sexual reproduction of *T. gondii* can only transpire in felines, there are likely potent selective pressures driving the parasite to evolve mechanisms that enhance transmission from intermediate hosts to the definitive feline host (Webster et al., 2013).

Notably, until relatively recently, latent adult-acquired toxoplasmosis in immunocompetent humans and animals was generally believed to be devoid of symptoms (Montoya and Liesenfeld 2004). In stark contrast, recent decades have witnessed several studies shedding light on the potential neural localization of bradyzoite cysts, leading to behavioral modifications (Webster et al., 2013). Extensive investigations conducted under various experimental settings have suggested that *T. gondii* alters the behavior of rodents in a manner that increases their susceptibility to predation by cats, the parasite's definitive host (Webster, 2007). These aforementioned studies have, for instance, revealed that *T. gondii* triggers heightened activity, diminished neophobia (innate fear of novelty), and reduced predator vigilance behaviors (Webster, 1994, 2001, 2007; Webster et al., 1994; Berdoy et al., 1995;

Lamberton et al., 2008). Each of these alterations can be postulated to facilitate the transmission of the parasite from the infected intermediate host to the feline definitive host. Furthermore, *T. gondii* infection in rodent hosts appears to subtly and specifically modify their cognitive perception of the risk of cat predation, effectively transforming their inherent aversion to the scent of cats into a 'suicidal' attraction to fatal feline encounters (Berdoy et al., 2000; Webster et al., 2006; Vyas et al., 2007; Webster and McConkey, 2010, Kaushik et al., 2014).

Analogous side effects, which can be nonadaptive or residual manipulative (Flegr et al., 2011), encompass a spectrum of behavioral consequences in *T. gondii*-infected humans, ranging from subtle shifts in personality traits to more severe outcomes such as an elevated susceptibility to schizophrenia (Flegr 2013a, 2013b), and association with an higher risk to develop other several neuropsychiatric disorders (Milne et al., 2020)

This association has been investigated also in other animal species during natural infection, especially in wildlife. Recent studies have brought attention to an intriguing phenomenon among wild red foxes, known as Dopey Fox Syndrome (DFS), characterized by unusual behavioral traits. Notably, the research findings have illuminated the pivotal role of *T. gondii* in this syndrome. It was found that DFS is associated with singular *T. gondii* infection, singular FoxCV infection, and the co-infection of *T. gondii* and FoxCV. Importantly, these behavioral changes in *T. gondii*-infected foxes, such as their heightened attraction to feline odor, mirror alterations observed in infected rodents (Milne et al., 2020). Another study, conducted on spotted hyenas, revealed that wild hyena cubs infected with *T. gondii* display a bold and risky behavior when interacting with lions. Notably, these infected cubs have a higher likelihood of experiencing lion-induced mortality compared to their uninfected counterparts (Gering et al., 2021). Recent research by Mayer et al. (2020) has demonstrated that the overlap of wolf territories with regions characterized by a high cougar population density serves as a significant predictor of infection in wolves. Furthermore, wolves that tested positive for the infection were found to be more inclined to make high-risk decisions, such as dispersing and assuming

leadership roles within packs. These decisions are crucial for individual fitness and overall wolf population dynamics. Considering the hierarchical structure within wolf packs, these findings suggest that the behavioural consequences of toxoplasmosis may contribute to a feedback loop, ultimately amplifying spatial overlap and disease transmission between wolves and cougars.

Considered the distinct influence of *T. gondii* infection observed in wild carnivores, our study delves into the potential correlation between serological evidence of exposure to the parasite and the manifestation of anxiety disorders in companion dogs. Anxiety itself represents an adaptive response to real or potential threats, essential for the survival of the animal. It is normal for a dog to react with fear or aggression to unfamiliar stimuli as a defense mechanism (Talegón and Delgado, 2011). The problem arises when these reactions become inappropriate in relation to the context (Steimer, 2002). Excessive levels of anxiety, therefore, can become maladaptive, transforming into a pathological condition that hinders an individual's ability to adapt due to an exaggerated perception of a threat that is not proportionate to reality (Ohl et al., 2008). Disorders related to anxiety is one of the most prevalent behavioural disorders (Ohl et al., 2008). The aetiology of this disorder is not fully understood; it is believed that various factors are involved, including neuroanatomical and receptor alterations, genetic factors, social factors, and life experiences (Newman et al., 2016). Crucial for the onset of anxiety are the neural circuits responsible for processing threatening stimuli. In fact, alterations in the neuroanatomy or receptors of these structures can lead to dysfunctional behaviours, including states of anxiety disorders (Overall, 1997), and numerous studies conducted on various animal species, including humans, have shown that these changes could also be induced by *T. gondii* (Hinze-Selch, 2015; El Saftawy et al., 2021). In this regard, it has been noted that the presence of the parasite inside amygdala neurons, which in the meantime plays an essential role in modulating anxiety, was correlated with an increase in inflammatory biomarkers and anti-apoptotic factors, which can lead to anatomical and physiological changes in the brain (El Saftawy et al., 2021). Therefore,

117 our prediction was to find a higher risk to develop anxiety disorders in dogs whose serological results
118 evidence a previous exposure to *T. gondii*.

119 In addition, we have considered the potential risk associated with the consumption of raw
120 meat concerning exposure to *T. gondii.* In recent years, the use of raw meat-based products or
121 complete raw meat diets has increased. However, it is essential to bear in mind that consuming raw
122 meat can pose contamination risks, and among various pathogens, raw meat may contain toxoplasma
123 (Schlesinger et al., 2011; Parr e Remillard, 2014; LeJeune and Hancock, 2001). Given the lack of
124 epidemiological studies exploring the relationship between the consumption of raw meat and the
125 exposure to *T. gondii*, we have chosen to investigate this aspect as well, with the prediction that the
126 consumption of raw meat could indeed lead to a higher risk of exposure to the parasite.

127

128 **2. Materials and Methods**

129 One hundred and twenty-four adult dogs (above 2 years of age) referred to a veterinary clinic were
130 randomly selected to participate in the study. The dogs had a mean age of 8.0 ± 4.5 years and a mean
131 weight of 15.88 ± 12.25 kg. Among these, there were 61 intact and 17 neutered males, and 63 intact
132 and 35 spayed females.

133 During routine medical check-ups, blood samples were collected from the dogs for various clinical
134 purposes, and, after a centrifugation of 7 minutes at 2500 rpm, an aliquot of serum was stored for our
135 study at -20 °C until the analysis of Indirect fluorescent antibody test (IFAT) for the diagnosis of *T.*
136 *gondii* infection. The blood sample was obtained through venipuncture, using butterfly and vacuum
137 tubes (Vacutest Kima). After the blood sampling, each dog underwent a behavioral assessment, to
138 identify subjects affected by anxiety disorders, and, in the same meeting, the owner was asked if
139 her/his dog ate raw meat.

140 2.1.***Behavioral examination***

To classify dogs as anxious/phobic or not, the owners were interviewed by a veterinary surgeon expert in animal behavior. The veterinarian employed two behavioral assessment scales as guide and tools to classify the dogs: the PANAS scales (positive and negative activation scale) and the Lincoln Canine Anxiety Scale. The Lincoln Canine Anxiety Scale is a validated scale used to rate a dog's anxiety concerning a specific trigger (e.g., thunder), and it is based on the observation, in specific contexts, of signs and behaviors related to anxiety, such as restlessness, drooling, freezing, tremors, hiding, crouching and/or panting. On the other hand, the PANAS scale, in its part related to negative activation, investigates fearfulness as a temperament trait of the subject and analyzes the level of sensitivity to reward and adverse/negative experiences (Sheppard and Mills, 2002; Mills et al., 2020). We classified as anxiety/anxiety disorders: generalized anxiety disorder and anxiety states related to specific stimuli (i.e., people, noises, places, animals, and objects).

The study was conducted in double-blind fashion, where the veterinarian who performed the behavioral assessment and sample collection remained unaware of the IFAT results until the study's conclusion. Conversely, the researchers conducting the laboratory analyses were not informed whether the dog was anxious or not, or if it consumed raw meat.

2.2 Indirect fluorescent antibody test (IFAT)

Toxoplasma gondii indirect fluorescent antibody test (IFAT) for IgG (MegaFLUO TOXOPLASMA g, MegaCor Diagnostik, Hoerbranz, Austria) was performed on serum samples, following the manufacturer's instructions. Briefly, slides coated with *T. gondii* infected cells were probed with 20µL of serum diluted in phosphate-buffered saline (PBS) with a starting dilution of 1:40. Slides were incubated for 30 min at 37 °C and washed two times with PBS. Internal canine positive and negative sera controls were included on each slide. The slides were therefore probed with 20µL of fluorescein isothiocyanate (FITC) conjugated anti-dog IgG antibody diluted in PBS at a concentration of 1:32 (Anti-Dog IgG-FITC antibody, Sigma-Aldrich, Saint Louis, MO) and incubated for 30 min at 37 °C. After two further washing steps with PBS, they were examined under a fluorescent microscope. The

166 highest dilution showing fluorescence was the final antibody titre. Serum samples with antibody titre
167 ≥1:40 were assessed positive, as 1:40 is the cut-off adopted for diagnostic purpose in different
168 diagnostic facilities in the same area (Dini et al., 2023a, Dini et al., 2023b).

169 ***2.3 Statistical analysis***

170 All data were analyzed using statistical software (JMP 17, SAS). A Receiver Operating Characteristic
171 (ROC) analysis was implemented to divide dogs according to size in small-breed and medium/large-
172 breed dogs (<15 kg and >15 kg). Furthermore, several nominal logistic models were implemented to
173 study the relationship between anxiety and raw meat, toxoplasmosis positivity, age, and breed. The
174 same procedure was implemented in previous studies (Masebo et al., 2023; Spadari et al., 2023).

175 **3 Results**

176 **3.1** ***Correlation between Anxiety and T. gondii infection***

177 The study population was divided into two groups based on dog size: those weighing less than 15 kg,
178 classified as small-breed dogs, and those weighing more than 15 kg, categorized as medium/large-
179 breed dogs. This division was determined using a ROC curve that assessed the relationship between
180 weight and predisposition to anxiety, with a sensitivity of 0.69 and specificity of 0.51.

181 The group of small-breed dogs consisted of 78 subjects, comprising 46 (59%) anxious subjects and
182 32 (41%) non-anxious dogs. Among these, 15 tested positive for *T. gondii* in the IFAT test, while 63
183 tested negative. Among the positive subjects, 8 exhibited anxiety states, while among the negatives,
184 there were 38 anxious individuals. Conversely, the group of medium/large breed dogs included 46
185 subjects, consisting of 17 (37%) dogs affected by anxiety states and 29 (63%) non-anxious dogs.
186 Among them, 14 tested positive for *T. gondii*, and 32 tested negatives. In this case, among the subjects
187 suffering from anxiety disorders, 8 had antibodies for the parasite, while 9 tested negative (see Table
188 1 and Figure 1).

First of all, the predisposition of dogs to develop anxiety in relation to their size was assessed. According to the results of the statistical analysis (as shown in Table 2), dogs weighing less than 15 kg exhibited a 2.3-fold higher risk of experiencing anxiety (p-value=0.01) compared to medium/large-breed dogs, regardless of their *Toxoplasma gondii* seropositivity.

Subsequently, always considering dogs based on their weight, the likelihood of developing anxiety in the presence of *T. gondii* seropositivity was examined. The findings indicated that small-breed dogs did not display an additional risk of being classified as anxious when testing positive for *T. gondii* IgG. On the contrary, dogs weighing more than 15 kg had a 3.4-fold risk, with a significant trend (p-value = 0.07), of developing anxiety when tested positive for *T. gondii*, as presented in Table 3. Among large/medium breed dogs weighing, it was found that 8 (57.1%) out of 14 seropositive subjects were also classified as anxious, while among the seronegative subjects, only 28.1% were affected by anxiety (9 dogs out of 32), while 71.9% did not exhibit any disorder. In small-breed dogs, the situation was reversed, as 60.3% of them tested negative for *T. gondii* was affected by anxiety (38 dogs out of 63), and, on the other hand, 53.3% (8 out of 15) of seropositive small-breed dogs are affected by anxiety disorders (Figure 1).

3.2 T. gondii infection related to raw meat intake

Considering the entire study population composed of 124 animals, 29 individuals were positive for IFAT for *T. gondii*, 74 dogs ate regularly raw meat, and 63 subjects were classified as anxious.

The possible correlation between the consumption of raw meat and the risk of contracting *T. gondii* was assessed. As previously mentioned, 74 dogs regularly consumed raw meat; among them, 22 tested positive for IFAT for *T. gondii*. In contrast, out of the remaining 50 dogs that were not fed raw meat, only 7 individuals had antibodies against the parasite. Therefore, when considering the 29 positive cases, 75.9% of them regularly consumed raw meat, while only 24.1% did not. On the other hand, among subjects that tested negative for *T. gondii* at IFAT (comprising 95 dogs), there was a smaller

difference between those who consumed raw meat (54.7%, n=52 dogs) and those who did not (45.3%, n=43 dogs) (see Fig. 2). According to the statistical analysis, dogs regularly fed raw meat showed a 2.6 times higher risk (p-value = 0.05) of contracting *T. gondii* (Table 4).

4 Discussion

Toxoplasma gondii, a worldwide intracellular parasite, infects warm-blooded animals, including mammals and certain birds, through various transmission routes (Tenter et al., 2000; Dubey, 2022). In immunocompetent individuals, the initial infection triggers an immune response that effectively suppresses tachyzoite replication, resulting in a persistent chronic infection characterized by tissue cysts housing bradyzoites. These cysts are distributed in various tissues, including the nervous system and striated muscles (Tenter et al., 2000). Patients with chronic toxoplasmosis can exhibit behavioural alterations, thereby demonstrating the potential role played by *T. gondii* in the pathogenesis of these disorders (Hinze-Selch, 2015). This study aims to investigate the relationship between exposure to *T. gondii* and the development of anxiety disorders in dogs. For this research, we employed indirect immunofluorescence, which allows the evaluation of an animal's exposure to *T. gondii* through IgG detection but does not represent an index of active infection (Molaei et al., 2022).

First and foremost, we explored the relationship between the habitual consumption of raw meat and the risk of develop an antibody titer for *T. gondii*. Dogs that regularly consume raw meat showed a 2.6 times higher risk (p-value = 0.05) of testing positive at IFAT. The consumption o raw or undercoocked meet has been widely recognized as an important risk factor for the infection in several carnivorous species, including humans (Tenter et al., 2000; Opsteegh et al., 2011; Bellucco et al., 2017). In the scientific literature, studies on the actual role of meat consumption in dogs as a risk factor are lacking, as the majority of epidemiological studies on seroprevalence in dogs do not include

237 the dog's diet as a variable, but rather focus on lifestyle. A recent study conducted in Italy has brought
238 attention to significant infection risks for dogs, such as living with cats, the habit of coprophagy, and
239 constant outdoor living. These risks demonstrate that, beyond the transmission route through oocysts,
240 factors like predatory behavior, which can be more actively pursued in outdoor settings, also play a
241 role (Dini et al., 2023b, under review).

242 Several studies have examined how behaviour may vary depending on a dog's size, indicating that
243 behaviour and the onset of behavioural disorders can be influenced by various factors such as training
244 techniques, owner interactions, and genetic differences between small and large-sized dogs (Arhant
245 et al., 2010; Zapata et al., 2022). To address a potential confounding factor, we divided our study
246 population based on weight in relation to their predisposition to anxiety, employing a ROC curve for
247 this purpose. The curve determined a weight threshold of 15 kg, leading to the categorization of dogs
248 as small-sized (weighing under 15 kg) or medium/large-sized (weighing over 15 kg). When
249 examining the likelihood of dogs developing anxiety disorders based on their size, regardless of *T.*
250 *gondii* exposure, it was found that small-sized dogs have a 2.34 higher risk ($p=0.01$) of developing
251 anxiety disorders compared to medium/large-sized dogs. This observation aligns with reports that
252 owners of small-sized dogs tend to be less consistent in training and interactions with their pets, and
253 they often promote insufficient socialization with other animals and humans, possibly because
254 behavioural problems in small-sized dogs are perceived as a less serious problem compared to their
255 larger conspecifics. Additionally, small-sized dogs may receive excessive protection, preventing them
256 from expressing their natural behavioural patterns (McGreevy et al., 2013). Moreover, genetic
257 differences between dog breeds should be considered. Smaller body size has been shown to
258 correspond with smaller brain regions, leading to reduced inhibition of subcortical areas, resulting in
259 heightened responsiveness to certain stimuli. These factors can contribute to the onset of behavioural
260 disorders, including anxiety, frustration, and increased aggression (Hecht et al., 2021). Therefore,
261 could have been a potential confounding factor. We proceeded to analyse the risk of anxiety in dogs

that had antibodies for *T. gondii*, dividing the subjects based on their weight. No significant difference was observed in the onset of anxiety related to toxoplasmosis among dogs weighing less than 15 kg. This can be explained by what was previously mentioned regarding the higher predisposition of small-sized dogs to develop behavioural problems, as anxiety. Conversely, dogs weighing over 15 kg showed a 3.41 higher risk, with a significant tendency ($p=0.07$), of experiencing anxiety in relation to anti-*T. gondii* IgG positivity. The results obtained are consistent with what has been reported in the literature concerning the association between *T. gondii* and behavioural alterations, examined in various animal species, including human-beings (Hinze-Selch, 2015; Adekunle and Lateef, 2022). Several studies have concluded that the persistence of the parasite, in the form of bradyzoites within tissue cysts, in the nervous system leads to structural and physiological changes in specific brain regions, as well as alterations in the metabolism of certain neurotransmitters responsible for behaviour modulation (Gatkowska et al., 2013; Evans et al., 2014; Parlog et al., 2014). In particular, it has been noted that the brain structure most involved in the pathogenesis of chronic toxoplasmosis is the amygdala, which, in the same time, plays a fundamental role in modulating responses to certain stimuli, especially negative stimuli that trigger fear and in disorders related to anxiety (El Saftawy et al., 2021). Indeed, the amygdala is the limbic structure that represents the most crucial part of the anxiety-fear circuit. It can be considered a repository of emotion-related memories and plays an essential role in activating the anxiety-fear circuit in response to negative stimuli (Davidson, 2002; Etkin, 2010; Duvarci and Pare, 2014). Alterations in the amygdala and hippocampus have often been observed in anxiety disorders (Overall, 1997).

In conclusion, the findings from this study do not definitively exclude the possible contribution of *T. gondii* in the development of anxiety disorders, partially supporting our initial predictions. However, it should always be considered that the probability of anxiety disorders onset is influenced by a myriad of factors, including not only neuroanatomical and receptor variations, but also genetic elements, social influences, and life experiences (Newman et al., 2016). Moreover, our research underlines that

287 the consumption of raw meat can be a contributing risk factor in dogs' exposure to *T. gondii*, and this
288 should be taken into serious consideration. The decision to opt for a raw meat-based diet or to use
289 raw-meat as threat necessitates a thorough and comprehensive evaluation, taking into account all
290 potential associated risks. Further investigations, such the direct confirmation of the presence of the
291 parasite in neural tissues of these animals, could be useful to provide additional insights into this
292 matter and deepened our results.

References

Adekunle, R., Lateef, A. 2022. Understanding the Effects of Toxoplasmosis on Host Behavior, Personality, and Cognition. In Towards New Perspectives on *Toxoplasma gondii*. In Towards New Perspectives on *Toxoplasma gondi*. IntechOpen

Arhant, C., Bubna-Littitz, H., Bartels, A., Futschik, A., Troxler, J. 2010. Behaviour of smaller and larger dogs: effects of training methods, inconsistency of owner behaviour and level of engagement in activities with the dog. Appl. Anim. Behav. Sci. 123(3-4), 131-142.

Attias, M., Teixeira, D. E., Benchimol, M., Vommaro, R. C., Crepaldi, P. H., & De Souza, W. 2020. The life-cycle of *Toxoplasma gondii* reviewed using animations. Parasit. Vectors 13(1), 588. https://doi.org/10.1186/s13071-020-04445-z

Belluco, S., Simonato, G., Mancin, M., Pietrobelli, M., Ricci, A. 2017. *Toxoplasma gondii* infection and food consumption: a systematic review and meta-analysis of case-controlled studies. Crit. Rev. Food Sci. 1–12

Berdoy, M., Webster, J.P., Macdonald, D.W. 1995: Parasite altered behaviour: is the effect of *Toxoplasma gondii* on *Rattus norvegicus* specific? Parasitology 111: 403–409

Berdoy, M., Webster, J.P., Macdonald, D.W. 2000. Fatal attraction in *Toxoplasma*-infected rats: a case of parasite manipulation of its mammalian host. Proc R Soc B. 2000;267:1591–1594

Davidson, R. J. 2002. Anxiety and affective style: role of prefrontal cortex and amygdala. Biol. Psychiatry. 51, 68-80.

Dini, F. M., Morselli, S., Marangoni, A., Taddei, R., Maioli, G., Roncarati, G., Balboni, A., Dondi, F., Lunetta, F., & Galuppi, R. 2023. Spread of *Toxoplasma gondii* among animals and humans in Northern Italy: A retrospective analysis in a One-Health framework. Food Waterborne Parasitol. 32, e00197. https://doi-org.ezproxy.unibo.it/10.1016/j.fawpar.2023.e00197

316 Dini, F.M., Stancampiano, L., Poglayen, G., Galuppi, R. 2023b. Risk factor for *Toxoplasma gondii*
317 infection in dogs: a serological survey. Under review in Acta Veterinaria Scandinavica.

318 Dubey, J.P. 2022 Toxoplasmosis of Animals and Humans, third edition, CRC Press, Boca Raton,
319 London New York.

320 Duvarci, S., Pare, D. 2014. Amygdala microcircuits controlling learned fear. Neuron, 82, 966-980.

321 El Saftawy, E. A., Shash, R. Y., Aboulhoda, B. E., Arsanyos, S. F., Albadawi, E. A., Abou-Fandoud,
322 S. M., Amin, N. M. 2021. Insights into immunopathology and triggering of apoptosis in chronic
323 cerebral toxoplasmosis using murine models. Trop. Biomed. 38(2), 53-62.

324 Etkin, A. 2010. Functional neuroanatomy of anxiety: a neural circuit perspective Curr Top Behav
325 Neurosci. 2010;2:251-77. doi: 10.1007/7854_2009_5. PMID: 21309113.

326 Evans, A. K., Strassmann, P. S., Lee, I. P., Sapolsky, R. M. 2014. Patterns of *Toxoplasma gondii* cyst
327 distribution in the forebrain associate with individual variation in predator odor avoidance and
328 anxiety-related behavior in male Long-Evans rats. Brain Behav. Immun.37, 122-133.

329 Flegr, J. 2013a. Influence of latent *Toxoplasma* infection on human personality, physiology and
330 morphology: Pros and cons of the *Toxoplasma*–human model in studying the manipulation
331 hypothesis. J. Exp. Biol. 216, 127–133

332 Flegr, J. 2013b. How and why *Toxoplasma* makes us crazy. Trends Parasitol. 29(4), 156–163.
333 https://doi.org/10.1016/j.pt.2013.01.007

334 Flegr, J., Lenochová, P., Hodný, Z., & Vondrová, M. 2011. Fatal attraction phenomenon in humans:
335 cat odour attractiveness increased for *Toxoplasma*-infected men while decreased for infected
336 women. PLOS Negl. Trop. Dis. *5*(11), e1389. https://doi.org/10.1371/journal.pntd.0001389

337 Gatkowska, J., Wieczorek, M., Dziadek, B., Dzitko, K., Dlugonska, H. 2013. Sex-dependent
338 neurotransmitter level changes in brains of Toxoplasma gondii infected mice. Exp. Parasitol.
339 133(1), 1-7.

340 Gering, E., Laubach, Z. M., Weber, P. S. D., Soboll Hussey, G., Lehmann, K. D. S., Montgomery, T.
341 M., Turner, J. W., Perng, W., Pioon, M. O., Holekamp, K. E., & Getty, T. (2021). *Toxoplasma*
342 *gondii* infections are associated with costly boldness toward felids in a wild host. Nat. Commun.
343 12(1), 3842. https://doi.org/10.1038/s41467-021-24092-x

344 Hecht, E. E., Zapata, I., Alvarez, C. E., Gutman, D. A., Preuss, T. M., Kent, M., Serpell, J. A. 2021.
345 Neurodevelopmental scaling is a major driver of brain–behavior differences in temperament
346 across dog breeds. Brain Struct Funct. 226, 2725-2739.

347 Hinze-Selch, D. 2015. *Toxoplasma gondii* infection and neuropsychiatric disease: current insight.
348 Rep. Parasitology, 4, 43-51.

349 Kaushik, M., Knowles, S. C., & Webster, J. P. 2014. What makes a feline fatal in *Toxoplasma gondii*'s
350 fatal feline attraction? Infected rats choose wild cats. Integr. Comp. Biol. 54(2), 118–128.
351 https://doi.org/10.1093/icb/icu060

352 Lamberton, P.H.L., Donnelly, C.A., Webster, J.P. 2008. Specificity of the *Toxoplasma gondii*-altered
353 behaviour to definitive versus non-definitive host predation risk. Parasitology 135: 1143–1150.

354 LeJeune, J.T., Hancock, D.D. 2001. Public health concerns associated with feeding raw meat diets
355 to dogs. J. Am. Vet. Med. Assoc., 219, 1222–1225.

356 McGreevy, P. D., Georgevsky, D., Carrasco, J., Valenzuela, M., Duffy, D. L., Serpell, J. A. 2013. Dog
357 behavior co-varies with height, bodyweight and skull shape. PloS One, 8(12), e80529.

358 Meyer, C. J., Cassidy, K. A., Stahler, E. E., Brandell, E. E., Anton, C. B., Stahler, D. R., & Smith, D.
359 W. 2022. Parasitic infection increases risk-taking in a social, intermediate host carnivore.
360 Commun. Biol. 5(1), 1180. https://doi.org/10.1038/s42003-022-04122-0

361 Milne, G., Fujimoto, C., Bean, T., Peters, H. J., Hemmington, M., Taylor, C., Fowkes, R. C.,
362 Martineau, H. M., Hamilton, C. M., Walker, M., Mitchell, J. A., Léger, E., Priestnall, S. L., &
363 Webster, J. P. 2020b. Infectious Causation of Abnormal Host Behavior: *Toxoplasma gondii* and

364 Its Potential Association With Dopey Fox Syndrome. Front. Psychiatry 11, 513536.
365 https://doi.org/10.3389/fpsyt.2020.513536

366 Milne, G., Webster, J.P., Walker, M. 2020. *Toxoplasma gondii*: An Underestimated Threat? Trends
367 Parasitol. Vol. 36, No. 12 https://doi.org/10.1016/j.pt.2020.08.005

368 Molaei, S., Dadkhah, M., Fathi, F. 2022. Toxoplasmosis diagnostic techniques: Current developed
369 methods and biosensors. Talanta. 252, 123828. https://doi-
370 org.ezproxy.unibo.it/10.1016/j.talanta.2022.123828

371 Montoya, J. and Liesenfeld, O. 2004. Toxoplasmosis. Lancet, 1965–1976

372 Newman, M. G., Shin, K., & Zuellig, A. 2016. Developmental risk factors in generalized anxiety
373 disorder and panic disorder. J. Affect. Disord. 94-102.

374 Ohl, F., Arndt, S. S., van der Staay, F. J. 2008. Pathological anxiety in animals. Vet. J. 175, 18-26.

375 Opsteegh, M., Prickaerts, S., Frankena, K., Evers, E.G. 2011a. A quantitative microbial risk
376 assessment for meatborne *Toxoplasma gondii* infection in The Netherlands. Int. J. Food
377 Microbiol. 150, 103–114.

378 Overall, K. L. 1997. Clinical behavioral medicine for small animals. Maryland Heights, Missouri:
379 Mosby-Year Book, Inc.

380 Parlog, A., Harsan, L. A., Zagrebelsky, M., Weller, M., von Elverfeldt, D., Marwin, C., Dunay, I. R.
381 2014. Chronic murine toxoplasmosis is defined by subtle changes in neuronal connectivity.
382 Disease models & mechanisms, 7(4), 459-469.

383 Parr, J.M. and Remillard, R.L. 2014. Handling alternative dietary requests from pet owners. Vet. Clin.
384 North Am. Small Anim. Pract., 44, 667–688.

385 Schlesinger, D.P. and Joffe, D.J. 2011. Raw food diets in companion animals: A critical review. Can.
386 Vet. J., 52, 50–54.

387 Spadari, A., Gialletti, R., Gandini, M., Valle, E., Cerullo, A., Cavallini, D., Bertoletti, A., Rinnovati,
388 R., Forni, G., Scilimati, N., & Giusto, G. 2023. Short-Term Survival and Postoperative
389 Complications Rates in Horses Undergoing Colic Surgery: A Multicentre Study. Animals,
390 13(6), 1107. https://doi.org/10.3390/ani13061107

391 Steimer, T. 2002. The biology of fear-and-anxiety-related behaviors. Dialogues Clin. Neurosci. 4,
392 231-249.

393 Talegón, M. I., Delgado, B. A. 2011. Anxiety Disorders in Dogs. In Anxiety Disorders (p. 261-280).
394 BoD-Books on Demand.

395 Tenter, A. M., Heckeroth, A., Weiss, L. 2000. Toxoplasma gondii: from animals to humans. Int. J.
396 Parasitol. 30, 1217-1258.

397 Vyas, A., Seon-Kyeong, K., Giacomini, N., Boothroyd, J.C., Sapolsky, R.M. 2007. Behavioural
398 changes induced by *Toxoplasma* infection of rodents are highly specific to aversion of cat
399 odours. Proc. Natl. Acad. Sci. U. S. A. 104: 6442–6447.

400 Webster, J. P. 2007. The effect of *Toxoplasma gondii* on animal behavior: playing cat and mouse.
401 Schizophr. Bull. 33(3), 752–756. https://doi.org/10.1093/schbul/sbl073

402 Webster, J. P., Kaushik, M., Bristow, G. C., & McConkey, G. A. 2013. *Toxoplasma gondii* infection,
403 from predation to schizophrenia: can animal behaviour help us understand human behaviour?.
404 J. Exp. Biol.216(Pt 1), 99–112. https://doi.org/10.1242/jeb.074716

405 Webster, J.P. 1994: The effect of *Toxoplasma gondii* and other parasites on activity levels in wild and
406 hybrid *Rattus norvegicus*. Parasitology 109: 583–589.

407 Webster, J.P. 2001: Rats, cats, people and parasites: the impact of latent toxoplasmosis on behaviour.
408 Microbes Infect. 3: 1–9.

409 Webster, J.P., Brunton, C.F.A., Macdonald, D.W. 1994. Effect of *Toxoplasma gondii* on neophobic
410 behaviour in wild brown rats, Rattus norvegicus. Parasitology. 109:37–43.

411 Zapata, I., Eyre, A. W., Alvarez, C. E., Serpell, J. A. 2022. Latent class analysis of behavior across
412 dog breeds reveal underlying temperament profiles. Scientific Reports, 12(1), 15627.

413

414

415

Table 1. The total number of small and medium/large breed dogs classified as anxious or non-anxious, and tested positive or negative for T. gondii to the IFAT.

	Anxiety	No Anxiety	Positive for *T. gondii*	Negative for *T. gondii*
Small breed (n=78)	46	32	15	63
Medium/large breed (n=46)	17	29	14	32

Table 2. Predisposition to anxiety in adult dogs weighing less than 15 kg (Small-breed dogs). Lower and Upper represent the limits of the 95% confidence interval (C.I.).

Level 1	Level 2	Odd Ratio	p-value	Lower	Upper
Small-breed	Large/Medium-breed	2.34	0.01	1.18	4.61
Large/Medium-breed	Small-breed	0.43	0.01	0.22	0.84

Table 3. Statistical risk analysis of being affected by anxiety in *T. gondii* seropositive (Pos) and seronegative (Neg) dogs of both small-breed and Large/medium-breed dogs. Lower and Upper represent the limits of the 95% confidence interval (C.I.).

	Level 1	Level 2	Odd Ratio	p-value	Lower	Upper
Large/Medium-breed dogs	Pos	Neg	3.41	0.07	0.92	12.62
	Neg	Pos	0.29	0.07	0.08	1.09
Small-breed dogs	Pos	Neg	0.75	0.62	0.24	2.33
	Neg	Pos	1.33	0.62	0.43	4.13

426 **Table 4**. Statistical risk analysis for *Toxoplasma gondii* infection in relation to raw meat
427 consumption. Lower and Upper are the limits of the 95% confidence interval (C.I.); Yes =
428 consumption of raw meat; No = not eating raw meat.
429

Level 1	Level 2	Odds Ratio	p-value	Lower	Upper
Yes	No	2.60	0.05	1.01	6.66
No	Yes	0.38	0.05	0.15	0.99

430

Figures

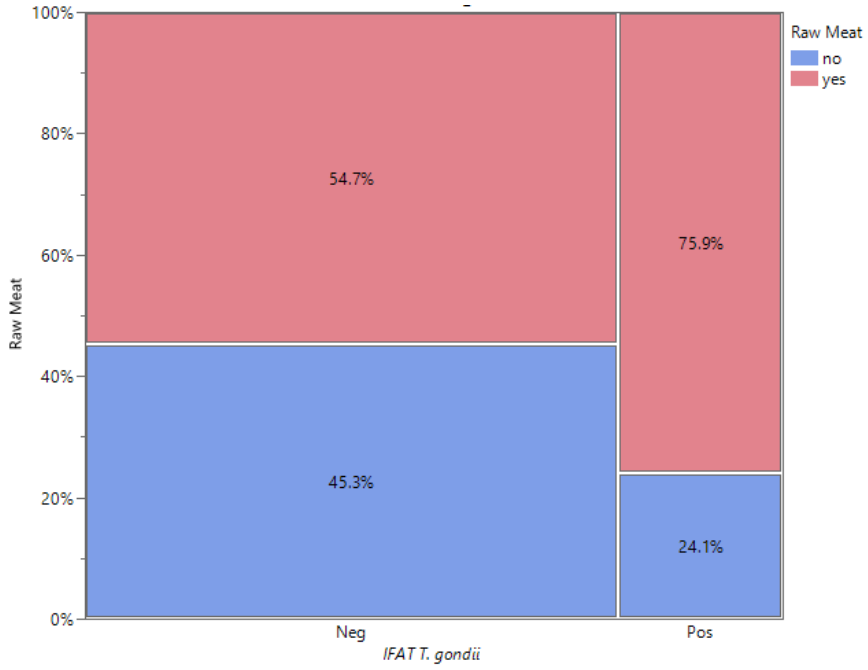


Figure 1. Percentage of dogs suffering or not from anxiety subdivided according to the results of IFAT for *T. gondii* (neg=negative; pos=positive) and divided considering their size.

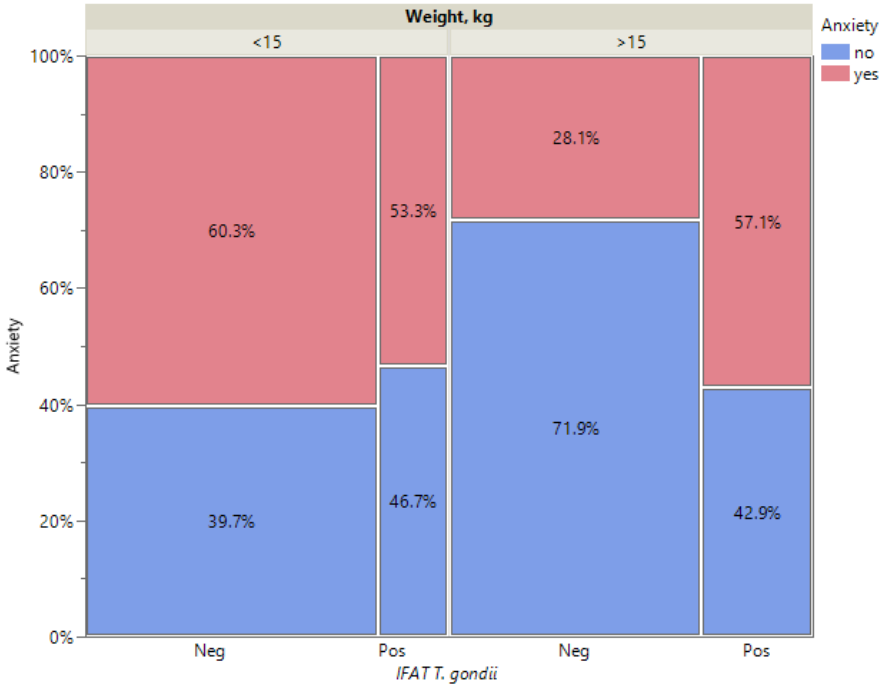


Figure 2. Percentage of negative (neg) and positive (pos) animals for *T. gondii* to the IFAT in relation to consumption or not of raw meat.

Chapter 6.1

Observational longitudinal study on *Toxoplasma gondii* infection in fattening beef cattle: serology and associated hematological findings

Dini FM, Jacinto JGP, Cavallini D, Beltrame A, Del Re FS, Abram L, Gentile A, Galuppi R. (2023). Observational longitudinal study on *Toxoplasma gondii* infection in fattening beef cattle: serology and associated hematological findings. *Parasitology Research* (under revision).

Original Paper

Observational longitudinal study on *Toxoplasma gondii* infection in fattening beef cattle: serology and associated haematological findings

Filippo M. Dini *[a1], Joana G.P. Jacinto [a1], Damiano Cavallini [a], Andrea Beltrame [b], Flavia S. Del Re [a], Laura Abram [a], Arcangelo Gentile [a], Roberta Galuppi [a]

[1] Both authors contributed equally to this work.

*Corresponding author at: Department of Veterinary Medical Sciences, Alma Mater Studiorum University of Bologna, Via Tolara di Sopra 50, 40064 Ozzano Emilia, BO, Italy. Email address: filippomaria.dini@unibo.it; telephone number: +39 051 2097056

Affiliations:

[a] *Department of Veterinary Medical Sciences, University of Bologna,* 40064 Ozzano Emilia, BO, Italy

[b] Bovine practitioner, DVM, Verona, Italy

ORCIDs:

F.M. Dini: https://orcid.org/0000-0001-7378-8650

J.G.P. Jacinto: https://orcid.org/0000-0002-6438-7975

D. Cavallini: https://orcid.org/0000-0002-1642-6722

A. Gentile: https://orcid.org/0000-0002-6091-8978

R. Galuppi: https://orcid.org/0000-0001-6959-8731

Abstract

Toxoplasmosis, caused by the protozoan parasite *Toxoplasma gondii*, is a globally distributed zoonotic infection with significant implications for human and animal health. This study investigated the prevalence of *T. gondii* infection in a population of beef cattle at three different stages of their productive lifespan and examined the impact of *T. gondii* serological status on blood parameters. A commercial beef fattening unit in Italy was the setting for this research, which involved a biosecurity assessment upon cattle arrival, blood sampling at three time points, and *Toxoplasma*-specific serological testing using indirect fluorescent antibody tests (IFAT). Results revealed a dynamic pattern of *T. gondii* seropositivity in cattle, with an initial prevalence of 30.6% at arrival (T0) that increased to 44.6% at 14 days (T1) and then decreased slightly to 39.3% at slaughter (T2). Interestingly, seroconversion was observed during the study, indicating ongoing infections, and antibody waning occurred in some animals. In terms of blood parameters, seropositive cattle exhibited significantly lower mean corpuscular volume (MCV) and a higher neutrophil-lymphocyte (N/L) ratio, suggesting an activation of the innate immune response. Furthermore, cattle with higher antibody titers displayed higher neutrophil counts. This study provides for the first time a longitudinal investigation on the serological status for *T. gondii* in naturally exposed beef cattle.. These findings provide valuable insights into the clinico-pathological aspects of natural *T. gondii* exposure in cattle and underscore the importance of monitoring and managing *T. gondii* infection in livestock production systems.

Keywords: antibody, bovine, *T. gondii*, IFAT, haematology, neutrophil

1. Introduction

Toxoplasmosis is a worldwide zoonotic protozoan infection caused by the Apicomplexa *Toxoplasma gondii*. Although the disease is usually asymptomatic in the most susceptible species, it can be life-threatening in immunocompromised individuals and can result in abortion or birth of an affected child if a primarily infected woman transmits the parasite to the foetus (Robert-Gangneux and Dardé, 2012; Havelaar et al., 2015). In Felidae definitive hosts, after a primary infection, sexual replication occurs in small intestine, resulting in the elimination of millions of environmentally resistant oocysts (Dabritz and Conrad, 2010). Virtually all warm-blooded species, including humans, can act as intermediate hosts. They will develop bradyzoite tissue cysts, after asexual replication, particularly in muscle and nervous tissues (Dubey, 2020). Herbivorous intermediate hosts are of particular importance for the epidemiology of the parasite. By acquiring the infection primarily through the environmental route, they are an indicator of environmental contamination with oocysts and, in the case of livestock species, a source of infection to humans through the consumption of raw/undercooked meat (Tenter et al., 2000, Shapiro et al., 2019). Cattle play a singular role in this parasitosis, with particular host-parasite dynamics and an unclear role in meat-borne transmission. Infection of cattle with *T. gondii* most likely occurs through ingestion of sporulated oocysts spread in pastures and other sources of feed and water (Stelzer et al., 2019). Exposure to the parasite is strongly influenced by livestock husbandry, farm and dietary management. Risk factors for infection identified in this species include extensive farming systems, the presence of cats and, drinking water sources (Gilot-Fromont et al., 2009; Magalhães et al., 2016), leading to seroprevalence rates in Europe ranging from 7.8 up to 83.3% (Klun et al. 2006; Gilot-Fromont et al. 2009; Berger-Schoch et al. 2011; Garcia-Bocanegra et al., 2013; Jokelainen et al. 2017; Blaga et al. 2019, Gazzonis et al., 2020). There is evidence of the importance of beef consumption in human infection, also in the context of outbreaks (Smith, 1993; Baril et al., 1999; Cook et al., 2000; Belluco et al., 2017). Indeed, based on quantitative risk assessment, beef was predicted to be the main source of meat-borne infections in the Netherlands

and Italy (Opsteegh et al., 2011a, Bellucco et al., 2018). Unfortunately, in contrast to other species, serological data on *Toxoplasma* exposure in cattle are of limited use for consumer protection, as no concordance has been shown between the detection of antibodies and the presence of viable tissue cysts (Opsteegh et al., 2011b, 2019). The observed phenomenon can be attributed to the hypothesis that cattle possess the ability to eliminate the parasite, resulting in the development of protective antibody titres, once the parasite has been cleared from their tissues (Opsteegh et al., 2011b). Additionally, available seroprevalence data in the literature suggest that cattle experience a moderate level of exposure to the parasite without any clinical evidence of infection, with only a limited number of congenital transmission cases documented, unlike in small ruminants that often present abortion and symptomatic congenital infection (Canada et al., 2002; Costa et al., 2010; Stelzer et al., 2019). The resistance mechanism observed in cattle infection is thought to be associated with the lethal impact of neutrophil extracellular traps (NETs) on tachyzoites, which merely have an immobilizing effect in sheep (Yildiz et al., 2017). Nevertheless, the specific pathological effects and dynamics of antibody production during natural *Toxoplasma* infections in cattle remain poorly understood so far.

The aims of this study were to investigate the seroprevalence of *T. gondii* infection at three different stages of the animals' productive lifespan in a population of beef cattle and to analyse the impact of *T. gondii* serological status on blood parameters.

2. Materials and Methods

2.1 Housing and Management

The research was conducted within a commercial fattening facility that housed Limousine bulls imported from France. This facility was situated in the province of Modena, in the Po Valley region of Italy, and the study period extended from November 2021 to May 2022. The housing system was a semi-closed barn with 44 pens configured in a free stall system. Each pen had the maximum capacity to stock 6 animals. The pens were arranged in close proximity, separated by iron bars to facilitate

91 interaction among animals in adjacent pens. The floor was slatted, with a pit beneath for manure
92 collection. Prior to introducing the animals, the pens underwent thorough cleaning and disinfection,
93 employing a pressure washer.

94 A total of 264 animals were delivered to the fattening unit facility in weekly shipments organized in
95 numerically diverse groups, spanning six consecutive weeks. These animals originated from various
96 farms across France, encompassing different regions within the country. The majority of these bulls
97 were primarily raised either on pastures or in indoor free stall systems with straw bedding.

98 Before their arrival in Italy, the bulls spent one day in a selection center in France, where they
99 underwent assessments related to their health status, age, and body weight. This selection process
100 aimed to create homogeneous groups of animals. Upon their entry into the fattening unit, the bulls
101 were approximately 11 months old and had an average weight of 400 kg.No quarantine period was
102 performed. At the arrival, animals were fed an adaptation diet in order to reduce dietary stressors
103 (Supplementary Table S1).

104 The production cycle lasted between 5 to 6 months. During this period, 14 bulls were euthanized due
105 to respiratory disease and 250 bulls finished the cycle and were slaughtered with 600kg.

106

107 ***2.2 Biosecurity assessment***

108 A biosecurity assessment was performed at arrival to the unit (T0) and 15 days after arrival (T1). An
109 adapted version of the Italian protocol for the assessment of beef cattle welfare included in the
110 ClassyFarm system (Bertocchi et al., 2020) was applied as previously reported (Masebo et al., 2023).
111 The used protocol included a list of 17 items: pests control measures, interaction with other animal
112 species, general precautions to the entrance of occasional visitors, general precautions to the entrance
113 of regular visitors, disinfection of vehicles upon entering the farm, possibility of contact between
114 foreign vehicles and farmed animals (< 20 m), carcass collection (< 20 m), live animal loading,

quarantine/housing management, control and prevention of most prevalent infectious diseases, health monitoring activities, control and prevention of endo/ectoparasites, control and analysis of water sources, cleaning of troughs/water point, storage buildings and rooms (hygiene, cleanliness and management of housing environments and bedding) and origin of the drinking water (Supplementary Table S2). For each item, a 2- or 3-point scale scoring system was applied (1=insufficient; 2=acceptable; 3=optimal). A value for each section was computed by summing the obtained score of each item from each section or area. The obtained values were further converted into percentages. A result below 59% indicated a poor status (=low), a result between 60 and 80% a medium status (=medium), and a result over 80% a good status (=high).

2.3 Haematological investigation

Blood samples from 88 animals were collected for haematological investigation at T0 and T1. Two animals were randomly selected from each pen at T0, and the same subjects were again sampled at T1. Samples were transferred to serum vacuum tubes for serological analyses and in EDTA vacuum tubes for complete blood count (CBC) and then to citrate tubes for fibrinogen analysis. The following parameters were analysed: RBC, haemoglobin, haematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), red cell distribution width (RDW), platelets (PLT), leukocytes (WBC), neutrophils, monocytes, lymphocytes, eosinophils, basophils and fibrinogen.

2.4 Sampling at slaughter

Five months after T0, cardiac blood samples were obtained at the slaughterhouse (T2), from 56 of the 88 animals that underwent a blood sampling at T0 and T1. Blood collection took place during the

heart excision process, where approximately 10-40 ml of blood was collected in a 50 ml falcon tube and kept at room temperature until further processing.

2.5 Indirect fluorescent antibody test (IFAT)

The blood samples were centrifuged at 2000 rpm for 25 minutes and the resulting serum was collected and stored at -20 °C until further analysis. *Toxoplasma gondii* indirect fluorescent antibody test (IFAT) for IgG was performed on serum samples, following the manufacturer's instructions (MegaFLUO TOXO-PLASMA g, MegaCor Diagnostik, Hoerbranz, Austria). As conjugated, anti-cattle IgG antibody diluted in PBS at concentration of 1:200 was used (Anti-Cattle IgG-FITC antibody, Sigma-Aldrich, Saint Louis, MO). Serum samples with antibody titer ≥ 1:40 were considered positive.

2.6 Statistical analysis

Data were entered into a statistics program (JMP Pro 17). Descriptive statistics were generated: mean, standard deviation (SD) and/or standard error (SE), median and interquartile range (25° and 75°) for continuous data, and count and percentage for categorical data. For continuous variables, normality was tested by the Shapiro-Wilk test and non-normally distributed variables were Box-Cox transformed before the analysis according to previous reports (Raspa et al., 2022). The evaluation of differences between the positive/negative to *T. gondii* and different IFAT titres was undertaken using the Mixed Model Procedure. Each bovine was set as an experimental unit within the arrival group and pen as nested factors. The seropositive/seronegative status for *T. gondii* (pos/neg) and different IFAT titer (1:40/1:80/≥ 1:160) was implemented as a fixed effect in separate models. After the analysis, the normal distribution of the data was checked again for the resulting residuals. Means are reported as least square mean and pairwise multiple comparisons were performed using Tukey-test as a post hoc test when a significance was detected. The nominal logistic model was used for

categorical variables using the same discriminant as before mentioned. A p-value ≤ 0.10 was considered a tendency; a p-value ≤ 0.05 was considered statistically significant; and a p-value ≤ 0.01 was considered highly significant.

Principal component analysis (PCA) (correlation matrix) was used to reduce the variables to factors as previously reported (Vinassa et al., 2020); data assumption for multivariate normality was checked using Keiser-Meyer-Olkin (KMO) and Barlett tests, which were performed to test the suitability of the data for structure detection.

3. Results

3.1. *Biosecurity assessment*

The biosecurity assessment did not vary between T0 and T1. Biosecurity was scored as medium with a 61% value in both T0 and T1 (Supplementary Table S1).

3.2. Distribution of serological status and IFAT titres for Toxoplasma gondii

The distribution of serological status for *T. gondii* is shown in Figure 1A. The percentage of seropositive animals at T0 was 30.6%, and increased at T1 to 44.6%, when the percentage of seropositive animals were almost equal to that of the seronegative group. Finally, at T2, the percentage of seropositive animals was 39.3%. Due to challenges faced during the slaughtering process, the total number of animals collected and tested at T2 was slightly lower (56/88) compared to the numbers obtained during the other two sampling events in the barn.

The distribution of IFAT titres in the seropositive group for *T. gondii* were as follow: at T0 51.2% (1:80) followed by 40.7% (1:40) and 7.4% (1:160) as the maximum titre; at T1, the animals with a titre of 1:40 slightly increased reaching the 43.2% followed by the ones with a titre of 1:160 (29.7%),

185 1:80 (16.2%), 1:320 (8.1%) and finally 1:1280 (2.7%); finally at T2, the percentage of animals with
186 a titre of 1:40 increased again as the most common category (54.6%), followed by animals with titres
187 ≥1:80. Seroconversion occurred in 13 animals (14.6%) from T0 to T1, and in 5 (6%) from T1 to T2.
188 No animals lost detectable antibody titre from T0 to T1, while at T2, 12 (14.5%) bulls previously
189 positive tested negative for IgG (Fig. 1B).

190

191 ### *3.3. Effect of the* **Toxoplasma gondii** *serological status on blood analysis*

192 The effect of *T. gondii* serological status (positive *vs* negative) on haematological analysis is shown
193 in Table 1. There was a statistically significant effect (p-value <0.05) on MCV and N/L ratio. MCV
194 was significantly lower and N/L ratio was significantly higher in seropositive compared with
195 seronegative cattle. There was also a trend effect (p-value <0.10) on MCH and neutrophils. MCH and
196 neutrophils tended to be higher in seropositive cattle.

197

198 ### *3.4. Effect of different IFAT titres of* **Toxoplasma gondii** *on blood analysis*

199 There was a significant effect (p-value <0.05) of the different titres on neutrophils and N/L ratio
200 (Table 2). The neutrophils and consequently the N/L ratio were significantly higher in cattle with
201 titres >1:160 compared to cattle with titres of 1:80 and 1:160.

202

203 ### *3.5. Principal component analysis*

204 PCA was performed to explain the variability in the blood analysis and to correlate each blood
205 parameter according to serological status for *T. gondii*. The suitability of the data for PCA was
206 evaluated (KMO = 0.80; Barlett's test, $p < 0.01$). Figure 2 shows that PCA separated the blood
207 parameters on the first principal component (PC1): component 1 explains 24.6% of the variance of

208 the data, and component 2 (PC2) another 16.3%, for a total of 40.9% of variability of variance. Table
209 3 shows the loadings of the variables of the first and second principal components, and how each
210 variable contributes to each component. Even though the obtained PCA showed a small effect, three
211 different patterns were identified. Cattle with titres >1:160 showed a greater number of eosinophils,
212 lymphocytes, WBC, basophils, monocytes and neutrophils, while cattle with a titre of 1:80 showed a
213 correlation with MCV and fibrinogen. Finally, cattle with titres of 1:40 or seronegative showed no
214 effect on the considered blood parameters.

215

216 **4. Discussion**

217 The present study consisted in a longitudinal investigation on the serological status for *T. gondii* in
218 beef cattle naturally exposed at three different stages of the productive cycle. We observed a relatively
219 high seroprevalence of *T. gondii* infection among cattle at T0, with 30.6% of the animals tested
220 positive. This initial prevalence suggest that animals were already infected at arrival to the fattening
221 unit. However, the most noteworthy finding was the increase in seropositivity observed at T1, where
222 44.6% of the cattle tested positive for *T. gondii* antibodies. Considering the kinetics of IgG, this rise
223 in seropositivity at T1 implies that some cattle may have become infected at the time of arrival in the
224 fattening unit, during the transport, or immediately before the shipping. Furthermore, we identified a
225 subset of animals (5 in total) that underwent seroconversion from T1 to T2, indicating that these
226 individuals likely acquired the infection during their time in the fattening unit rather than before their
227 arrival. Interestingly, when comparing T1 with T2, a slight decrease in seroprevalence at T2 was
228 noticed. This reduction suggests that some cattle may have lost detectable antibody titres by the time
229 of slaughter, potentially indicating a waning of the immune response or clearance of the infection in
230 these individuals. Overall, these findings highlight the dynamic nature of *T. gondii* infection in
231 fattening cattle, with evidence of both new infections and antibody waning over the course of their
232 productive cycle.

233 Estimates of seroprevalence in cattle, when obtained by highly specific assays, may be useful for
234 monitoring exposure of bovines to *T. gondii*. Different serological techniques have been
235 recommended and considered suitable for the confirmation of exposure to *T. gondii* in cattle such as
236 IFAT, modified agglutination test (MAT) and enzyme linked immunosorbent assay (ELISA) (WOAH,
237 2017). Nevertheless, results of seropositivity should be interpreted with caution, as studies using
238 bioassay experiments on naturally exposed cattle indicate that the overwhelming majority of
239 seropositive cattle do not show evidence of viable *T. gondii* infection (Boch et al., 1965; Dubey et al.,
240 2005; Dubey and Streitel, 1976; Jacobs and Moyle, 1963; Opsteegh et al., 2019). On the contrary,
241 there are a limited number of studies of naturally exposed cattle in which positive *T. gondii* bioassays
242 indicate viable infection (Arias et al., 1994; Catar et al., 1969; de Macedo et al., 2012; Dubey, 1992;
243 Jacobs et al., 1960). Therefore, identification of *T. gondii* genomic material without positive bioassays
244 should not be considered as conclusive of infection and consequently does not provide an indication
245 of risk for the consumer (Opsteegh et al., 2019; Stelzer et al., 2019).

246 Herein within the seropositive group, the most prevalent IFAT titre at T0 was 1:80, and at T1 and T2
247 was 1:40. Moreover, seroconversion occurred in 14.6% of cattle from T0 to T1, and in 6% from T1
248 to T2. No animals lost detectable antibody titre from T0 to T1, while at T2 14.5% became seronegative
249 for IgG. Therefore, our results suggest that cattle might become infected with *T. gondii* but are able
250 to clear the infection followed by a decline of antibody titres. The host-*T. gondii* interaction in cattle
251 is poorly understood and only few studies have investigated the antibody kinetics (Dubey et al., 1985;
252 Opsteegh et al., 2011a). It has been observed that the dynamics of anti-*T. gondii* antibody levels in
253 cattle are influenced by age, with infected adult cattle typically exhibiting low antibody titres (Dubey
254 et al., 1985). Additionally, it could be postulated that calves exposed to low doses of parasite early in
255 life, which may result in the generation of a relatively weak protective immunological response, could
256 experience seronegativization during their relatively short lifespan, as observed in our case (16-18
257 months).

The fact that an increase in seroprevalence was observed during the five months of the fattening period may indicate that some risk factors may have been present in the investigated fattening farm leading to *T. gondii* infection of the cattle. In the biosecurity assessment, the main risk factors identified were inadequate rodent and insect control measures, the possibility of contact with other animal species such as cats, and the possibility of contamination of drinking water in case of failure of the central water supply (use of a storage tank). The presence of cats (Gilot-Fromont et al., 2009; Magalhaes et al., 2016; Sun et al., 2015) and rodents (Sun et al., 2015) on farms are considered important risk factors for infection of cattle. In addition, access to water from a reservoir has also been identified as a potential risk factor (Magalhaes et al., 2016).

In respect to the blood parameters, MCV was significantly lower and N/L ratio was significantly higher in seropositive compared with seronegative cattle and the MCH and neutrophils tended to be higher in seropositive cattle. Furthermore, the neutrophils and consequently the N/L ratio were also significantly higher in cattle with titres >1:160 compared to cattle with lower titres. These results related to neutrophils indicate an activation of the innate immune response in the *T. gondii* positive animals. An effective innate immune response plays a crucial role in the early recognition of *T. gondii* (Wilson, 2012). Neutrophils, essential components of the innate immune system, are produced in the bone marrow and despite their relatively short lifespan, they rapidly accumulate at the site of infection employing diverse strategies to fight invading pathogens (Mantovani et al., 2011). Their primary function revolves around phagocytosis, wherein pathogens taken up by neutrophils are subsequently eradicated within the phagolysosome through the actions of enzymes and proteins (Brinkmann and Zychlinsky, 2012). Neutrophilia, a common occurrence during numerous infections, was observed in our study in association with seropositivity and higher antibody titres. This phenomenon may be linked to recent toxoplasmic infections, where IgGs have yet to be fully developed. A limitation of our study lies in the absence of IgM research, which is more closely related to the acute phase of infection. In the context of toxoplasmosis, neutrophils appear to hold particular significance. *T. gondii*

283 tachyzoites have been shown to induce the formation of extracellular trap structures in murine and
284 human neutrophils (NETs) (Abi Abdallah et al., 2011; Manda et al., 2014). These extracellular traps
285 not only physically entrap tachyzoites, preventing host cell invasion, but also exert detrimental effects
286 on the viability of the trapped tachyzoites (Abi Abdallah et al., 2011). Furthermore, it was
287 demonstrated that NET structures released from sheep polymorphonuclear leukocytes (PMNs) led to
288 the mechanical immobilization of *T. gondii* tachyzoites, while NET structures released from cattle
289 PMNs appeared to have lethal effects on the tachyzoites (Yildiz et al., 2017). It might be premature
290 and speculative to establish a direct link between our research findings and the role of NETosis in
291 bovine toxoplasmosis. However, our study does provide valuable insights suggesting a discernible
292 association between neutrophils and seropositivity for *T. gondii* antibodies in cattle. This observation
293 contributes to a better understanding of the clinico-pathological aspects of naturally exposure of *T.*
294 *gondii* in cattle.

295

296 **5. Conclusion**

297 This study evaluates for the first time the antibody kinetics for *T. gondii* at three different time points
298 of the production cycle of fattening cattle. The high seroprevalence of *T. gondii* infection among cattle
299 at T0, with an increase in T1 and a slight decrease in T2 emphasize the dynamic nature of *T. gondii*
300 infection in cattle, with evidence of both new infections and antibody decay during the production
301 cycle. In addition, it was observed that the same titer varies in different samplings over time,
302 suggesting that this species has a peculiar antibody dynamic.

303 Regarding blood parameters, the N/L ratio was significantly higher and the number of neutrophils
304 tended to be higher in seropositive cattle. In addition, the number of neutrophils was significantly
305 higher in cattle with higher titers for *T. gondii*. Overall, our findings suggest an activation of the innate
306 immune response in the *T. gondii* seropositive animals. Further studies are needed to better understand
307 the specific behavior of neutrophils in cattle exposed to *T. gondii*.

CRediT authorship contribution statement

Filippo M. Dini: Conceptualization, Formal analysis, Investigation, Methodology, Validation, Writing – original draft, Writing – review & editing. Joana G.P. Jacinto: Conceptualization, Data curation, Investigation, Resources, Writing – original draft, Writing – review & editing, Funding acquisition, Resources. Damiano Cavallini: Investigation, Methodology, Validation; Andrea Beltrame: Investigation, Methodology, Validation; Flavia S. Del Re: Investigation, Methodology, Validation; Laura Abram: Investigation, Methodology, Validation; Arcangelo Gentile: Conceptualization, Investigation, Methodology, Validation; Roberta Galuppi: Conceptualization, Formal analysis, Investigation, Methodology, Validation, Writing – review & editing, Funding acquisition, Resources, Project administration.

Declaration of Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. The authors declare no competing interests.

Ethical Approval

Not applicable

Funding

Not applicable

Availability of data and materials

Not applicable

Acknowledgments

330 The authors acknowledge Giovanni Testa for assistance at the abattoir, Valentina Luci and Alessia
331 Cantori for the exceptional laboratory assistance.

332

333 **Supplemental Information (SI)**

334 **Supplementary Table S1**: Analysis of the adaptation TMR diet (T0 and T1) and chemical analysis.

335 **Supplementary Table S2:** Check-list used to perform the biosecurity assessment.

336 **References**

337 Abi Abdallah DS, Lin C, Ball CJ, King MR, Duhamel GE, Denkers EY (2011) *Toxoplasma gondii*
338 triggers release of human and mouse neutrophil extracellular traps. Infect. Immun. 80, 768–
339 777.

340 Arias ML, Chinchilla M, Reyes L, Sabah J, Guerrero OM (1994) Determination of *Toxoplasma*
341 *gondii* in several organs of cattle by carbon immunoassay (CIA) testing.Vet. Parasitol. 55,
342 133–136.

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

345 Belluco S, Patuzzi I, Ricci A (2018) Bovine meat versus pork in *Toxoplasma gondii* transmission in
346 Italy: a quantitative risk assessment model. Int. J. Food Microbiol. 269, 1–11.

347 Belluco S, Simonato G, Mancin M, Pietrobelli M, Ricci A (2017) *Toxoplasma gondii* infection and
348 food consumption: a systematic review and meta-analysis of case-controlled studies. Crit.
349 Rev. Food Sci. 1–12

350 Berger-Schoch AE, Bernet D, Doherr MG, Gottstein B, Frey CF (2011) *Toxoplasma gondii* in
351 Switzerland: a serosurvey based on meat juice analysis of slaughtered pigs, wild boar, sheep

352 and cattle. Zoonoses Public Hhlt 58, 472–478. https://doi.org/10.1111/j.1863-
353 2378.2011.01395.x

354 Bertocchi L, Fusi F, Lorenzi V (2020) Valutazione Del Benessere Animale e Della Biosicurezza,
355 Nell'allevamento Bovino Da Carne: Manuale Di Autocontrollo. CReNBA (Centro di
356 Referenza Nazionale per il Benessere Animale), Istituto Zooprofilattico Sperimentale della
357 Lombardia e dell'Emilia Romagna, Brescia, Italy.

358 Blaga R, Aubert D, Thébault A, Perret C, Geers R, Thomas M, Alliot A, Djokic V, Ortis N, Halos L,
359 Durand B, Mercier A, Villena I, Boireau P (2019) *Toxoplasma gondii* in beef consumed in
360 France: regional variation in seroprevalence and parasite isolation. Parasite 26,
361 77.https://doi.org/10.1051/parasite/2019076

362 Boch J, Janitschke K, Rommel M, Sommer R (1965) Untersuchungen über das Vorkommen von
363 *Toxoplasma*-Infektionen bei Schlachtrindern. Wiener tierarztliche Monatsschrift, 12, 1477–
364 1480.

365 Brinkmann V, Zychlinsky A (2012) Neutrophil extracellular traps: is immunity the second function
366 of chromatin? J. Cell Biol. 198, 773–783.

367 Canada N; Meireles CS; Rocha A, da Costa JMC, Erickson MW, Dubey JP (2002) Isolation of viable
368 *Toxoplasma gondii* from naturally infected aborted bovine fetuses. J. Parasitol. 2002, 88,
369 1247–1248.

370 Catar G, Bergendi L, Holkova R (1969) Isolation of *Toxoplasma gondii* from swine and cattle. J.
371 Parasitol. 55, 952–955.

372 Cook AJ, Gilbert RE, Buffolano W, Zufferey J, Petersen E, Jenum PA, Foulon W, Semprini AE, Dunn
373 DT (2000) Sources of *Toxoplasma* infection in pregnant women: European multicentre case-
374 control study. European Research Network on Congenital Toxoplasmosis. BMJ 321, 142–147.

375 Costa G H, da Costa A J, Lopes WD, Bresciani K D, dos Santos T R, Esper CR, Santana AE (2011)
376 *Toxoplasma gondii*: infection natural congenital in cattle and an experimental inoculation of
377 gestating cows with oocysts. Exp. Parasitol. 127,277–281.
378 https://doi.org/10.1016/j.exppara.2010.08.005

379 Dabritz HA, Conrad PA (2010) Cats and *Toxoplasma*: implications for public health. Zoonoses Public
380 Hlth. 57, 34–52.

381 de Macedo MF, de Macedo CA, Ewald MP, Martins GF, Zulpo DL, da Cunha IA, Taroda A, Cardim
382 ST, Su C, Garcia JL (2012) Isolation and genotyping of *Toxoplasma gondii* from pregnant
383 dairy cows (*Bos taurus*) slaughtered. Rev. Bras. Parasitol. Vet. 21, 74–77.

384 Dubey JP (1992) Isolation of *Toxoplasma gondii* from a naturally infected beef cow. J. Parasitol. 78,
385 151–153.

386 Dubey JP (2020) The history and life cycle of *Toxoplasma gondii*. In *Toxoplasma gondii*; Elsevier:
387 Amsterdam, The Netherlands, Dpp. 1–19

388 Dubey JP, Hill DE, Jones JL, Hightower AW, Kirkland E, Roberts JM, Marcet PL, Lehmann T, Vianna
389 MC, Miska K, Sreekumar C, Kwok OC, Shen SK, Gamble HR (2005). Prevalence of viable
390 *Toxoplasma gondii* in beef, chicken, and pork from retail meat stores in the United States: risk
391 assessment to consumers. J. Parasitol. 91, 1082–1093.

392 Dubey JP, Streitel RH (1976) Prevalence of *Toxoplasma* infection in cattle slaughtered at an Ohio
393 abattoir. J. Am. Vet. Med. Assoc. 169, 1197–1199

394 García-Bocanegra I; Cabezón O; Hernández E; Martínez-Cruz MS; Martínez-Moreno Á; Martínez-
395 Moreno J. (2013) *Toxoplasma gondii* in ruminant species (cattle, sheep, and goats) from
396 Southern Spain. J. Parasitol. 99, 438–440

Gazzonis A L, Marino AMF, Garippa G, Rossi L, Mignone W, Dini V, Giunta RP, Luini M, Villa L, Zanzani SA, Manfredi MT (2020) *Toxoplasma gondii* seroprevalence in beef cattle raised in Italy: a multicenter study. Parasitology research, 119, 3893–3898. https://doi-org.ezproxy.unibo.it/10.1007/s00436-020-06878-y

Gilot-Fromont E, Aubert D, Belkilani S, Hermitte P, Gibout O, Geers R, Villena I (2009) Landscape, herd management and within-herd seroprevalence of *Toxoplasma gondii* in beef cattle herds from Champagne-Ardenne, France. Vet. Parasitol. 161, 36–40.

Havelaar AH, Kirk MD, Torgerson PR, Gibb HJ, Hald T, Lake RJ, Praet N, Bellinger DC, de Silva NR, Gargouri N, Speybroeck N, Cawthorne A, Mathers C, Stein C, Angulo FJ, Devleesschauwer B (2015) World Health Organization global estimates and regional comparisons of the burden of foodborne disease in 2010. PLoS Med. 12, e1001923

Jacobs L, Moyle GG (1963) The prevalence of toxoplasmosis in New Zealand sheep and cattle. Am. J. Vet. Res. 24, 673–675

Jacobs L, Remington JS, Melton ML (1960) A survey of meat samples from swine, cattle, and sheep for the presence of encysted *Toxoplasma*. J. Parasitol. 46, 23–28

Jokelainen P, Tagel M, Mõtus K, Viltrop A, Lassen B (2017) *Toxoplasma gondii* seroprevalence in dairy and beef cattle: large-scale epidemiological study in Estonia. Vet Parasitol 236,137–143. https://doi.org/10.1016/j.vetpar.2017.02.014

Klun I, Djurković-Djaković O, Katić-Radivojević S, Nikolić A (2006) Cross-sectional survey on *Toxoplasma gondii* infection in cattle, sheep and pigs in Serbia: seroprevalence and risk factors. Vet Parasitol 135,121–131. https://doi.org/10.1016/j.vetpar.2005.08.010

Magalhaes FJ, Ribeiro-Andrade M, Alcantara AM, Pinheiro JWJ, Sena MJ, Porto WJ, Vieira RF, Mota RA (2016) Risk factors for *Toxoplasma gondii* infection in sheep and cattle from Fernando de Noronha Island, Brazil. Rev. Bras. Parasitol. Vet. 25,511–515.

421 Manda A, Pruchniak MP, Araźna M, Demkow UA (2014) Neutrophil extracellular traps in physiology
422 and pathology. Cent. Eur. J. Immunol. 39, 116–121.

423 Mantovani A, Cassatella MA, Costantini C, Jaillon S (2011) Neutrophils in the activation and
424 regulation of innate and adaptive immunity. Nat. Rev. Immunol. 11, 519–531

425 Masebo NT, Marliani G, Cavallini D, Accorsi PA, Di Pietro M, Beltrame A, Gentile A, Jacinto JGP
426 (2023) Health and welfare assessment of beef cattle during the adaptation period in a
427 specialized commercial fattening unit. Res. Vet. Sci., 158, 50–55. https://doi-
428 org.ezproxy.unibo.it/10.1016/j.rvsc.2023.03.008"https://doi-
429 org.ezproxy.unibo.it/10.1016/j.rvsc.2023.03.008

430 Opsteegh M, Prickaerts S, Frankena K, Evers EG (2011a) A quantitative microbial risk assessment
431 for meatborne *Toxoplasma gondii* infection in The Netherlands. Int. J. Food Microbiol. 150,
432 103–114.

433 Opsteegh M, Spano F, Aubert D, Balea A, Burrells A, Cherchi S, Cornelissen JBWJ, Dam-Deisz C,
434 Guitian J, Györke A, Innes E A, Katzer F, Limon G, Possenti A, Pozio E, Schares G, Villena
435 I, Wisselink HJ, van der Giessen JWB (2019) The relationship between the presence of
436 antibodies and direct detection of *Toxoplasma gondii* in slaughtered calves and cattle in four
437 European countries. Int. J. Parasitol. 49, 515–522.
438 https://doi.org/10.1016/j.ijpara.2019.01.005

439 Opsteegh M, Teunis P, Zuchner L, Koets A, Langelaar M, van der Giessen J (2011b) Low predictive
440 value of seroprevalence of *Toxoplasma gondii* in cattle for detection of parasite DNA. Int. J.
441 Parasitol. 41,343–354.

442 Raspa F, Vervuert I, Capucchio MT, Colombino E, Bergero D, Forte C, Greppi M, Cavallarin L,
443 Giribaldi M, Antoniazzi S, Cavallini D, Valvassori E, Valle E (2022) A high-starch vs. high-

444 fibre diet: effects on the gut environment of the different intestinal compartments of the horse
445 digestive tract. BMC Vet. Res., 18(1). https://doi.org/10.1186/s12917-022-03289-2

446 Robert-Gangneux F, Darde´ ML (2012) Epidemiology of and diagnostic strategies for toxoplasmosis.
447 American Society of Microbiology 25, 264–296.

448 Smith JL (1993) Documented outbreaks of toxoplasmosis: transmission of *Toxoplasma gondii* to
449 humans. J. Food Protect. 56, 630–639.

450 Stelzer S, Basso W, Benavides Silván J, Ortega-Mora L. M, Maksimov P, Gethmann J, Conraths FJ,
451 Schares G (2019) *Toxoplasma gondii* infection and toxoplasmosis in farm animals: Risk
452 factors and economic impact. Food Waterborne Parasitol, 15, e00037.
453 https://doi.org/10.1016/j.fawpar.2019.e00037

454 Sun WW, Meng QF, Cong W, Shan XF, Wang CF, Qian AD (2015) Herd-level prevalence and
455 associated risk factors for *Toxoplasma gondii*, *Neospora caninum*, *Chlamydia abortus* and
456 bovine viral diarrhoea virus in commercial dairy and beef cattle in eastern, northern and
457 northeastern China. Parasitol. Res. 114, 4211–4218.

458 Vinassa M, Cavallini D, Galaverna D, Baragli P, Raspa F, Nery J, Valle E (2020) Palatability
459 assessment in horses in relation to lateralization and temperament. Appl. Anim. Behav. Sci..
460 232, 105110

461 Wilson E (2012) Apicomplexa: *Toxoplasma gondii*. In: Lamb, T.J. (Ed.), Immunity to Parasitic
462 Infection. Wiley-Blackwell, pp. 107–120.

463 World Organisation For Animal Health – OIE. Terrestrial manual: toxoplasmosis [online]. Paris: OIE;
464 2017 [cited 2023 Aug 2]. Available from:
465 http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.09.09_TOXO.pdf

466 Yildiz K, Gokpinar S, Gazyagci AN, Babur C, Sursal N, Azkur AK (2017) Role of NETs in the
467 difference in host susceptibility to *Toxoplasma gondii* between sheep and cattle. Vet Immunol
468 Immunopathol 189, 1-10. http://dx.doi.org/10.1016/j.vetimm.2017.05.005. PMid:28669381

469

470

Figures

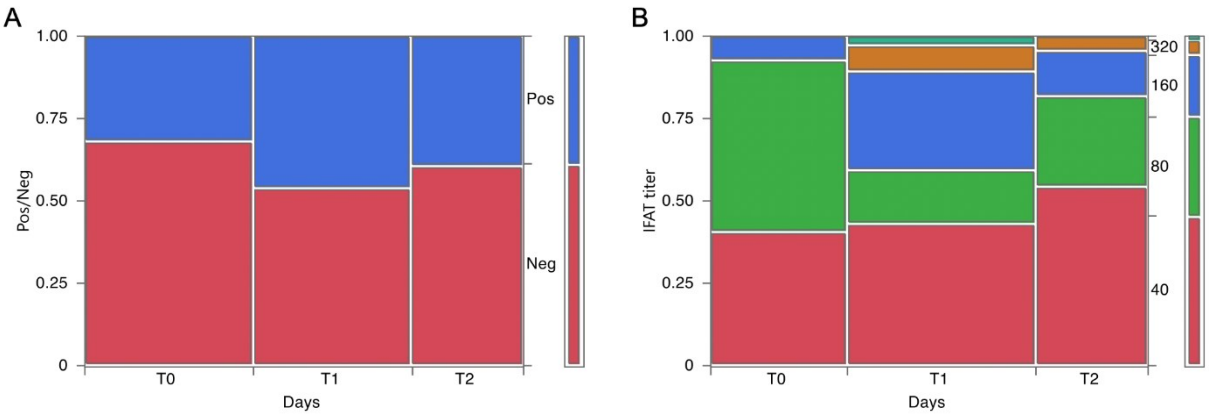
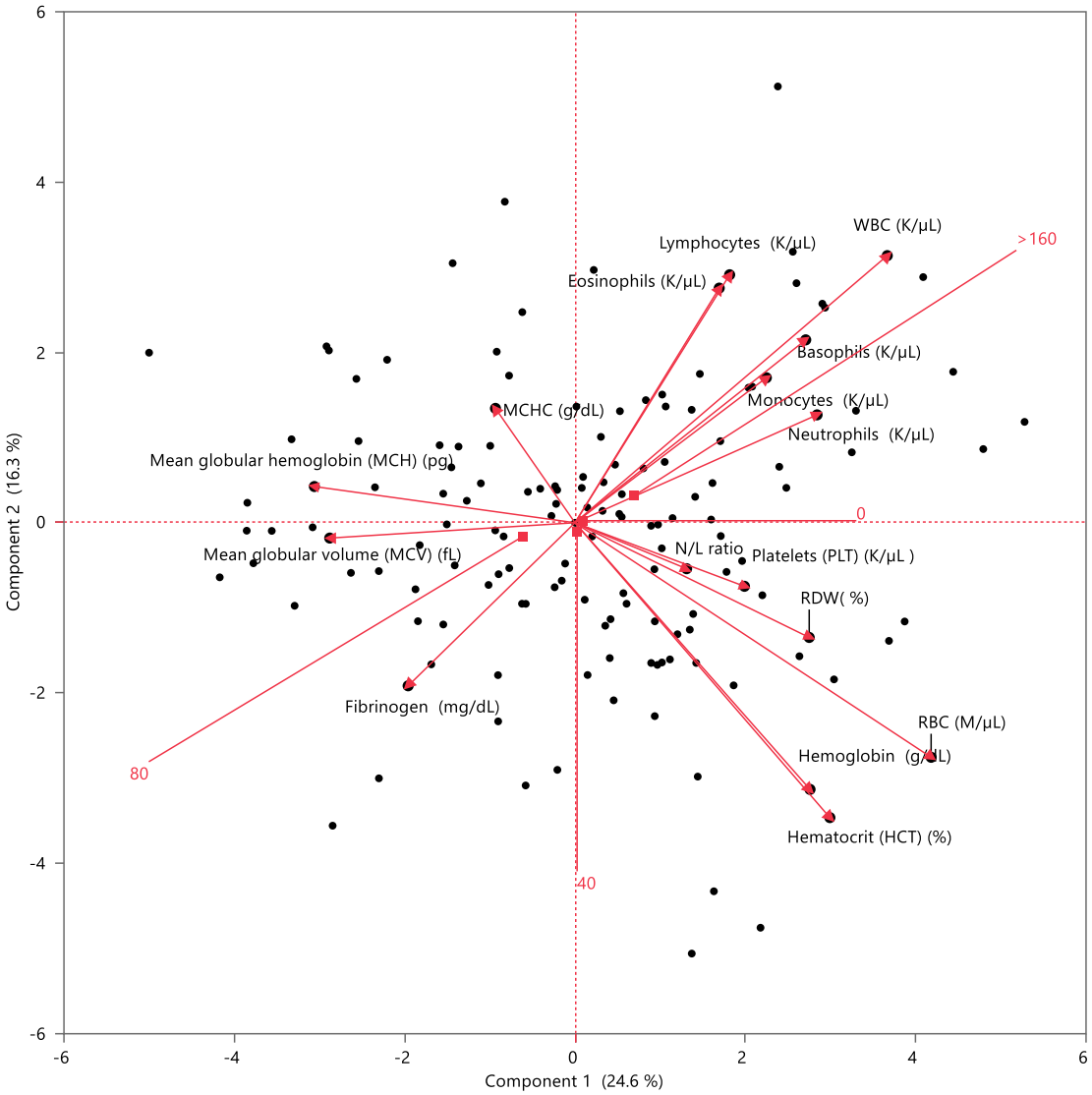


Figure 1: Contingency Analysis comparing the distribution of serological status for Toxoplasma gondii (A) and the IFAT titers in the seropositive cattle (B) at T0, T1 and T2.



476

477 **Figure 2: Principal component analysis biplot (PC1 and PC2) performed on blood parameters**

478 **according to serological status for *Toxoplasma gondii***

479

480 **Table 1: Effect of the serological status for *Toxoplasma gondii* on the blood analysis**

Parameter		*T. gondii* Neg.	*T. gondii* Po.s	p-value
RBC (M/µL)	Mean± SD	9.82 ± 1.34	9.71±1.04	0.65
HGB (g/dL)	Mean± SD	12.07±1.21	11.94±1.13	0.83
HCT (%)	Mean± SD	39.76±4.31	38.67±3.66	0.7
MCV (fL)	Mean± SD	40.6±3.06	40±2.37	0.04
MCH (pg)	Median [Min.-Max]	12.2 [11.6-13.08]	12.4 [11.7-12.95]	0.07
MCHC (g/dL)	Mean± SD	30.4±1.33	30.9±1.1	0.13
RDW (%)	Mean± SD	24.06±1.84	24.06±1.86	0.91
PLT (K/µL)	Median [Min.-Max]	283 [150-408.5]	151.5 [151.5-428]	0.29
WBC (K/µL)	Median [Min.-Max]	9.24 [7.81-11.73]	9.29 [7.73-10.64]	0.72
NEU (K/µL)	Median [Min.-Max]	3.42 [2.76-4.65]	3.77 [2.93-5.2]	0.08
MONO (K/µL)	Median [Min.-Max]	1.23 [0.95-1.46]	1.1 [0.9-1.35]	0.38
LYM (K/µL)	Mean± SD	4.37±1.64	3.83±1.42	0.2
EOS (K/µL)	Median [Min.-Max]	0.13 [0.05-0.29]	0.1 [0.06-0.3]	0.59
BASO (K/µL)	Median [Min.-Max]	0.07 [0.05-0.1]	0.07 [0.05-0.11]	0.93
FIBR (mg/dL)	Median [Min.-Max]	916.2 [661.8-1348.2]	826.5 [636-1111.8]	0.28
N/L ratio	Median [Min.-Max]	0.85 [0.59-1.18]	1.04 [0.69-1.74]	0.03

Abbreviations: Neg=negative, Pos=positive, RBC= Red blood cell, HGB= Hemoglobin, HTC=Hematocrit, MCV = Mean corpuscular volume, MCH= Mean corpuscular hemoglobin, MCHC= Mean corpuscular hemoglobin concentration, RDW= Red blood cell distribution width, PLT= Platelets, NEU= Neutrophils, WBC= white blood cells, MONO=Monocytes, LYM=Lymphocytes, EOS=Eosinophils, BASO= Basophils, FIBR=Fibrinogen, N/L ratio= Neutrophils : Lymphocytes ratio, M/µL= 10^6 per microliter ,%,=percentage, K/µL=10^3 per microliter, g/dL= grams per deciliter, fL= femtoliter, pg= picogram, mg/dL=milligram per decilitre

Table 2: Effect of different serological titre *Toxoplasma gondii* on the blood analysis

Parameter		40	80	≥160	p-value	Reference Range
NEU (K/µL)	Median [Min.-Max]	3.37 [2.6-3.87]	4.01 [2.93-4.55]	5.39 [3.75-8.16]	0.03	1.8-6.3
N/L ratio	Median[Min.-Max]	0.65 [0.85-1.23]	0.85 [0.59-1.18]	1.02 [1.97-1.41]	0.02	0.4-2.34

Abbreviations: NEU= Neutrophils, N/L ratio= Neutrophils : Lymphocytes ratio, K/µL=10^3 per microliter

Table 3: Principal component analysis loadings of blood parameters according to serological status for *Toxoplasma gondii*

Parameter	PCA1 (24.6%)	PCA2 (16.3%)
RBC (M/µL)	39.9%	-32.3
HGB (g/dL)	26.3%	-36.7%
HCT (%)	28.6%	-40.6%
MCV (fL)	-27.5%	-2.1%

Parameter	PCA1 (24.6%)	PCA2 (16.3%)
MCH (pg)	-29.2%	5%
MCHC (g/dL)	-8.9%	15.7%
RDW (%)	26.3%	-15.8%
PLT (K/µL)	19%	-8.7%
WBC (K/µL)	35%	36.8%
NEU (K/µL)	27.1%	14.9%
MONO (K/µL)	21.4%	20%
LYM (K/µL)	17.3%	34.1%
EOS (K/µL)	16.2%	32.3%
BASO (K/µL)	25.9%	25.2%
FIBR (mg/dL)	-18.7%	-22.4%
N/L ratio	12.5%	-6.3%

Abbreviations: RBC= Red blood cell, HGB= Hemoglobin, HTC=Hematocrit, MCV = Mean corpuscular volume, MCH= Mean corpuscular hemoglobin, MCHC= Mean corpuscular hemoglobin concentration, RDW= Red blood cell distribution width, PLT= Platelets, NEU= Neutrophils, WBC= white blood cells, MONO=Monocytes, LYM=Lymphocytes, EOS=Eosinophils, BASO= Basophils, FIBR=Fibrinogen, N/L ratio= Neutrophils : Lymphocytes ratio, M/µL= 10^6 per microliter ,%,=percentage, K/µL=10^3 per microliter, g/dL= grams per deciliter, fL= femtoliter, pg= picogram, mg/dL=milligram per decilitre

Supplementary Table S1. Analysis of the adaptation TMR diet (T0 and T1) and chemical analysis.

TMR	Feed, kg af
Wheat silage	3.5
Meadow hay[1]	1.2
Wheat straw	1.1
Beat pulp	1.3
Corn, finely ground[2]	1.1
Soybean meal	0.5
Cane molasses[3]	0.5
Min&Vit Premix	0.3
	Nutrients, %DM
DM	70.07
UFC	0.81
CP[4]	11.25
Ash	8.78
EE[5]	2.06
Starch	13.57
Sugars	7.37
NDF[6]	39.21
ADF[7]	25.79
ADL[8]	3.45

[1] the quality of the hay was checked to ensure the absence of molds and spores (Cavallini et al. 2022). [2] the corn was below the EU maxim tolerable level (Girolami et al. 2022). [3] molasses were properly characterized (Palmonari et la. 2021). [4] Crude protein. [5] ether extract. [6] neutral detergent fiber. [7] acid detergent fiber. [8] acid detergent lignin.

Cavallini D, Penazzi L, Valle E, Raspa F, Bergero D, Formigoni A, Fusaro I. 2022. When changing the hay makes a difference: A series of case reports. J Equine Vet Sci. 113:103940.

Girolami F, Barbarossa A, Badino P, Ghadiri S, Cavallini D, Zaghini A, Nebbia C. 2022. Effects of turmeric powder on aflatoxin M1 and aflatoxicol excretion in milk from dairy cows exposed to aflatoxin b1 at the EU maximum tolerable levels. Toxins 14: 430.

Palmonari A, Cavallini D, Sniffen CJ, Fernandes L, Holder P, Fusaro I, Giammarco M, Formigoni A, Mammi LME. 2021. In vitro evaluation of sugar digestibility in molasses. Ital. J. Anim. Sci. 20:571-577.

Supplementary Table S2. Check-list used to perform the biosecurity assessment.

			T0	T1
	AREA	**BIOSECURITY**		
	Items	**Level**		
1.	Rodent and insect control measures	Total absence of control measures		
		Presence of rough and minor control measures (absence of written plans)	2	2
		Presence of defined and effective procedures supported by written manual and recording system		
2.	Contact with other animal species	Yes, the contact is frequent and evident		
		No, contact may occur but is not evident at the time of the visit such as cats	2	2
		No, the farm is well protected (fences, etc.); no other animal species are present on the farm perimeter, and no contact with herds of the same species or other animals		
3	General precautions at the entrance of occasional visitors	Total absence of measures		
		Presence of minor procedures (absence of written plans)	2	2
		Presence of defined and effective procedures supported by written and recording system manual		
4	General precautions at the entrance of regular visitors	Total absence of measures		
		All visitors are required to wear disposable footwear before entering the farm or use boots that are on the farm for their exclusive personal use	2	2
		All visitors must pass through a changing area and are required to wear disposable footwear and clothes provided by the farm or use clothing and boots that remain on the farm for their exclusive personal use		
5.	Disinfection of vehicles upon entering the farm	Absence of disinfection facilities	1	1
		Presence of non-specific disinfection facilities or used of disinfection aids only when necessary		
		Presence of specific, fixed and routinely used disinfection facilities		
6.	Possibility of contact between foreign vehicles and farmed animals (< 20 m)	Yes	1	1
		No		
7.	Carcass collection (< 20 m)	Yes, vehicles used to remove the carcasses have direct/indirect contact with cattle (< 20 m distance)	1	1

		No, vehicles used to remove the carcasses are stopped at the border of the farm w (>20m distance)		
8.	Live animal loading (i.e. for sale)	Loading is carried out close to the housing premises where the animals are kept (<20 m)	1	1
		Loading is carried out away from the housing premises where the animals are kept (>20 m)		
9.	Quarantine/Housing management	No quarantine for new entering animals	1	1
		Partial/minor quarantine measures (i.e. designated area not separated from the areas where the other cattle are kept, quarantine is too short, no biological tests)		
		Proper quarantine measures, adequate in time and facilities (i.e. designated area separated from the areas where the other cattle are kept, adequate duration of the quarantine, biological tests)		
10.	Control and prevention of most prevalent infectious diseases	No knowledge of most prevalent infectious diseases or no information of the herd health status		
		Partial knowledge and/or presence of undefined plans (i.e., approximate, random, and not continuous over time)		
		Knowledge of at least three diseases prevalence in the herd; in addition, application of proper operational plans of prevention and control on at least two of them (vaccination plan, plan for dealing of infected animals, eradication plan, etc.).	3	3
11.	Health monitoring activities (Verify the farm's habit of submitting pathological material, fetuses, carcasses, and blood samples to the reference testing laboratory; the farmer must be in possession of an analytical result from the last 12 months)	Absence		
		Presence of analysis on pathological material	2	2
12.	Control and prevention of endo/ectoparasites	No knowledge and absence of prevention/control plans		
		Partial knowledge and/or presence of random control and prevention plans (i.e. approximate, random, and not continuous over time)		
		Knowledge of most prevalent parasites on the farm and prevention performed following laboratory tests	3	3
13.	Control and analysis of water sources	Absence of water analysis		

		Drinking water comes from the central supply system or from other sources and the quality of the water is checked at least once a year	2	2
14.	Cleaning of troughs/water point	Presence of dirt on the surface and walls of troughs/water point		
		Presence of food only on the water surface or only on the bottom. The water still remains clear	2	2
		Absence of dirt, clean troughs/water point and clear water		
15.	Storage buildings and rooms: hygiene, cleanliness and management of housing environments and bedding	Inadequate: Dirty, unmanaged and/or animal-harmful housing and bedding environments		
		Adequate: Fairly clean and sufficiently managed housing and/or bedding environments and/or clean grid in almost all groups	2	2
		Optimum: Clean, dry and optimally managed housing and bedding environments with frequent material changes		
16.	Origin of the drinking water	only one drinking water source and no storage tank		
		Only one drinking water source but presence of a storage tank that guarantees a sufficient water supply in case of disruption of the water source	2	2
		presence of two or more drinking water sources		
TOTAL obtained			27	27
TOLTAL obtained in percentage			61%	61%

Abbreviations: T0 = Assessment at day 2 after arrival; T1=Assessment at day 15 after arrival.

Chapter 6.2

A Case of Bovine Eosinophilic Myositis (BEM) Associated with Co-Infection by *Sarcocystis hominis* and *Toxoplasma gondii*

Dini FM, Caffara M, Jacinto JGP, Benazzi C, Gentile A, Galuppi R. (2023). A Case of Bovine Eosinophilic Myositis (BEM) Associated with Co-Infection by *Sarcocystis hominis* and *Toxoplasma gondii*. *Animals*. 13 (2): 311. doi:10.3390/ani13020311.

animals

MDPI

Article

A Case of Bovine Eosinophilic Myositis (BEM) Associated with Co-Infection by *Sarcocystis hominis* and *Toxoplasma gondii*

Filippo Maria Dini *, Monica Caffara, Joana G. P. Jacinto, Cinzia Benazzi, Arcangelo Gentile and Roberta Galuppi

Department of Veterinary Medical Sciences, University of Bologna, Ozzano dell'Emilia, 40064 Bologna, Italy
* Correspondence: filippomaria.dini@unibo.it

Simple Summary: In this study, a peculiar case of bovine eosinophilic myositis (BEM) observed in a beef cattle is described. BEM is a specific inflammatory myopathy, often associated with *Sarcocystis* spp., with multifocal gray-green lesions that can lead to considerable economic losses and public health issues. Through histological, molecular, and serological analyses, we confirmed the first detection of *T. gondii* DNA in a case of BEM, associated with the coinfection by *S. hominis*. Molecular results highlighted DNA of both pathogens within the lesion, in healthy muscle and or in the meat juice pellets, drawing attention to the possible role that a co-infection of *T. gondii* with *Sarcocystis* sp. may play in evoking BEM lesions.

Abstract: Bovine eosinophilic myositis (BEM) is a specific inflammatory myopathy, often associated with *Sarcocystis* spp., with multifocal gray-green lesions leading to carcass condemnation with considerable economic losses. Here is described a peculiar case of BEM that occurred in an adult (16 month) cattle, born in France, bred, and slaughtered in Italy at the end of 2021. On inspection, muscles showed the typical multifocal gray-green lesions that were sampled for, cytological, histological, and molecular investigations, while meat juice was subjected to IFAT for *Toxoplasma* IgG. Genomic DNA was extracted from lesions, portions of healthy muscle and from meat juice pellet and analyzed by PCR targeting 18S rDNA, COI mtDNA and B1 genes, and sequenced. The cytology showed inflammatory cells mostly referable to eosinophils; at histology, protozoan cysts and severe granulomatous myositis were observed. A BEM lesion and meat juice pellet subjected to PCR showed, concurrently, sequences referable both to *S. hominis* and *T. gondii*. Meat juice IFAT resulted negative for *T. gondii* IgG. Our findings highlight the first detection of *T. gondii* DNA in association with *S. hominis* in a BEM case, suggesting a multiple parasite infection associated with this pathology, although the actual role of *T. gondii* infection in the pathophysiology of the diseases should be clarified.

Keywords: *Sarcocystis hominis*; *Toxoplasma gondii*; BEM; cattle; meat-safety; Apicomplexa

Citation: Dini, F.M.; Caffara, M.; Jacinto, J.G.P.; Benazzi, C.; Gentile, A.; Galuppi, R. A Case of Bovine Eosinophilic Myositis (BEM) Associated with Co-Infection by *Sarcocystis hominis* and *Toxoplasma gondii*. *Animals* **2023**, *13*, 311. https://doi.org/10.3390/ani13020311

Academic Editor: Maurizio Francesco Brivio

Received: 24 December 2022
Revised: 11 January 2023
Accepted: 13 January 2023
Published: 16 January 2023

1. Introduction

Sarcocystis is an Apicomplexan parasite infecting several hosts including humans. Among the more than 200 *Sarcocystis* species at least six are recognized as infecting bovine muscular tissue, namely, *S. hirsuta*, *S. bovifelis*, *S. bovini*, *S. cruzi*, *S. hominis* and *S. heydorni*. Felids serve as definitive hosts of the first three species, canids are definitive hosts of *S. cruzi*, while *S. hominis* and *S. heydorni* are zoonotic [1–3].

Over the past few years, several species of *Sarcocystis* associated with eosinophilic lesions in bovine muscles have been reported. Their identification has been based on histological observation of the cyst wall thickness, separating the species forming thick-walled (2–7 µm) sarcocysts and thin-walled (<1 µm) sarcocysts [4]. The most common *Sarcocystis* species in cattle belonging to the first group are *S. hominis*, *S. bovifelis*, *S. bovini* and *S. hirsuta* [4–6], while the second group includes *S. cruzi* and *S. heydorni* [7,8]. As some of the abovementioned species forming thick- (*S. bovifelis* and S. *bovini*) and thin- (*S. heydorni*)

walled cysts have been recently described, misidentification may have occurred in the past with reference to etiology of bovine eosinophilic myositis [9].

Bovine eosinophilic myositis (BEM) is a specific inflammatory myopathy characterized by typical grey-green lesions in cattle muscles, visible during *post-mortem* inspection [4]. Although the etiology of the eosinophilic myositis remains uncertain [9], this condition in cattle is often associated with *Sarcocystis* sp. infection and can lead to carcass condemnation [10]. It has been hypothesized that the pathogenesis is linked with cysts' degeneration together with the establishment of a hypersensitivity response towards the parasite [11]. Supporting this hypothesis is the finding of intralesional *Sarcocystis* species inside the eosinophilic granulomatous lesions [4,5,7] and the ability of *Sarcocystis* sp. antigens to induce an eosinophilic granulocyte-mediated immune response [12]. However, except for the latter study, BEM has never been reported during experimental infection with *Sarcocystis* [11]. The prevalence of BEM is very low worldwide, ranging from 0.002 up to 5% [5], while the prevalence of *Sarcocysts* sp. infection in cattle is extremely high, with values in Italy ranging from 67.8% up to 95% [1,13–16]. A possible explanation of this discrepancy could be that BEM may be associated with one or more specific *Sarcocystis* species [5], including those that are zoonotic. For this reason, the correct identification of the species involved in BEM is crucial in order to assess the risk for the consumer of eating raw or undercooked meat [13,17].

So far sequencing the 18S rDNA has been widely used for *Sarcocystis* species identification, even if several authors pointed out that misidentification may occur due to high conservative characters of this gene, as for example among *S. bovini*, *S. bovifelis* and *S. hominis* [6,18–20]. Therefore, cytochrome C oxidase subunit I mitochondrial (COI mtDNA) gene has recently been exploited as a useful genetic marker for the Sarcocystidae and has proved to be useful in resolving unclear species boundaries of closely related *Sarcocystis* spp. in different hosts [2,18,21,22]. In the wake of this evidence, molecular techniques have been recently developed to clarify the identification of *Sarcocystis* species infecting cattle, confirming the higher discriminatory power of COI mitochondrial gene for *Sarcocystis* species identification [23]. A novel species-specific multiplex PCR assay for the simultaneous identification of all the species of the genus *Sarcocystis* reported in cattle in Italy has been recently developed by Rubiola et al. [19]. Through this new molecular approach, during an investigation on the presence of *Sarcocystis* species in BEM cases, the presence of *S. bovifelis* and *S. hominis* has been observed and seems to be considerably higher in specimens isolated from BEM condemned carcasses than in samples isolated from randomly sampled slaughter cattle [1]; anyway, *Sarcocystis* species seem to be predominant in different geographical areas [9].

Furthermore, possible co-infections between *Sarcocystis* spp. and other Apicomplexa parasites, such as *Toxoplasma gondii* have been described in cattle [24–27]. *Toxoplasma gondii* is a widespread zoonotic protozoon that can also lead to important economic impacts in livestock, causing mainly reproductive failure in small ruminants [28]. In contrast to small ruminants, cattle appear to be largely resistant to *T. gondii* infections and rarely showed the presence of tissue cysts [29]. Reports on clinical toxoplasmosis in naturally infected cattle are rare and comprised only abortions in association with the isolation of *T. gondii* from the fetuses [30].

Our study presents a severe case of BEM in a Limousine bull imported from France and fattened and slaughtered in Italy by characterizing in detail the gross-pathological, cytological and histological features and by identifying the associated etiology by serologic and molecular analysis.

2. Materials and Methods

2.1. Source Material

Portions of muscle (*gluteus*, *semimembranosus*, and *semitendinosus*) from a clinically healthy 16-month-old Limousine bull were conferred at the Department of Veterinary Medical Sciences of Bologna University because of suspected sarcosporidiosis. The bull was

born in France and imported to a fattening unit in the province of Verona (Italy) at the age of 10 months to be fattened and then slaughtered. No signs of disease were observed during the entire period of fattening in Italy and no differences in body weight were noticed when compared to the cohort animals. The carcass was rejected at slaughterhouse inspection due to the presence of macroscopic nodular green lesions on most muscle masses.

2.2. Gross Pathology, Cytology and Histology Investigation

A gross-pathologic examination of the skeletal muscles of the Limousine bull was carried out. Smears and impressions of different lesion were performed, fixed and stained with May-Grunwald Giemsa. Samples from both affected areas and healthy muscle were collected for histology. The samples were fixed in 10% neutral buffered formalin, trimmed, embedded in paraffin wax, sectioned at 3–4 µm, and stained with hematoxylin and eosin (H&E) for histological evaluation.

2.3. Meat Juice Extraction and Serology

Portions of the skeletal muscle (approximately 1 kg) were frozen in a plastic bag at −20 °C immediately after sampling and thawed at +4 °C overnight for collecting meat juice. After defrosting, approximately 1.5 mL of meat juice from the bag was transferred into sterile tubes (Eppendorf, Hamburg, Germany). Meat juice tubes were then centrifuged at 2500 rpm for 15 min to remove coarse particles, and the supernatant were tested by Immunofluorescence Antibody test (IFAT) by commercial antigen (Mega Cor Diagnostik, Horbranz, Osterreich) consisting of tachyzoites cultured on Vero cells and, as a conjugate, rabbit anti-bovine IgG (Sigma Immunochemicals, St. Louis, MO, USA) bound to fluorescein isothiocyanate (FITC) and diluted 1/300. An initial dilution of 1:4 (cut off) was used for the meat juice. The pellet was stored at −20 °C for downstream analyses.

2.4. Molecular Investigations

Genomic DNA was purified from different parts of the muscle: four samples from macroscopic lesions (ML) and four from muscle macroscopically healthy (HM), of 25 mg each, and one meat juice pellet (MJ), approximately 200 µL, using Pure Link ® Genomic DNA Mini kit (Invitrogen by Thermo Fisher), according to the manufacturer's protocol.

A first screening end-point PCR targeting 18S rDNA gene of Apicomplexa was performed on all of the samples with the primers COC-1 and COC-2, as described by Hornok et al. [31]. Briefly, a reaction volume of 25 µL, containing 12.5 µL 2× Dream Taq Hot Start Green PCR Master Mix (Thermo Scientific), 9.5 µL ddH2O, 0.25 µL (1 µM final concentration) of each primer, and 2.5 µL template DNA were used. For amplification, an initial denaturation step at 94 °C for 10 min was followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 54 °C for 30 s and an extension at 72 °C for 30 s. A final extension was performed at 72 °C for 10 min.

Additional Multiplex PCR assay, described by Rubiola et al. [19] was performed to simultaneously identify all of the species of the genus *Sarcocystis* actually reported in cattle in Italy, targeting 18S rDNA and COI mtDNA. The multiplex-PCR contained 2.5 µL of template DNA, 0.25 µL (0.5 mM) of each primer, Sarco Rev, Sar F, Hirsuta, Cruzi, COI HB, COI H and COI B, 12.5 µL 2× Dream Taq Hot Start Green PCR Master Mix to a total volume of 25 µL. The PCR assay involved a denaturation step at 95 °C for 3 min, followed by 35 cycles at 95 °C for 60 s, 58 °C for 60 s and 72 °C for 30 s and a final extension of 72 °C for 3 min.

Finally, a Nested PCR targeting the glycerol-3-phosphate dehydrogenase (B1 gene) of *Toxoplasma gondii* was performed as described by Jones et al. [32]. First round of amplification included a denaturation step at 96 °C for 2 min, followed by 40 cycles at 93 °C for 10 s, 57 °C for 10 s, and 72 °C for 30 s. The second round of amplification involved a denaturation step at 95 °C for 2 min, followed by 40 cycles at 93 °C for 10 s, 62.5 °C for 10 s, and 72 °C for 30 s. Amplifications were performed in a T-personal thermal cycler

(Biometra, Goettingen, Germany). In all of the abovementioned PCRs, water was included as a negative control.

The PCR products were electrophoresed on a 1% (for the first two assays) and 2% (for B1 nested PCR), agarose gel stained with SYBR Safe DNA Gel Stain (Thermo Fisher Scientific, Carlsbad, CA, USA) in 0.5× TBE. For sequencing, the amplicons were excised and purified by Nucleo-Spin Gel and PCR Clean-up (Mackerey-Nagel, Düren, Germany) and sequenced with an ABI 3730 DNA analyzer (StarSEQ, Mainz, Germany). All of the primers used in this study are reported in Table 1.

The trace files were assembled with Contig Express (VectorNTI Advance 11 software, Invitrogen, Carlsbad, CA, USA), and the consensus sequences were compared with published data by BLAST tools (https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 5 December 2022). Sequence alignments were carried out by BioEdit 7.2.5 [33], while p-distance and maximum-likelihood (ML) tree (K2+G substitution model for both genes and bootstrap of 1000 replicate) were calculated by MEGA 7 [34]. The sequences obtained in this study were deposited in GenBank under accession numbers OQ184854-56 (18SrDNA) and OQ190466-67 (COI mtDNA).

Table 1. Forward and reverse primers used in the different PCR assays.

	Primers	Gene	Primer Sequences	Product Length	Reference
18S Apicomplexa	COC-1 COC-2	18S	AAGTATAAGCTTTTATACGGCT CACTGCCACGGTAGTCCAATA	300 bp	[31]
Sarcocystis spp. Multiplex PCR	Sarco_Rev SarF Hirsuta Cruzi COI_HB COI_H COI_B	18S COI 	AACCCTAATTCCCCGTTA TGGCTAATACATGCGCAAATA CATTTCGGTGATTATTGG ATCAGATGAAAATCTACTACATGG AATGTGGTGCGGTATGAACT GGCACCAACGAACATGGTA TCAAAAACCTGCTTTGCTG	 200–250 bp 108 bp 300 bp 420 bp 700 bp	[15] [35] [15] [19]
B1 *Toxoplasma* nested PCR	I Round for I Round rev II Round for II Round rev	B1	GGAACTGCATCCGTTCATGAG TCTTTAAAGCGTTCGTGGTC- TGCATAGGTTGCAGTCACTG GGCGACCAATCTGCGAATACACC	96bp	[32]

3. Results

3.1. Gross-Pathological Findings

On gross pathology, skeletal muscles showed multifocal, firm, cohalescent green-yellowish round lesions with a diameter ranging from 0.1 to 1.5 cm. Some of the larger lesions (1.0–1.5 cm in diameter) had necrotic yellow-green content (Figure 1a), and the smaller (0.2–0.5 cm in diameter) were whitish in color and appeared solid or released only little yellowish-white material when squeezed (Figure 1b). Based on the gross pathology, a diagnosis of severe multifocal chronic myositis was formulated.

3.2. Cytological and Histological Findings

On cytology, inflammatory cells mostly referable to eosinophils were noticed.

Histologically, the muscle revealed extensive multifocal areas of necrosis with mineralization surrounded by fibrosis and inflammatory cells, mostly lymphoid cells (Figure 2a,b). In some areas, single muscle fibers were noted to be atrophic or with initial necrosis (in a longitudinal section made visible by hypertrophy, loss of transverse striation, and initial fragmentation) immersed in connective tissue with macrophages, giant cells, and lymphoid cells. Occasionally, the foci presented degenerate parasitic cysts surrounded by variable numbers of inflammatory cells (among which many eosinophils), or, at a later state, macrophages and giant cells forming a granulomatous lesion (Figure 2c). Intact protozoan

cysts (morphologically referable to *Sarcocystis* sp.) were detected in the unaffected muscle (Figure 2d).

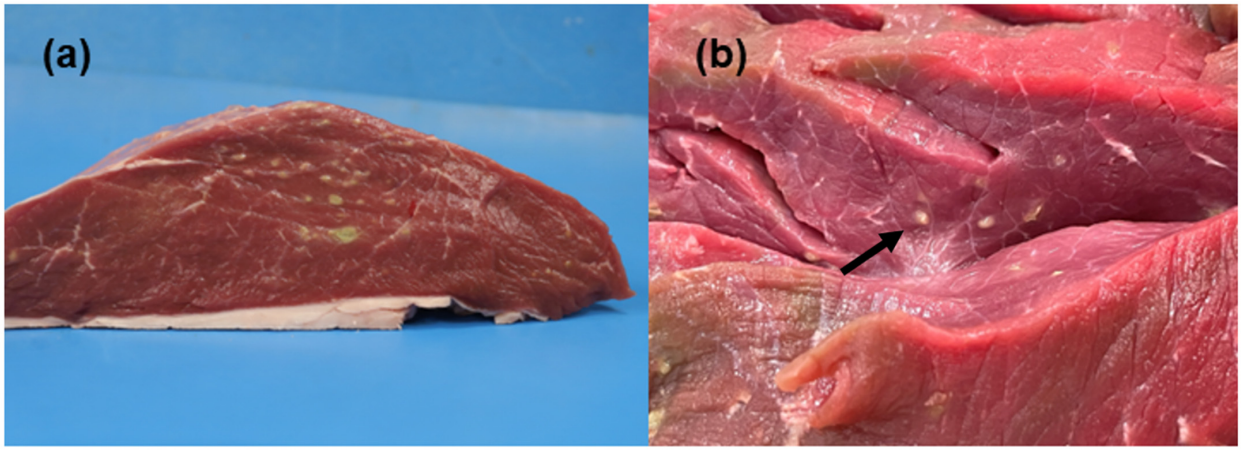


Figure 1. Macroscopic appearance of the infected skeletal muscle. (**a**) Note the multifocal yellowish-green round lesions, ranging from 0.1 to 1cm of diameter, with a necrotic content in a cross-section of skeletal muscle. (**b**) Note the white round lesions and a green discoloration area of the muscle (arrow).

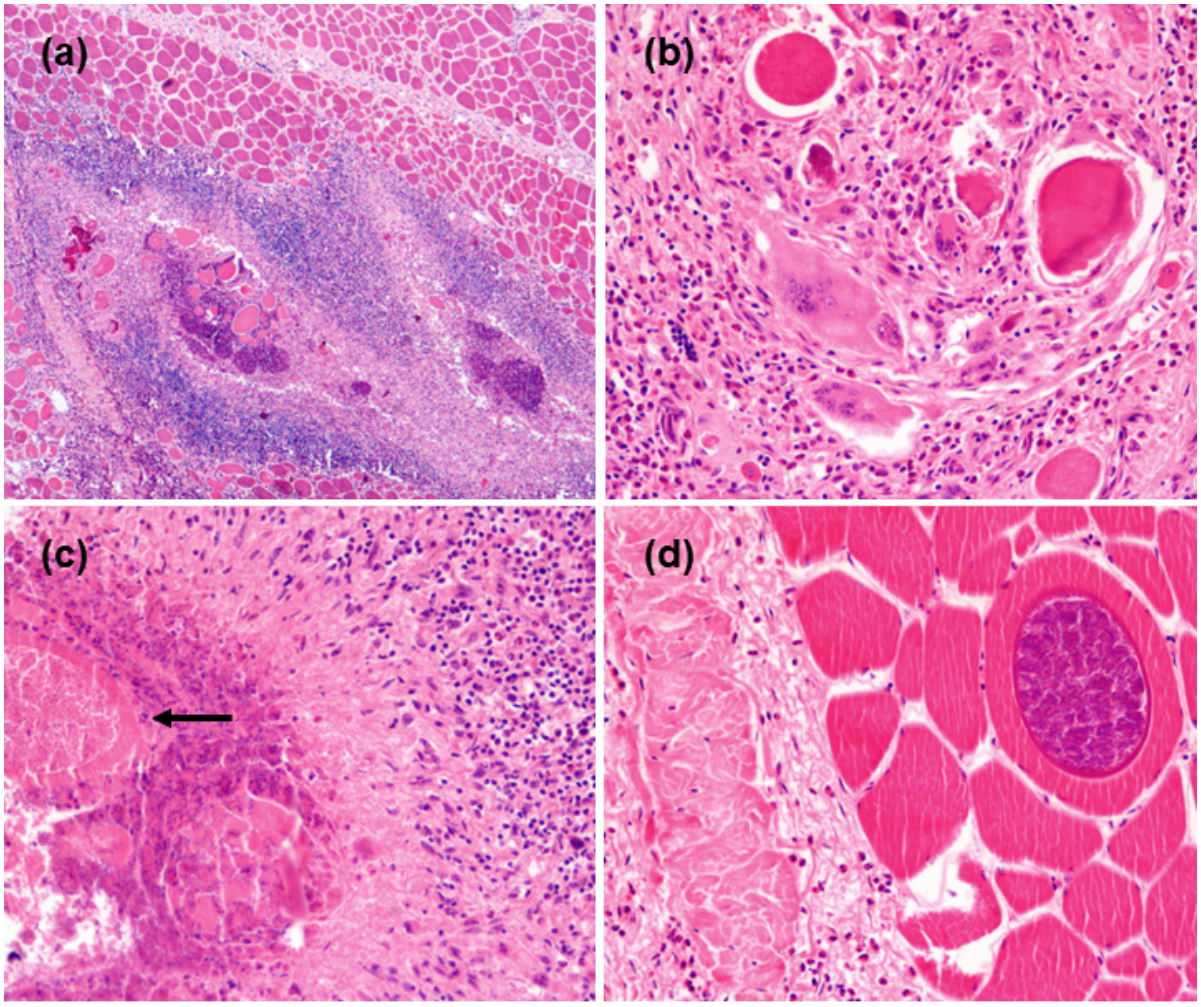


Figure 2. Histological appearance of the infected skeletal muscle. (**a**) A vast lesion showing mineralized foci of necrotic tissue surrounded by inflammation, mostly mononuclear elements. H.&E. 4× (**b**) Higher

magnification shows a few necrotic fibers in the center of inflammatory tissue composed by palisades epithelioid macrophages, multinucleated giant cells, admixed with eosinophils and lymphoid tissue. H.&-E. 20× (**c**) On the left, extensive necrotic material in which the debris of a protozoan cyst are visible (arrow). Around them a layer of necrotic inflammatory cells surrounded by granulomatous tissue. H.&E. 10× (**d**) Top right: A cyst in the middle of a viable skeletal muscle fiber, with no inflammation. Left: Thick connective tissue (fibrosis) with scattered eosinophils. H.&E.10×.

Histologically, the retrieved findings were compatible with a severe granulomatous myositis.

3.3. Serological and Molecular Results

The meat juice IFAT resulted negative for *T. gondii* specific IgG at a 1:4 dilution.

Concerning the molecular analyses, all of the PCR assays successfully amplified all of the three matrices examined. In detail, in the PCR targeting the Apicomplexa 18S rDNA, all nine specimens were positive, with a band of ~ 300 bp. Sequences were obtained from six samples (4 ML, 1 HM, 1 MJ) and a BLAST search gave 99.6% identity with *S. hominis* in five (3 ML, 1 HM, 1 MJ), and 99.6–99.3% *Hammondia hammondi*/*T. gondii* only from ML.

The same samples tested with the multiplex PCR specific for cattle *Sarcocystis* showed a band of 420 bp of *S. hominis*. To confirm the results, the COI mtDNA of two samples (1 ML, 1 MJ) were sequenced. A BLAST search gave a 99.5% (ML) and a 99.7% (MJ) identity with *S. hominis*.

Finally, the nested PCR targeting B1 gene of *T. gondii* showed amplification of all the nine samples, with an amplicon of approximately of 96 bp. Unfortunately, only one specimen (MJ) gave a readable sequence showing 100% identity with *T. gondii* (Table 2).

Table 2. Results of PCR and Sequencing on tested samples.

Sample	18s PCR [31]	Multiplex Sarcocystis PCR [19]	B1 Toxoplasma Nested-PCR [32]
ML (4 samples)	4 PCR positive * (3 *S. hominis*, 1 *Toxoplasma/Hammondia*)	4 *S. hominis* PCR positive (1 *S. hominis*)	4 PCR Positive (no sequence)
HM (4 samples)	4 PCR Positive (1 *S. hominis*)	4 *S. hominis* PCR positive	4 PCR Positive (no sequence)
MJ (1 sample)	1 PCR Positive (1 *S. hominis*)	1 *S. hominis* PCR positive (1 *S. hominis*)	1 PCR Positive (1 *T. gondii*)

* In round brackets the results of Sanger sequencing.

The p-distance of the 18S rDNA *S. hominis* specimens (HM, ML and MJ) showed 0% genetic variability to the same species retrieved from GenBank and used for building the ML tree. The interspecific p-distance was 0.2–0.3% with *S. bovini* and *S. bovifelis*, respectively, reaching 1.2–1.3 and 1.4% with *S. hirsuta*, *S. heydorni* and *S. cruzi*, respectively. Concerning the more variable COI mtDNA the intraspecific variability among *S. hominis* was 0.1–0.2%, while the interspecific divergence was 0.9–1% with *S. bovini* and *S. bovifelis*, respectively, and 1.6–2.5 and 2.3 % with *S. hirsuta*, *S. heydorni* and *S. cruzi*, respectively.

The ML tree of both molecular markers showed the same topology (Figure 3a,b); our specimens (both HM and MJ) are included in the *S. hominis* cluster and closely related to *S. bovini* and *S. bovifelis*. Moreover, the *T. gondii* sample formed a cluster with the other member of the family Sarcocystidae (*H. hammondi*/*T. gondii* and *N. caninum*).

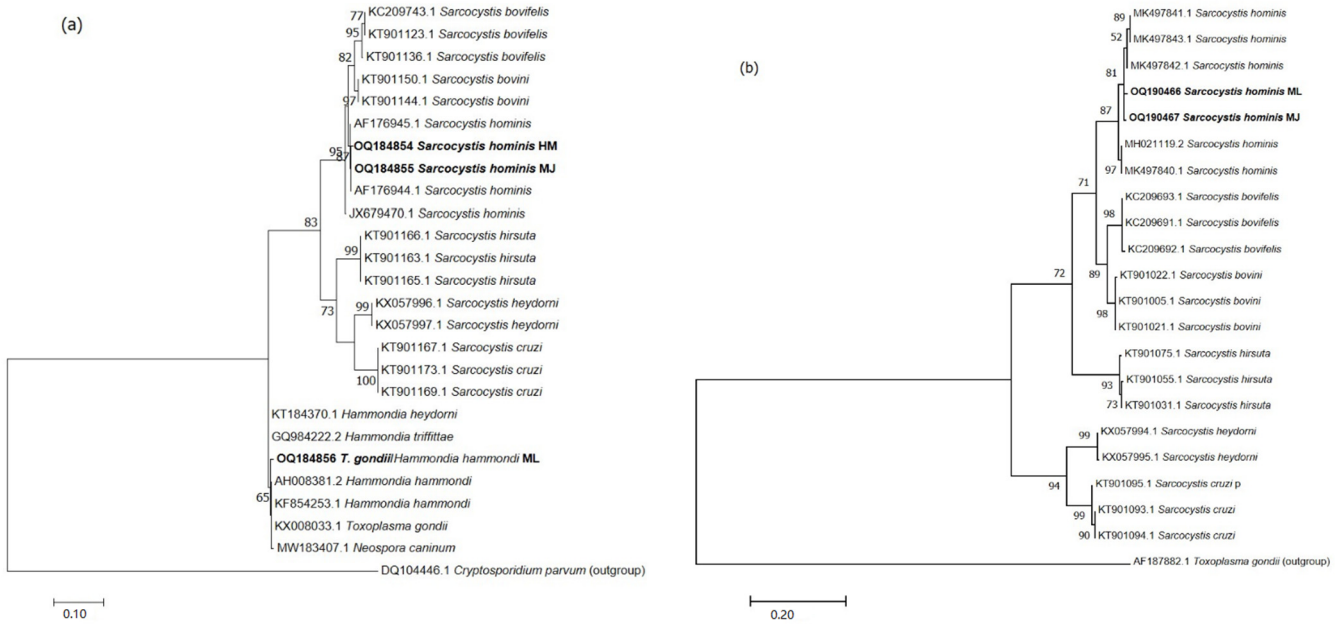


Figure 3. Maximum Likelihood trees inferred from Sarcocystis homins and Toxoplasma gondii 18S rDNA (**a**) and COI mtDNA (**b**) from this study and sequences retrieved from GenBank. The evolutionary history was computed with the Kimura 2-parameter model. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. There were a total of 207 position for 18S rDNA and 348 for COI mtDNA in the final data sets. Our specimens are in bold. HM = healthy muscle; ML = macroscopic lesion; MJ = meat juice pellet.

4. Discussion

This paper reports for the first time co-infection by *S. hominis* and *T. gondii* in a severe case of BEM, resulting in carcass condemnation at slaughter. *Sarcocystis* spp. infection is common in cattle, but the development of BEM only occurs in some cases and seems to be linked to the presence of some *Sarcocystis* spp. [4]. In Italy, it has been seen to be associated mainly with *S. bovifelis*, *S. hominis* and *S. cruzi* [1]. Occasionally, as shown in the present report, muscular involvement can be extremely severe, causing carcass discard and high economic losses. The histopathological lesions observed in this study were similar to those described by Wouda [4] in a case of BEM in a beef cow due to *S. hominis*.

The correct identification of the etiological agents involved in BEM are of primary importance as the cattle muscle can be infected by some important zoonotic parasites, i.e., *Sarcocystis* spp. and *T. gondii*. In this view, the identification at species level of the former genus should be considered of primary importance in order to discriminate the zoonotic from the non-zoonotic species. In fact, *S. hominis* and *S. heydorni* are meat-borne zoonotic parasites that can be transmitted by eating raw or undercooked meat, a very common practice in all the southern regions of Europe [13,17,18].

In our study, depending on the method used, both *S. hominis* and *T. gondii* were detected in all of the three matrices tested (ML, HM and MJ). In particular by the species-specific multiplex PCR for *Sarcocystis* spp. [19], the *S. hominis*-related amplicon was detected in all matrices, as well as with the nested PCR targeting B1 gene of *T. gondii* [32].

The less specific PCR targeting of the 18S rDNA of Apicomplexa [32], did not allow for properly identifying *T. gondii* (BLAST result *H. hammondi*/*T. gondii*), which was confirmed only by the B1 specific gene.

Despite the two Apicomplexa detected in all matrices examined, sequences of good quality were obtained mostly for *S. hominis*, probably due to the high presence of this species also in healthy tissues (observed in histological sections).

Lastly, in our study, the sediment of meat juice was used as a target for the DNA extraction and amplification, and to the best of our knowledge this is the first time that

such a matrix has been used for this purpose. Interestingly, only from this matrix were we able to obtain a readable sequence of *T. gondii* B1 gene, probably because the staring amount of parasite was higher than in lesions and in healthy muscle.

Therefore, based on our results, meat juice sediment proved to be a promising matrix to be tested for the molecular diagnosis of cysts-forming protozoans infecting bovine muscle, independently of the presence of BEM lesions.

Co-infections in cattle between *T. gondii* and *Sarcocystis* spp. have been already described [24–27]. To the best of our knowledge, this is the first report of the presence of *T. gondii* DNA in a case of BEM. However, its role in evoking the disease is unclear, as the infection in cattle is usually asymptomatic [28] and the reported cases are mainly related to reproductive failure [30]. Nevertheless, ingestion of *T. gondii* tissue cysts from infected meat is a major route of infection for humans, with consumption of raw or undercooked meat from infected animals considered a significant public health risk [36]. There is evidence suggesting the important role of the beef as a source of human infection [37–39]. The reported seroprevalence for *T. gondii* infection in cattle varies between different countries, for example, 83.3% (n = 504) in southern Spain [40], 45.6% (n = 406) in Switzerland [41], and 10.2% in Italian beef cattle [42].

Contrastingly to what happens in other animal species, in cattle the seroprevalence does not give a valid indication of the risk for human infection by eating meat because cattle can eliminate their tissue cysts while remaining seropositive [43]. Possibly, only recently infected animals, which have not yet developed antibodies, have a parasite load high enough to be detectable by direct assays [44]. This is confirmed also in the presented study in which the animal was seronegative for IgG against *T. gondii* by IFAT on meat juice, while *T. gondii* DNA was observed in muscle.

The finding of *T. gondii* DNA within the BEM lesions draws attention to the possible role that a co-infection of this parasite with *Sarcocystis* sp. may play in evoking BEM lesions. In the human context, although toxoplasmosis is not a well-recognized cause of eosinophilia, the literature suggests that associated factors, such as coinfection with other parasites or drug hypersensitivity, may play a role in the development of eosinophilia with acquired toxoplasmosis [45,46]. However, this is only a possible pathogenetic analogy, as these two host species (bovine and human) have a totally different susceptibility to *T. gondii*, with very different consequences of infection.

5. Conclusions

In conclusion, we described in this paper the first detection of *T. gondii* DNA in a severe case of bovine eosinophilic myositis associated with a coinfection by *S. hominis*. Molecular results highlighted DNA of both pathogens within the lesion in healthy muscle and or in the meat juice pellets, laying the groundwork for a possible etio-pathogenetic correlation between co-infection and the development of BEM. However, the role of *T. gondii* in the pathogenesis of eosinophilic myositis is completely unclear, and further studies on co-infection of *T. gondii* and *Sarcocystis* sp. in BEM cases are necessaryto understand the possible role of *Toxoplasma* in this condition that impacts food safety and the economy of the livestock sector.

Author Contributions: Conceptualization, F.M.D. and J.G.P.J.; methodology, F.M.D., M.C. and R.G.; software, M.C.; validation, R.G., M.C. and C.B.; formal analysis, F.M.D. and M.C.; investigation, J.G.P.J., A.G., R.G. and F.M.D.; resources, J.G.P.J. and A.G.; data curation, F.M.D. and M.C.; writing—original draft preparation, F.M.D.; writing—review and editing, M.C., C.B. and R.G.; visualization, M.C. and A.G.; supervision, M.C. and R.G.; project administration, F.M.D. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: This study did not require official or institutional ethical approval as it was not an experimental study but part of a veterinary diagnostic case.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: GenBank accession numbers OQ184854-56 (18SDNA), OQ190466-67 (COI mtDNA).

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Rubiola, S.; Civera, T.; Panebianco, F.; Vercellino, D.; Chiesa, F. Molecular Detection of Cattle *Sarcocystis* spp. in North-West Italy highlights their association with bovine eosinophilic myositis. *Parasites Vectors* **2021**, *14*, 1–8. [CrossRef]
2. Gjerde, B. Molecular Characterisation of *Sarcocystis bovifelis*, *Sarcocystis bovini* sp., *Sarcocystis hirsuta* and *Sarcocystis cruzi* from Cattle (*Bos taurus*) and *Sarcocystis sinensis* from Water Buffaloes (*Bubalus bubalis*). *Parasitol. Res.* **2016**, *115*, 1473–1492. [CrossRef] [PubMed]
3. Dubey, J.P.; van Wilpe, E.; Calero-Bernal, R.; Verma, S.K.; Fayer, R. *Sarcocystis heydorni*, n. sp. (Apicomplexa: Sarcocystidae) with cattle (*Bos taurus*) and human (*Homo sapiens*) cycle. *Parasitol. Res.* **2015**, *114*, 4143–4147. [CrossRef] [PubMed]
4. Wouda, W.; Snoep, J.J.; Dubey, J.P. Eosinophilic myositis due to *Sarcocystis hominis* in a beef cow. *J. Comp. Pathol.* **2006**, *135*, 249–253. [CrossRef] [PubMed]
5. Vangeel, L.; Houf, K.; Geldhof, P.; De Preter, K.; Vercruysse, J.; Ducatelle, R.; Chiers, K. Different *Sarcocystis* spp. Are present in bovine eosinophilic myositis. *Vet. Parasitol.* **2013**, *197*, 543–548. [CrossRef]
6. Hoeve-Bakker, B.J.A.; van der Giessen, J.W.B.; Franssen, F.F.J. Molecular identification targeting Cox1 and 18s genes confirms the high prevalence of *Sarcocystis* spp. in cattle in the Netherlands. *Int. J. Parasitol.* **2019**, *49*, 859–866. [CrossRef]
7. Gajadhar, A.A.; Marquardt, W.C. Ultrastructural and transmission evidence of *Sarcocysis cruzi* associated with eosinophilic myositis in cattle. *Can. J. Vet. Res.* **1992**, *56*, 41. [PubMed]
8. Waine, K.; Bartley, P.M.; Cox, A.; Newsome, R.; Strugnell, B.; Katzer, F. Molecular detection of *Sarcocystis cruzi* in three beef carcases with eosinophilic myositis lesions and in unaffected beef from animals in the same herd. *Vet. Parasitol. Reg. Stud. Rep.* **2022**, *33*, 100751. [CrossRef]
9. Dubey, J.P.; Rosenthal, B.M. Bovine sarcocystosis: *Sarcocystis* species, diagnosis, prevalence, economic and public health considerations, and association of *Sarcocystis* species with eosinophilic myositis in cattle. *Int. J. Parasitol.* 2022, *in press*. [CrossRef]
10. Lindsay, D.S.; Dubey, J.P. Neosporosis, Toxoplasmosis, and Sarcocystosis in ruminants: An update. *Vet. Clin. N. Am. Food Anim. Pract.* **2020**, *36*, 205–222. [CrossRef]
11. Dubey, J.P.; Calero-Bernal, R.; Rosenthal, B.M.; Speer, C.A.; Fayer, R. *Sarcocystosis of Animals and Humans*, 2nd ed.; CRC Press: Boca Raton, FL, USA, 2015; ISBN 9781498710152.
12. Vangeel, L.; Houf, K.; Geldhof, P.; Nollet, H.; Vercruysse, J.; Ducatelle, R.; Chiers, K. Intramuscular inoculation of cattle with *Sarcocystis* antigen results in focal eosinophilic myositis. *Vet. Parasitol.* **2012**, *183*, 224–230. [CrossRef]
13. Bucca, M.; Brianti, E.; Giuffrida, A.; Ziino, G.; Cicciari, S.; Panebianco, A. Prevalence and distribution of *Sarcocystis* spp. cysts in several muscles of cattle slaughtered in Sicily, Southern Italy. *Food Control* **2011**, *22*, 105–108. [CrossRef]
14. Domenis, L.; Peletto, S.; Sacchi, L.; Clementi, E.; Genchi, M.; Felisari, L.; Felisari, C.; Mo, P.; Modesto, P.; Zuccon, F.; et al. Detection of a morphogenetically novel *Sarcocystis hominis*-like in the context of a prevalence study in semi-intensively bred cattle in Italy. *Parasitol. Res.* **2011**, *109*, 1677–1687. [CrossRef] [PubMed]
15. Chiesa, F.; Muratore, E.; Dalmasso, A.; Civera, T. A New Molecular approach to assess the occurrence of *Sarcocystis* spp. in cattle and products thereof: Preliminary data. *Ital. J. Food Saf.* **2013**, *2*, 148–151. [CrossRef]
16. Meistro, S.; Peletto, S.; Pezzolato, M.; Varello, K.; Botta, M.; Richelmi, G.; Biglia, C.; Baioni, E.; Modesto, P.; Acutis, P.; et al. *Sarcocystis* spp. prevalence in bovine minced meat: A histological and molecular study. *Ital. J. Food Saf.* **2015**, *4*, 85–87. [CrossRef] [PubMed]
17. Dubey, J.P. Foodborne and waterborne zoonotic sarcocystosis. *Food Waterborne Parasitol.* **2015**, *1*, 2–11. [CrossRef]
18. Moré, G.; Schares, S.; Maksimov, A.; Conraths, F.J.; Venturini, M.C.; Schares, G. Development of a multiplex real time PCR to differentiate *Sarcocystis* spp. affecting cattle. *Vet. Parasitol.* **2013**, *197*, 85–94. [CrossRef]
19. Rubiola, S.; Civera, T.; Ferroglio, E.; Zanet, S.; Zaccaria, T.; Brossa, S.; Cipriani, R.; Chiesa, F. Molecular Differentiation of Cattle *Sarcocystis* spp. by Multiplex PCR Targeting 18S and COI Genes Following Identification of Sarcocystis hominis in Human Stool Samples. *Food Waterborne Parasitol.* **2020**, *18*, e00074. [CrossRef]
20. Prakas, P.; Strazdaitė-Žielienė, Ž.; Januškevičius, V.; Chiesa, F.; Baranauskaitė, A.; Rudaitytė-Lukošienė, E.; Servienė, E.; Petkevičius, S.; Butkauskas, D. Molecular identification of four *Sarcocystis* species in cattle from Lithuania, including *S. hominis*, and development of a rapid molecular detection method. *Parasit. Vectors.* **2020**, *13*, 610. [CrossRef]
21. Gjerde, B. Phylogenetic Relationships among *Sarcocystis* Species in Cervids, Cattle and Sheep inferred from the mitochondrial cytochrome c oxidase subunit i gene. *Int. J. Parasitol.* **2013**, *43*, 579–591. [CrossRef]
22. Zeng, H.; Van Damme, I.; Kabi, T.W.; Šoba, B.; Gabriël, S. *Sarcocystis* species in bovine carcasses from a Belgian abattoir: A cross-sectional study. *Parasit. Vectors.* **2021**, *14*, 271. [CrossRef]
23. Rubiola, S.; Chiesa, F.; Zanet, S.; Civera, T. Molecular Identification of *Sarcocystis* spp. in Cattle: Partial Sequencing of Cytochrome C Oxidase Subunit 1 (COI). *Ital. J. Food Saf.* **2018**, *7*, 204–208. [CrossRef]
24. Moré, G.; Basso, W.; Bacigalupe, D.; Venturini, M.C.; Venturini, L. Diagnosis of *Sarcocystis cruzi*, *Neospora caninum*, and *Toxoplasma gondii* infections in cattle. *Parasitol. Res.* **2008**, *102*, 671–675. [CrossRef] [PubMed]

25. Amairia, S.; Rouatbi, M.; Rjeibi, M.R.; Gomes, J.; Rekik, M.; Darghouth, M.A.; Gharbi, M. Molecular detection of *Toxoplasma gondii* and *Sarcocystis* spp. co-infection in tunisian merguez, a traditional processed sausage beef meat. *Food Control* **2021**, *121*, 107618. [CrossRef]
26. Langoni, H.; Generoso, D.; Hayasaka, Ê.Y.; Mantovan, K.B.; Menozzi, B.D.; Richini-Pereira, V.B.; da Silva, R.C. Molecular characterization of *Toxoplasma gondii* and *Sarcocystis* spp. in raw kibbeh and other meat samples commercialized in Botucatu, Southeastern Brazil. *Rev. Bras. Parasitol. Vet.* **2021**, *30*, 1–9. [CrossRef] [PubMed]
27. Hooshyar, H.; Chehrazi, F.; Arbabi, M. Molecular identification and frequency of cyst-forming coccidia (*Sarcocystis*, *Toxoplasma gondii*, and *Neospora caninum*) in native slaughtered cattle in Kashan, Central Iran. *Int. Arch. Health Sci.* **2021**, *8*, 301. [CrossRef]
28. Stelzer, S.; Basso, W.; Benavides Silván, J.; Ortega-Mora, L.M.; Maksimov, P.; Gethmann, J.; Conraths, F.J.; Schares, G. *Toxoplasma gondii* infection and toxoplasmosis in farm animals: Risk factors and economic impact. *Food Waterborne Parasitol.* **2019**, *15*, e00037. [CrossRef]
29. Dubey, J.P. A Review of Toxoplasmosis in cattle. *Vet. Parasitol.* **1986**, *22*, 177–202. [CrossRef] [PubMed]
30. Canada, N.; Meireles, C.S.; Rocha, A.; da Costa, J.M.C.; Erickson, M.W.; Dubey, J.P. Isolation of viable *Toxoplasma gondii* from naturally infected aborted bovine fetuses. *J. Parasitol.* **2002**, *88*, 1247–1248. [CrossRef]
31. Hornok, S.; Mester, A.; Takács, N.; Baska, F.; Majoros, G.; Fok, É.; Biksi, I.; Német, Z.; Hornyák, Á.; Jánosi, S.; et al. *Sarcocystis*-infection of cattle in Hungary. *Parasites Vectors* **2015**, *8*, 4–9. [CrossRef]
32. Jones, C.D.; Okhravi, N.; Adamson, P.; Tasker, S. Comparison of PCR detection methods for B1, P30, and 18S rDNA genes of *T. gondii* in aqueous humor. *Investig. Ophthalmol. Vis. Sci.* **2000**, *41*, 634–644.
33. Hall, T.A. BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp. Ser.* **1999**, *41*, 95–98.
34. Kumar, S.; Stecher, G.; Tamura, K. MEGA7: Molecular Evolutionary Genetics Analysis version 7 for bigger datasets. *Mol. Biol. Evol.* **2016**, *33*, 1870–1874. [CrossRef] [PubMed]
35. Vangeel, L.; Houf, K.; Chiers, K.; Vercruysse, J.; D'Herde, K.; Ducatelle, R. Molecular-based identification of *Sarcocystis hominis* in Belgian minced beef. *J. Food Prot.* **2007**, *70*, 1523–1526. [CrossRef]
36. Guo, M.; Dubey, J.P.; Hill, D.; Buchanan, R.L.; Ray Gamble, H.; Jones, J.L.; Pradhan, A.K. Prevalence and risk factors for *Toxoplasma gondii* infection in meat animals and meat products destined for human consumption. *J. Food Prot.* **2015**, *78*, 457–476. [CrossRef]
37. Baril, L.; Ancelle, T.; Goulet, V.; Thulliez, P.; Tirard-Fleury, V.; Carme, B. Risk Factors for *Toxoplasma* Infection in pregnancy: A case-control study in France. *Scand. J. Infect. Dis.* **1999**, *31*, 305–309. [CrossRef] [PubMed]
38. Cook, A.J.; Gilbert, R.E.; Buffolano, W.; Zufferey, J.; Petersen, E.; Jenum, P.A.; Foulon, W.; Semprini, A.E.; Dunn, D.T. Sources of *Toxoplasma* infection in pregnant women: European multicentre case-control study. european research network on congenital toxoplasmosis. *BMJ* **2000**, *321*, 142–147. [CrossRef] [PubMed]
39. Jones, J.L.; Dargelas, V.; Roberts, J.; Press, C.; Remington, J.S.; Montoya, J.G. Risk Factors for *Toxoplasma gondii* Infection in the United States. *Clin. Infect. Dis. Off. Publ. Infect. Dis. Soc. Am.* **2009**, *49*, 878–884. [CrossRef]
40. García-Bocanegra, I.; Cabezón, O.; Hernández, E.; Martínez-Cruz, M.S.; Martínez-Moreno, Á.; Martínez-Moreno, J. *Toxoplasma gondii* in ruminant species (cattle, sheep, and goats) from Southern Spain. *J. Parasitol.* **2013**, *99*, 438–440. [CrossRef]
41. Berger-Schoch, A.E.; Bernet, D.; Doherr, M.G.; Gottstein, B.; Frey, C.F. *Toxoplasma gondii* in Switzerland: A serosurvey based on meat juice analysis of slaughtered pigs, wild boar, sheep and cattle. *Zoonoses Public Health* **2011**, *58*, 472–478. [CrossRef]
42. Gazzonis, A.L.; Marino, A.M.F.; Garippa, G.; Rossi, L.; Mignone, W.; Dini, V.; Giunta, R.P.; Luini, M.; Villa, L.; Zanzani, S.A.; et al. Toxoplasma gondii seroprevalence in beef cattle raised in Italy: A multicenter study. *Parasitol. Res.* **2020**, *119*, 3893–3898. [CrossRef]
43. Opsteegh, M.; Teunis, P.; Züchner, L.; Koets, A.; Langelaar, M.; van der Giessen, J. Low predictive value of seroprevalence of *Toxoplasma gondii* in cattle for detection of parasite DNA. *Int. J. Parasitol.* **2011**, *41*, 343–354. [CrossRef] [PubMed]
44. Dubey, J.P. Isolation of *Toxoplasma gondii* from a naturally infected beef cow. *J. Parasitol.* **1992**, *78*, 151–153. [CrossRef] [PubMed]
45. Guneratne, R.; Mendis, D.; Bandara, T.; Fernando, S.D. *Toxoplasma*, *Toxocara* and tuberculosis co-infection in a four year old child. *BMC Pediatr.* **2011**, *11*, 44. [CrossRef] [PubMed]
46. Yusuf, I.H.; Sahare, P.; Hildebrand, G.D. DRESS Syndrome in a child treated for *toxoplasma* retinochoroiditis. *J. AAPOS Off. Publ. Am. Assoc. Pediatr. Ophthalmol. Strabismus* **2013**, *17*, 521–523. [CrossRef] [PubMed]

Chapter 7

Genotype characterization of *Toxoplasma gondii* in Italy: multiple typing approach from captive and livestock species confirm the circulation of type II variant

Dini FM, Salas-Fajardo M, Caffara M, Taddei R, Galuppi R. (2023). Genotype characterization of *Toxoplasma gondii* in Italy: multiple typing approach from captive and livestock species confirm the circulation of type II variant. Submitted to *Parasitology Research*.

Genotype characterization of *Toxoplasma gondii* in Italy: multiple typing approach from captive and livestock species confirms the circulation of Type-II variants

Filippo Maria Dini[1], Martha Ynés Salas-Fajardo[2], Monica Caffara[1], Roberta Taddei[3], Roberta Galuppi[1]

[1]Department of Veterinary Medical Sciences (DIMEVET), *Alma Mater Studiorum* University of Bologna, Via Tolara di Sopra 50, 40064 Ozzano Emilia (BO), Italy

[2]SALUVET, Department of Animal Health, Faculty of Veterinary, Complutense University of Madrid, Madrid, 28040, Spain.

[3]Istituto Zooprofilattico Sperimentale Della Lombardia e della Emilia-Romagna

Abstract

Toxoplasma gondii is a widespread foodborne parasite affecting both humans and animals worldwide. Genetic characterization of this parasite has become of crucial importance for epidemiological and clinical implications. This study focused on the direct genetic characterization of *T. gondii*-positive DNA samples in Italy, with different standardized genotyping methods. An attempt to quantify a total of 87 DNA samples that had tested positive for *T. gondii* and were obtained from livestock and wild animals in Northern Italy was undertaken using quantitative PCR. Only 11 were positive with specific qPCR and only three gave an adequate typing by microsatellite analysis, PCR-RFLP, and partial sequencing of GRA6 and SAG3 genes**.** Predominantly, Type II strains were observed, with non-clonal variants identified in sheep and a lemur. Sequencing of SAG3 revealed specific single nucleotide polymorphism previously documented in European Type-II variants. This study provides valuable insights into the epidemiology of *T. gondii* in Italy. Understanding the diversity and transmission dynamics of this parasite is crucial for managing its impact on both human and animal health.

Keywords: *Toxoplasma gondii*, Genotyping, Microsatellite analysis, RFLP, Zoonosis

1. Introduction

The protozoan parasite *Toxoplasma gondii*, a member of the Apicomplexa, family Sarcocystidae, is known to infect a wide range of warm-blooded animals. This pathogen poses a specific threat to food safety within the European Union (EFSA, 2018) and ranks as the second leading cause of foodborne illnesses in the United States (Scallan et al., 2011). Infection primarily result from the consumption of undercooked meat containing viable tissue cysts or the ingestion of food and water contaminated with oocysts (Pereira et al., 2010; Guo et al., 2015). This infection are closely linked to harmful effects, such as reproductive failure in pregnant women, neurological manifestations in immunocompromised individuals, and ocular diseases in otherwise healthy humans.

Toxoplasma gondii has significant implications for animal health as well, especially in the ovine industry, resulting in substantial global economic losses (Katzer et al., 2011; Stelzer et al., 2019; Dubey, 2022a). Some animal species are highly susceptible to fatal toxoplasmosis, such as lemurs, New World non-human primates (NWNHPs), and Australian marsupials, often experiencing sudden death upon infection (Carrossino et al., 2021; Rocchigiani et al., 2022; Salas-Fajardo et al., 2023). The genotyping of *T. gondii* strains plays a pivotal role in understanding the global population structure of the parasite. Genomic diversity within *T. gondii* may significantly influence its epidemiology, impacting factors such as host adaptation in definitive and intermediate hosts (Lilue et al., 2013; Khan et al., 2014; Hamidovic et al., 2021). Furthermore, some *T. gondii* genotypes are known to exhibit higher virulence towards specific hosts compared to others (Khan et al., 2006; Behnke et al., 2011). These differences in virulence may not only exist between different host species but also at the intra-individual level (Calero Bernal et al., 2022).

In the last decade, Italy has witnessed numerous studies employing diverse genotyping techniques to analyze clinical and epidemiological *T. gondii* isolates identifying primarily the Type II variants and, to a lesser extent, Type III.

Type II and III have been indeed reported in two different reports on striped dolphin on Italian coasts, through Multilocus Sequencing genotyping (Di Guardo et al., 2011) and through PCR-Restriction Fragment Length Polymorphism (PCR-RFLP) genotyping and subtyping with PCR-Sequencing (Fernández-Escobar et al., 2022) In a terrestrial context, *T. gondii* Type II, and less frequently Type III, have been widely observed in various hosts through different typing methods: in sheep flocks following abortion outbreaks (Chessa et al., 2014), from slaughter samples (Vismarra et al., 2017), and in wild animals such as corvids (Mancianti et al., 2020), red deer (Rocchigiani et al., 2016) and Eurasian otter (Viscardi et al., 2021). Few other reports have identified genetic variants rarely described in Europe, such as Type I variant in wild and domestic mammals from Northern (Battisti et al., 2018) and Southern regions (Sgroi et al., 2020), though involving the analysis few numbers of typing loci. These findings contrast with results from other European countries where Type II is the most prevalent *T. gondii* genotype (Richomme et al., 2009; Aubert et al., 2010; Calero-Bernal et al., 2015).

Given the limited information available on *T. gondii* genotype characterization in Italy, this study aims to genotype samples collected from both epidemiological surveys and clinical outbreaks of toxoplasmosis in animals from northern Italy, employing a complementary analysis of three distinct genotyping approaches: Microsatellite analysis, PCR-RFLP, and Multilocus Sequencing.

2. Materials and Methods

In this study, we carried out genetic characterization of *T. gondii* on positive samples tested during epidemiological surveys and routine diagnostic procedures. The 87 specimens were collected from both wild and domestic animals, all of which were screened for *T. gondii* using a nested PCR targeting B1 gene of *T. gondii* (Dini et al., 2023) (see Table 1 for details).

Toxoplasma-specific DNA quantification was performed using a duplex qPCR assay, adapted from Slany et al. (2019). It included the amplification of the species-specific 529 bp-RE locus and an internal amplification control (IAC) to aid the identification of false negative results (Slana et al., 2008). Reaction details, amplification condition and quantification acquisition were performed following Fernández-Escobar et al. (2022)

Samples with Ct values < 36 were subjected to genotyping by 15 microsatellite (MS) markers analyzed in a multiplex PCR assay (Ajzenberg et al. 2010). For *T. gondii* MS typing, we used a set of up to 15 markers located on 11 different chromosomes of the *T. gondii* genome, including eight lineage typing markers (B18, M33, TUB2, XI.1, TgM-A, W35, IV.1, and B17) and seven fingerprinting markers (N61, M48, N83, N82, N60, M102, and AA), used to resolve different isolates, applicable to both archetypal (type I, II, or III) and non-archetypal lineages (Ajzenberg et al., 2010, Joeres et al., 2023). Multiplex PCR, fragment size measurement, and typing follow Joeres et al. (2023).

Additionally, samples with complete microsatellite profile were subjected to further genotyping analysis. DNA extracts were subjected to Mn-PCR restriction fragment length polymorphism (RFLP) method, with the markers SAG1, SAG2 (50–30 SAG2, and alt. SAG2), SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1, and Apico (Su et al., 2010). ToxoDB RFLP genotype was identified according to http://toxodb.org/toxo/ (accessed 12 September 2023).

Finally, sequencing of the SAG3 and GRA6 markers was performed for all qPCR positive samples. Sequencing were carried at the Center for Genomic Technologies of the Complutense University of Madrid, Spain, with the BigDye® Terminator kit v 3.1 by Applied Biosystems (Foster City, CA, USA). The resulting sequences were then analyzed using an ABI 3130 Genetic Analyzer (Applied Biosystems).

A Maximum-Likelihood (ML) phylogenetic tree was built based on SAG3 sequences (HKY model with 1000 bootstrap) together with sequences retrieved from GenBank, selected based on their

geographical origin, representing Europe and Africa to contextualize the Mediterranean region. Furthermore, we included sequences from well-established clonal reference strains, such as TgRH (type I), TgMe49 (type II), and TgNED (type III), from GenBank.

3. Results

3.1 qPCR

Out 87 samples only 11 successfully amplified the 529bp RE region. These samples yielded cycle threshold (Ct) values ranging from 27.8 to 39.9, indicative of varying levels of parasite DNA amount. The maximum quantification of *T. gondii* zoites in these samples was observed at 82.5 zoites per milligram of tissue. Notably, the majority of these positive cases derived from domestic small ruminants, all of which were sourced from instances of abortion outbreaks.

Moreover, one sample from muscle of a ring-tailed lemur exhibited a parasitic load of 37 zoites per milligram of tissue while a second one from the heart of a red fox showed a notably low parasitic burden (Table 2).

3.2 Microsatellite Analysis

The results of genotyping and fingerprinting using microsatellite (MS) markers are reported in Table 3. Among the seven samples subjected to MS typing, five amplified a minimum of five typing markers. Among these three specimens sourced from sheep (two placental samples and one central nervous system [CNS] foetus), one muscle sample from *Lemur catta*, and one CNS sample from an aborted goat foetus. Two of these samples (Lemur muscle and sheep placenta [1016]) yielded a comprehensive MS profile encompassing all 15 markers. Consensus profiles were determined for three samples out of five, including only the samples with a complete profile or with at least 7/8 genotyping markers. Specifically, these three samples (two sheep placenta [1016 and 956] and lemur muscle) exhibited non-clonal Type II strains. (Table 3).

3.3 RFLP results

The two samples subjected to PCR-RFLP procedures (sheep placenta [1016], and lemur muscle) yielded a complete profile corresponding to the ToxoDB#3. The results are shown in Table 3.

3.4 Results of Sequencing and Phylogenetic analysis

After sequencing all GRA6 sequences, corresponding to type II, displayed a remarkable 99.7-100% similarity with sequences of *T. gondii* from various sources, including ON814571 (dolphin, Italy), MT370491 (sheep, Spain), MG587975 (pig, Italy), MG587959 (pig, Italy), and numerous others available in GenBank. For SAG3 sequences the type II allele showed a single nucleotide polymorphism (SNP), namely G1691T, which effectively separated our type II and type II-like samples into two distinct groups.

The first group, represented by sample 493 (muscle from a congenitally infected goat; IIa SAG3 allele), exhibited 100% identity with the sequences MT361125 (sheep, Spain), KU599489 (cat, Turkey), KU599478 (chicken, Portugal), ON814566 (Me49 reference strain), ON814568 (dolphin, Italy), and others present in GenBank. The second group, characterized by the G1691T SNP, included samples 16, 621, 1016, 956, and 630 (IIb SAG3 allele) and displayed 100% similarity with the sequences MT361126 (sheep, Spain), KU599488 (cat, Turkey), KU599479 (pig, Portugal), KU599412 (sheep, France), ON814569 (dolphin, Italy), among various other deposited sequences. Notably, this SNP results in an amino acid change at codon 368 from Methionine (Met) to Isoleucine (Ile), a phenomenon previously documented in a significant number of samples collected from sheep abortion cases in Spain (Fernández-Escobar et al., 2020a) and stranded dolphins in Italy (Fernández-Escobar et al., 2022).

The ML tree showed 3 clearly separated cluster composed by the three types: Type I, Type II, and Type III, supported by high bootstrap values ranging from 90% to 99%. It's worth noting that

144 within the Type II cluster, we identified two well-defined sub clusters, namely Type IIa and Type IIb,
145 as illustrated by the previously mentioned SNP observed in the alignment of SAG3 sequences.

146 **Discussion**

147 Genetic characterization, employing three distinct genotyping methodologies, has revealed
148 the predominance of Type-II variants within our sample set, primarily encompassing sheep and lemur.
149 Type-II strains are notably prevalent in European domestic livestock (Fernández-Escobar 2020a,
150 2020b), wildlife (Richomme et al., 2019), and humans (Jokelainen et al., 2018). It is important to note
151 that the current understanding of the genetic diversity of *T. gondii* populations in Italy remains limited.
152 Existing studies often employ a reduced number of typing loci (Mancianti et al., 2020, Battisti et al.,
153 2018, Sgroi et al., 2020), which may introduce bias in the characterization of the infecting strain.

154 Phylogenetic analyses of highly variable loci responsible for encoding virulence factors,
155 including surface and secretory antigens, which often experience significant selective pressure, have
156 been extensively employed to elucidate potential genetic population structure models, evolutionary
157 relationships among *T. gondii* populations, reservoirs, and transmission dynamics, among other
158 crucial factors (Jiang et al., 2018; Bertranpetit et al., 2017).

159 Our findings suggest a potential genetic relatedness between *T. gondii* populations in Italy,
160 Spain, and France. This inference is based on limited SAG3 sequences of sheep origin available in
161 GenBank database. Our SAG3 sequences grouped into two distinct clusters, which were delineated
162 by the specific SNP (G1691T) as described by Fernández-Escobar et al. (2020a).

163 The exclusive identification of *T. gondii* Type II strains within our collection of small ruminant
164 samples, primarily composed of sheep, aligns with the well-documented literature that underscores
165 the prevalence of *T. gondii* Type II alleles in European sheep populations (Dubey, 2009).
166 Nevertheless, *T. gondii* is recognized as a prominent contributor to ovine reproductive failures,

resulting in significant economic losses to the global sheep industry (Katzer et al., 2011, Stelzer et al., 2019, Dubey, 2022).

The clinical manifestations of ovine toxoplasmosis are influenced by various factors, including strain virulence and the parasite's developmental stage at the time of infection (Dubremetz and Lebrun, 2014; Benavidez et al., 2014). It is noteworthy that all the sheep samples analysed in this study originated from abortion outbreaks, comprising both placental infected tissue and congenitally infected lambs.

The prevalence of *T. gondii* Type II strains among European animals suggests that zoo species in Europe may encounter a relatively restricted set of strain genotypes compared to those found in their natural habitats (Denk et al., 2022). Our study findings corroborate this trend, as we observed that the single primate specimen in our sample set hosted a Type IIb variant, which was observed to cluster in the phylogenetic tree alongside the small ruminant samples displaying the distinctive SNP (G1691T).

Lemurs and New World monkeys are especially vulnerable to develop severe clinical manifestations and succumb to acute toxoplasmosis. Many outbreaks have been described in captive species, but those accompanied by genotypic data are scarce (Dubey et al 2022b). In an Italian zoo, a possible type II strain determined by 8 PCR-RFLP markers was confirmed in one individual during a fatal outbreak in ring-tailed lemur (*Lemur catta*) (Rocchigiani et al. 2022). Lately, another lethal case occurring in a zoo-housed black-capped squirrel monkey (*Saimiri boliviensis*) in Portugal have been described. Genotyping of 13 microsatellite markers confirmed a systemic *T. gondii* infection linked to a non-clonal type II strain (Salas-Fajardo et al., 2023).

Apart from *T. gondii* cell culture isolates, where the parasitic burden is often considerably high and permits feasible DNA genotyping, typing and subtyping discrimination in *T. gondii*-positive samples can be challenging, especially in epidemiological surveys. Studies such as Dubey et al. (2008) and Fernández-Escobar et al. (2020a,b), which include a bioassay step prior to genotyping,

have yielded more favourable results in terms of marker amplification compared to those that carried out PCR-RFLP or MS typing without prior bioassay, as observed by several authors (Herrmann et al., 2012; Calero-Bernal et al., 2015; Fernández-Escobar et al., 2022; Salas-Fajardo et al., 2023; and present study). These findings underscore the critical importance of high-quality DNA to obtain a satisfactory percentage of marker amplification. However, in epidemiological investigations, particularly in wildlife settings, challenges arise. Sampling activities in the wild may occur days after the animals' demise, making it difficult to recover viable *T. gondii*. Moreover, conducting a bioassay is both costly and time-consuming, rendering it impractical for epidemiological studies involving a large number of samples (Battisti et al., 2018).

Quantitative PCR assay results showed a reduction in the number of positive samples by amplification of the 529 bp repeat element marker compared to B1. These could be explained either by false positive results by B1 PCR assay or due to lower sensitivity of the former marker (Veronesi et al., 2017). Furthermore, some authors have reported instances of non-reproducibility in qPCR results for *T. gondii*, highlighting the potential for limited agreement between various direct molecular assays (Opsteegh et al., 2019).

Hence, the insights of this study hold significant importance in the context of *T. gondii* epidemiology within Italy. Notably, they mark the first instance of comprehensive microsatellite profiles, confirmed by RFLP and further detailed by MLS analysis, originating from two distinct species, each bearing unique epidemiological implications: captive ring-tailed lemurs and sheep. The exhaustive characterization of these strains circulating in diverse ecological settings undoubtedly contributes valuable insights to enhance our understanding of the epidemiology and transmission dynamics of this remarkably successful protozoan.

Conclusion

By employing a combination of three distinct genotyping methods, we have generated the first comprehensive microsatellite profiles for *T. gondii* in Italy. Our findings underscore the prevalence

of Type II strains, particularly in cases of ovine abortion, and in a fatal toxoplasmosis in captive *Lemur catta*. This multifaceted approach not only enhances our knowledge of *T. gondii*'s genetic variants but also provides valuable insights into its transmission dynamics. This comprehensive analysis is crucial for effectively managing the impact of this pathogen on both human and animal health in Italy.

References

Ajzenberg D, Collinet F, Mercier A, Vignoles P, Dardé ML (2010) Genotyping of *Toxoplasma gondii* isolates with 15 microsatellite markers in a single multiplex PCR assay. J Clin Microbiol 48:4641–4645. https://doi.org/10.1128/JCM.01152-10

Aubert D, Ajzenberg D, Richomme C, Gilot-Fromont E, Terrier ME, de Gevigney C, Game Y, Maillard D, Gibert P, Dardé ML, Villena I. (2010) Molecular and biological characteristics of *Toxoplasma gondii* isolates from wildlife in France. Vet. Parasitol. 171, 346–349.

Battisti E, Zanet S, Trisciuoglio A, Bruno S, Ferroglio E. (2018) Circulating genotypes of *Toxoplasma gondii* in Northwestern Italy. Vet Parasitol. 253, 43–47. https://doi.org/10.1016/j.vetpar.2018.02.023

Behnke MS, Khan A, Wootton JC, Dubey JP, Tang K, Sibley LD (2011) Virulence diferences in *Toxoplasma* mediated by amplifcation of a family of polymorphic pseudokinases. Proc Natl Acad Sci U S A 108:9631–9636. https://doi.org/10.1073/pnas.1015338108

Benavides J, Fernández M, Castaño P, Ferreras MC, Ortega-Mora L, Pérez V. (2017) Ovine toxoplasmosis: a new look at its pathogenesis. J Comp Pathol. 157, 34–8.

Bertranpetit E, Jombart T, Paradis E, Pena H, Dubey J, Su C, Mercier A, Devillard S, Ajzenberg D. (2017). Phylogeography of *Toxoplasma gondii* points to a South American origin. Infect Genet Evol. 48, 150-155. doi: 10.1016/j.meegid.2016.12.020. Epub 2016 Dec 24. PMID: 28028000.

240 Calero-Bernal R, Fernández-Escobar M, Katzer F, Su C, Ortega-Mora LM (2022) Unifying virulence
241 evaluation in *Toxoplasma gondii*: a timely task. Front Cell Infect Mi 12:868727.
242 https://doi.org/10.3389/fcimb.2022.868727

243 Calero-Bernal R, Saugar JM, Frontera E, Pérez-Martín JE, Habela MA., Serrano F J, Reina D, Fuentes
244 I. (2015) Prevalence and genotype identification of Toxoplasma gondii in wild animals from
245 southwestern Spain. J. Wildl. Dis. 51(1), 233–238. https://doi.org/10.7589/2013-09-233

246 Carossino M, Bauer R, Mitchell MA, Cummings CO, Stöhr AC, Wakamatsu N, Harper K, Langohr
247 IM, Schultz K, Mitchell MS, Howe DK, Balasuriya UBR. (2021) Pathologic and
248 immunohistochemical findings in an outbreak of systemic toxoplasmosis in a mob of red
249 kangaroos. J Vet Diagn Invest. 2021 33(3), 554-565. doi: 10.1177/10406387211001869. Epub
250 2021 Mar 19. PMID: 33739178; PMCID: PMC8120092.

251 Chessa G, Chisu V, Porcu R, Masala G. (2014) Molecular characterization of *Toxoplasma gondii* Type
252 II in sheep abortion in Sardinia, Italy. Parasite. 21, 6. https://doi.org/10.1051/parasite/2014007

253 Cook AJ, Gilbert RE, Buffolano W, Zufferey J, Petersen E, Jenum PA, Foulon W, Semprini AE, Dunn
254 DT. (2000) Sources of *Toxoplasma* infection in pregnant women: European multicentre case-
255 control study. European Research Network on Congenital Toxoplasmosis. BMJ. 321(7254),
256 142-7. doi: 10.1136/bmj.321.7254.142. PMID: 10894691; PMCID: PMC27431.

257 Denk D, De Neck S, Khaliq S, Stidworthy MF (2022) Toxoplasmosis in Zoo Animals: a Retrospective
258 Pathology Review of 126 cases. Animals 12, 619. https://doi.org/10.3390/ani12050619

259 Di Guardo G, Di Cesare A, Otranto D, Casalone C, Iulini B, Mignone W, Tittarelli C, Meloni S,
260 Castagna G, Forster F, Kennedy S, Traversa D. (2011) Genotyping of *Toxoplasma gondii*
261 isolates in meningo-encephalitis affected striped dolphins (*Stenella coeruleoalba*) from Italy.
262 Vet. Parasitol. 183(1-2), 31–36. https://doi.org/10.1016/j.vetpar.2011.07.004

263 Dini FM, Graziosi G, Lupini C, Catelli E, Galuppi R. (2023) Migratory Wild Birds as Potential Long-
264 Distance Transmitters of *Toxoplasma gondii* Infection. Pathogens. 18;12(3):478. doi:
265 10.3390/pathogens12030478. PMID: 36986400; PMCID: PMC10051421.

266 Dubey JP. (2009)Toxoplasmosis in sheep - the last 20 years. Vet Parasitol.163,1–14.

267 Dubey JP. (2022) Toxoplasmosis of animals and humans. 3rd ed. Boca Raton: CRC Press, Taylor &
268 Francis Group.

269 Dubey JP. (2022) Clinical toxoplasmosis in zoo animals and its management. Emerg. Anim. Species.
270 2, 100002

271 Dubey JP, Huong LT, Lawson BW, Subekti DT, Tassi P, Sundar N, Velmurugan GV, Kwock OC, Su
272 C. (2008). Seroprevalence and isolation of *Toxoplasma gondii* from free-range chickens in
273 Ghana, Indonesia, Italy, Poland, and Vietnam. J. Parasitol. 94 (1), 68–71.

274 Dubremetz JF, Lebrun M. (2012) Virulence factors of *Toxoplasma gondii*. Microbes Infect. 14, 1403–
275 10.

276 European Food Safety Authority (EFSA). (2018). Public health risks associated with food-borne
277 parasites. EFSA J. 16(12) :5495. doi: 10.2903/j.efsa.2018.5495.

278 Fernández-Escobar M, Calero-Bernal R, Regidor-Cerrillo J, Vallejo R, Benavides J, Collantes-
279 Fernández E, Ortega-Mora LM. (2020b) Isolation, Genotyping, and Mouse Virulence
280 Characterization of *Toxoplasma gondii* From Free Ranging Iberian Pigs. Front Vet Sci.
281 7:604782. doi: 10.3389/fvets.2020.604782.

282 Fernández-Escobar M, Giorda F, Mattioda V, Audino T, Di Nocera F, Lucifora G, Varello K,
283 Grattarola C, Ortega-Mora LM, Casalone C, Calero-Bernal R. (2022) *Toxoplasma gondii*
284 Genetic Diversity in Mediterranean Dolphins. Pathogens. 11(8), 909. doi:
285 10.3390/pathogens11080909.

Fernández-Escobar M, Calero-Bernal R, Benavides J, Regidor-Cerrillo J, Guerrero-Molina MC, Gutiérrez-Expósito D, Collantes-Fernández E, Ortega-Mora LM. (2020a) Isolation and genetic characterization of *Toxoplasma gondii* in Spanish sheep flocks. Parasit Vectors. 2020 13(1),396. doi: 10.1186/s13071-020-04275-z.

Hamidovic A, Etougbetche JR, Tonouhewa ABN, Galal L, Dobigny G, Houemenou G, Da Zoclanclounon H et al (2021) A hotspot of Toxoplasma gondii Africa 1 lineage in Benin: how new genotypes from West Africa contribute to understand the parasite genetic diversity worldwide. PLoS Negl Trop Dis 15:e0008980. https://doi.org/10.1371/journal.pntd.0008980

Herrmann DC, Maksimov P, Maksimov A, Sutor A, Schwarz S, Jaschke W, Schliephake A, Denzin N, Conraths F J, Schares G. (2012) *Toxoplasma gondii* in foxes and rodents from the German Federal States of Brandenburg and Saxony-Anhalt: seroprevalence and genotypes. Vet. Parasitol. 185(2-4), 78–85. https://doi.org/10.1016/j.vetpar.2011.10.030

Jiang T, Shwab EK, Martin RM, Gerhold RW, Rosenthal BM, Dubey JP, Su C. (2018) A partition of *Toxoplasma gondii* genotypes across spatial gradients and among host species, and decreased parasite diversity towards areas of human settlement in North America. Int J Parasitol. 48(8), 611-619. doi: 10.1016/j.ijpara.2018.01.008. Epub 2018 Mar 23. PMID: 29577892.

Joeres M, Cardron G, Passebosc-Faure K, Plaut N, Fernández-Escobar M, Hamilton C, O'Brien-Anderson L, Calero-Bernal R, Galal L, Lutterman C, Maksimov P, Conraths FJ, Dardé ML, OrtegaMora L, Jokelainen P, Mercier A, Schares G (2023) A ring trial to harmonize *Toxoplasma gondii* microsatellite typing: comparative analysis of results and recommendations for optimization. Eur J Clin Microbiol Infect Dis 42, 803–818. https://doi.org/10.1007/ s10096-023-04597-7

Jokelainen P, Murat JB, Nielsen HV. (2018) Direct genetic characterization of *Toxoplasma gondii* from clinical samples from Denmark: not only genotypes II and III. Eur J Clin Microbiol Infect Dis. 37, 579–86. doi: 10.1007/s10096-017-3152-z

Katzer F, Brülisauer F, Collantes-Fernández E, Bartley PM, Burrells A, Gunn G, Maley SW, Cousens C, Innes EA. (2011) Increased *Toxoplasma gondii* positivity relative to age in 125 Scottish sheep flocks; evidence of frequent acquired infection. Vet Res.42(1), 121. doi: 10.1186/1297-9716-42-121.

Khan A, Ajzenberg D, Mercier A, Demar M, Simon S, Dardé ML, Wang Q, Verma SK, Rosenthal BM, Dubey JP, Sibley LD. (2014) Geographic separation of domestic and wild strains of *Toxoplasma gondii* in French Guiana correlates with a monomorphic version of chromosome1a. PLoS Negl Trop Dis. 8(9):e3182. doi: 10.1371/journal.pntd.0003182.

Khan A, Jordan C, Muccioli C, Vallochi AL, Rizzo LV, Belfort R Jr, Vitor RW, Silveira C, Sibley LD. (2006) Genetic divergence of *Toxoplasma gondii* strains associated with ocular toxoplasmosis, Brazil. Emerg Infect Dis.12(6),942-9. doi: 10.3201/eid1206.060025.

Lilue J, Müller UB, Steinfeldt T, Howard JC (2013) Reciprocal virulence and resistance polymorphism in the relationship between *Toxoplasma gondii* and the house mouse. eLife 2:e01298. https://doi.org/10.7554/eLife.01298

Mancianti F, Terracciano G, Sorichetti C, Vecchio G, Scarselli D, Perrucci S. (2020). Epidemiologic Survey on *Toxoplasma gondii* and *Trichinella pseudospiralis* Infection in Corvids from Central Italy. Pathogens 9(5), 336. https://doi.org/10.3390/pathogens9050336

Opsteegh M, Spano F, Aubert D, Balea A, Burrells A, Cherchi S, Cornelissen JBWJ, Dam-Deisz C, Guitian J, Györke A, Innes EA, Katzer F, Limon G, Possenti A, Pozio E, Schares G, Villena I, Wisselink HJ, van der Giessen JWB. (2019) The relationship between the presence of

antibodies and direct detection of *Toxoplasma gondii* in slaughtered calves and cattle in four European countries. Int J Parasitol. 49(7), 515-522. doi: 10.1016/j.ijpara.2019.01.005

Richomme C, Aubert D, Gilot-Fromont E, Ajzenberg D, Mercier A, Ducrot C, Ferté H, Delorme D, Villena I. (2009) Genetic characterization of *Toxoplasma gondii* from wild boar (Sus scrofa) in France. Vet Parasitol. 164(2-4):296-300. doi: 10.1016/j.vetpar.2009.06.014.

Rocchigiani G, Fonti N, Nardoni S, Cavicchio P, Mancianti F, Poli A (2022) Toxoplasmosis in Captive Ring-Tailed Lemurs (*Lemur catta*). Pathogens. 11, 1142. https://doi.org/10.3390/ pathogens11101142

Rocchigiani G, Nardoni S, D'Ascenzi C, Nicoloso S, Picciolli F, Papini RA, Mancianti F. (2016) Seroprevalence of *Toxoplasma gondii* and *Neospora caninum* in red deer from Central Italy. Ann Agric Environ Med. 23(4):699-701. doi: 10.5604/12321966.1226870.

Salas-Fajardo MY, Benavides J, Azevedo A, Figueiras P, Monteiro M, Orge L, Mendonça P, Carvalho P, Waap H, Ortega-Mora LM, Calero-Bernal R. (2023) Fatal toxoplasmosis in a captive squirrel monkey (*Saimiri boliviensis*) in Portugal. Vet Res Commun. 21. doi:10.1007/s11259-023-10179-x

Scallan E, Hoekstra RM, Angulo FJ, Tauxe RV, Widdowson MA, Roy SL, Jones JL, Griffin PM. (2011) Foodborne illness acquired in the United States--major pathogens. Emerg Infect Dis. 17(1), 7-15. doi: 10.3201/eid1701.p11101.

Sgroi G, Viscardi M, Santoro M, Borriello G, D'Alessio N, Boccia F, Pacifico L, Fioretti A, Veneziano V, Fusco G. (2020) Genotyping of *Toxoplasma gondii* in wild boar (*Sus scrofa*) in southern Italy: Epidemiological survey and associated risk for consumers. Zoonoses Public Health. 67(7),805-813. doi: 10.1111/zph.12762. Epub 2020 Sep 4.

353 Slana I; Kralik P; Kralova A; Pavlik I. (2008) On-farm spread of *Mycobacterium avium* subsp.
354 *paratuberculosis* in raw milk studied by IS900 and F57 competitive real time quantitative PCR
355 and culture examination. Int. J. Food Microbiol. 128, 250–257

356 Slany M, Dziedzinska R, Babak V, Kralik P, Moravkova M, Slana I. (2019) *Toxoplasma gondii* in
357 vegetables from fields and farm storage facilities in the Czech Republic. FEMS Microbiol Lett.
358 366(14):fnz170. doi: 10.1093/femsle/fnz170..

359 Stelzer S, Basso W, Benavides Silván J, Ortega-Mora LM, Maksimov P, Gethmann J, Conraths FJ,
360 Schares G. (2019) *Toxoplasma gondii* infection and toxoplasmosis in farm animals: Risk factors
361 and economic impact. Food Waterborne Parasitol. 15:e00037. doi:
362 10.1016/j.fawpar.2019.e00037..

363 Su C; Shwab EK; Zhou P; Zhu XQ. (2010) Dubey, J.P. Moving towards an integrated approach to
364 molecular detection and identification of *Toxoplasma gondii*. Parasitology. 137, 1–11.

365 Veronesi F, Santoro A, Milardi GL, Diaferia M, Morganti G, Ranucci D, Gabrielli S (2017) Detection
366 of *Toxoplasma gondii* in faeces of privately owned cats using two PCR assays targeting the B1
367 gene and the 529-bp repetitive element. Parasitol. Res. 116(3), 1063–1069.
368 https://doi.org/10.1007/s00436-017-5388-z

369 Viscardi M, Santoro M, Cozzolino L, Borriello G, Fusco G. (2022) A type II variant of *Toxoplasma*
370 *gondii* infects the Eurasian otter (*Lutra lutra*) in southern Italy. Transbound. Emerg. Dis. 69 (2),
371 874–880. https://doi.org/10.1111/tbed.14012

372 Vismarra A, Barilli E, Miceli M, Mangia C, Genchi M, Brindani F, Kramer L, Bacci C (2017)
373 *Toxoplasma gondii* in the Cornigliese sheep breed in Italy: Meat juice serology, in vitro isolation
374 and genotyping. Vet Parasitol. 243, 125–129. https://doi.org/10.1016/j.vetpar.2017.06.013

375

376

377 Table 1. Samples analysed by qPCR. In the "tissues examined" column in brackets the number of
378 samples if more than one matrices were analysed. All the DNA extraction were performed using Pure
379 Link ® Genomic DNA Mini kit (Invitrogen by Thermo Fisher), according to the manufacturer's
380 protocol.

381

Animal Species	**Specimens N**	**Tissues examined (n)**	**Amount of tissue for DNA extraction**
Canis lupus italicus	7	CNS (7), Heart Tissue (5)	25 mg
Capreolus capreouls	6	Spleen	25 mg
Vulpes vulpes	6	Heart Tissues (5), Spleen (1)	25 mg
Talpa europaea	1	CNS, Heart Tissue, Tongue	25 mg
Mustela nivalis	1	CNS, Heart Tissue, Tongue	25 mg
Erinaceus europaeus	1	CNS, Heart Tissue, Tongue	25 mg
Sciurus vulgaris	2	CNS, Heart Tissue, Tongue, Muscle	25 mg
Sus scrofa (wild)	2	Muscle	10 g (200 µL of pellet after Peptic Digestion)
Spatula clypeata	3	Heart Tissue	25mg
Anas crecca	1	Heart Tissue	25mg
Anas platyrhynchos	2	Heart Tissue	25mg
Vanellus vanellus	1	Heart Tissue	25mg
Lemur catta	1	Muscle	25mg
Gallus gallus domesticus	12	CNS (4)	2g (200 µL of pellet after Peptic Digestion)
		Heart Tissue (3)	4g (200 µL of pellet after Peptic Digestion)
		Skeletal Muscle (8)	25g (200 µL of pellet after Peptic Digestion)
Bos taurus	13	Heart Tissue	25mg
Capra hircus	4	Liver (1), CNS (Aborted Fetuses) (2), Muscle (Aborted Fetus) (1)	25mg
Ovis aries	7	CNS (Aborted Fetuses) (2), Placenta (2), Muscles (Aborted Fetuses) (2), Heart Tissue (1)	25mg

382

383

384 Table 2 Real time PCR positive samples

Sample ID	Host	Locality	Tissue	qPCR results	
				Ct	Zoites/mg tissue
630	*Lemur catta*	Ravenna (RA)	Muscle	26.9	37
956	*Ovis aries*	Parma (PR)	Placenta	29.7	24
1011		Mantova (MN)	CN Sfoetus	34	1.5
1016		Mantova (MN)	Placenta	27.8	82.5
22235		Parma (PR)	CNS foetus	35.8	0.5
812-1		Bologna (BO)	CNS foetus	39.5	0.04
812-1		Bologna (BO)	CNS foetus	39.9	0.03
621		Bologna (BO)	CNS foetus	33.9	1.6
16	*Capra hircus*	Modena (MO)	CNS foetus	30.3	16.2
493		Forlì-Cesena (FC)	Muscle	36.2	0.35
268624	*Vulpes vulpes*	Modena (MO)	Heart	36.5	0.3

385

386

Table 3. Microsatellite (MS) typing profile of the samples analysed and RFLP results

		Microsatellites alleles															Consensus profiles	
Sample	**Species**	**N61**	**B18**	**M33**	**M48**	**TUB2**	**N83**	**XI.1**	**N82**	**TgM-A**	**W35**	**IV.1**	**B17**	**N60**	**M102**	**AA**	**MS genotype**	**RFLP**
1016	*Ovis aries*	93	158	169	211	289	310	356	129	207	242	274	336	140	176	263	II	ToxoDB#3
630/19	*Lemur catta*	97	158	169	219	289	310	356	111	207	242	274	336	140	174	261	II	ToxoDB#3

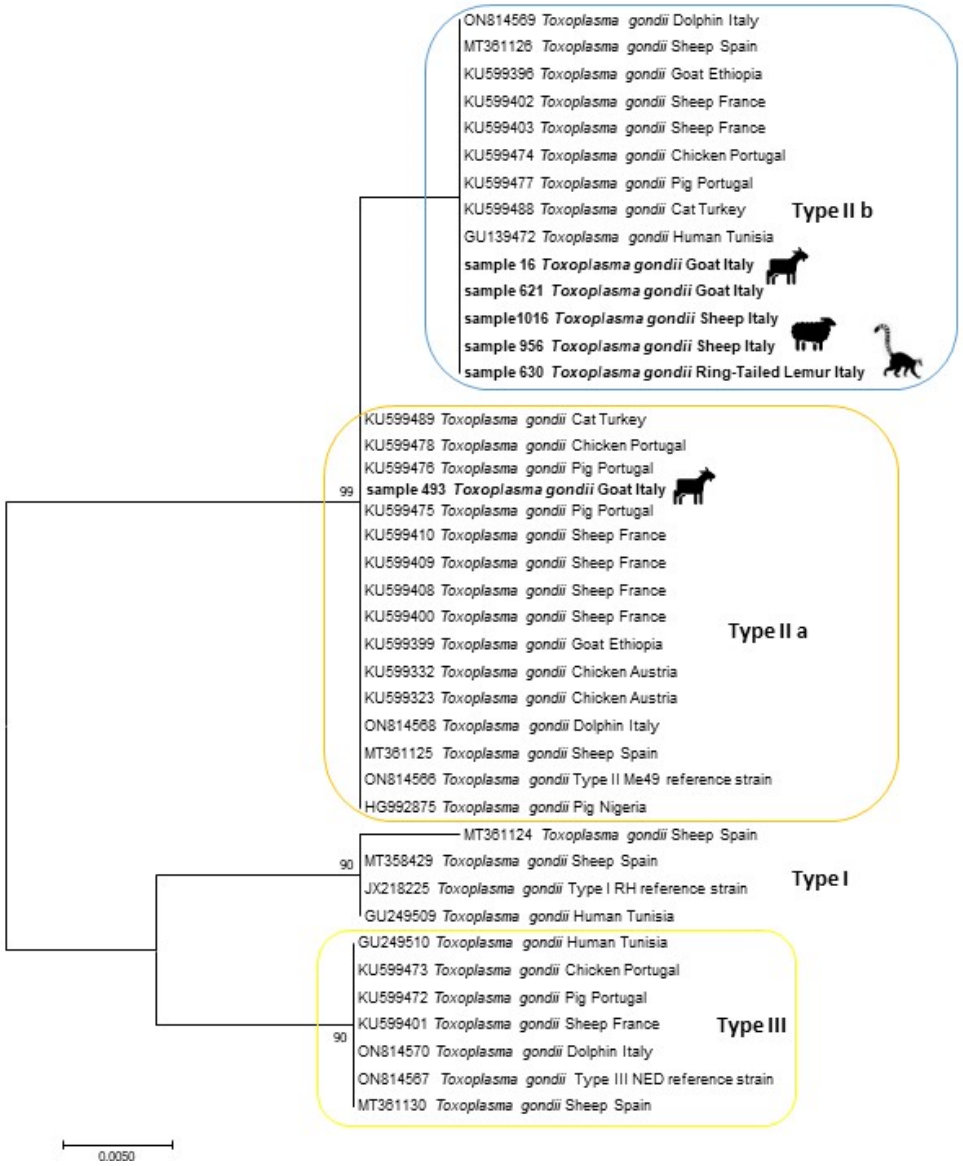


Figure 1. Phylogenetic tree with sequences of SAG3 in the Mediterranean context. It yielded a clear classification into the three distinct genotypes: Type I, Type II, and Type III. The robustness of this clustering is supported by high bootstrap (BP) values, ranging from 90% to 99%. Within the Type II cluster, a more detailed examination revealed the presence of two well-defined subclusters, specifically Type IIa and Type IIb. These subclusters were distinguished by a previously mentioned single nucleotide polymorphism (SNP) observed in the alignment of SAG3 sequences. This SNP likely represents a critical genetic variation that separates these two subclusters within the Type II group.

CHAPTER 8 - Discussions and Conclusion

The One Health approach embodies the essence of transdisciplinary collaboration, uniting efforts to address multifaceted challenges spanning human health, animal well-being, plant health, and the environment. Some authors underline the necessity for One Health to proactively engage in examining, mitigating, and preventing substantial challenges both in the present and the foreseeable future. This urgency is particularly emphasized by the fact that a majority of emerging infectious diseases in humans have their origins in animals, often taking the form of zoonoses. As such, it is imperative to integrate human, animal, and ecosystem health under a common framework. Recent global issues, including environmental shifts, biodiversity decline, habitat fragmentation, globalization, and the emergence of infectious diseases, have converged to create a demand for integrative approaches that transcend traditional disciplinary boundaries and coalesce under the banner of 'One Health.' These converging problems highlight the need for holistic and collaborative strategies. Critical to One Health will be effective monitoring of toxoplasmosis and *T. gondii* prevalence.

- The evidence presented in the third chapter of this thesis paints a compelling picture of the widespread distribution of the parasite *Toxoplasma gondii* in the study area. Specific antibodies were found in various animal species, as well as in human populations, indicating the constant presence of this parasite across diverse environments. Furthermore, the presence of seropositivity in both humans, wild, and domestic animals underscores the parasite's adaptation to anthropized environments. The interplay between human activities and environmental factors plays a pivotal role in shaping the epidemiology of this parasitic infection and influencing its spread. Given the inherently interdisciplinary nature of this issue, a One Health approach is indispensable. This approach is not only pertinent in prevalence surveys, as demonstrated in this study, but is equally crucial in the development of strategies for control, education, and prevention campaigns. It reflects the need for a unified effort to address the complex interdependencies between human, animal, and environmental health, ultimately striving for a more holistic and effective approach to these pressing health challenges.
- In the holistic perspective of the One Health approach, the environment plays an irreplaceable and significant role. This is also the case in the epidemiology of Toxoplasmosis, where the environment is a fundamental component in the spread of the

parasite, which recognizes it as an important natural reservoir for one of its infectious forms, the oocysts. These oocysts, when released into the environment, serve as a source of infection through environmental sources such as water and soil for various hosts, including certain wildlife, which can act as indicators of environmental contamination by oocysts. Migratory aquatic birds, synanthropic rodents, wolves, and wild boars were the subjects of this chapter (4.1-4-3), in which *Toxoplasma* was investigated using both molecular and serological methods, with serological methods being the most indicative of parasite exposure. The positive findings in migratory aquatic birds reflect how these species can serve as indicators of an aquatic environment contaminated by oocysts. Furthermore, as migratory birds, they have the potential to easily transport parasite strains from specific geographic areas. The rodents, sampled in human-influenced environments, revealed that there is a certain degree of contamination by apicomplexan oocysts shed by felids, such as *T. gondii*, *Hammondia hammondi*, and *Sarcocystis gigantea*, in the sampling areas. Interestingly, for the first time in Italy in these hosts, also *Besnoitia besnoiti* has been recovered, for which the definitive host is unknown in Europe. The seropositivity for *T. gondii* found in Italian wild boars and wolves, sampled in more natural settings, indicates that parasite transmission consistently occurs in the wild, where these hosts become infected by ingesting oocysts present in the environment or through the consumption of other intermediate hosts. The results of this study also provide an initial and comprehensive insight into seroprevalence in wolves.

- The fifth chapter of the thesis delved into the role of dogs as intermediate hosts of veterinary importance, assessing the risk factors in various categories of dogs and examining the correlation between seropositivity and pathological anxiety. The results underscore the significance of specific factors in amplifying the risk of *T. gondii* infection among dogs. Cohabitation with cats, coprophagy behaviours, and continuous outdoor habitation were identified as pivotal factors contributing to the likelihood of infection. This comprehensive understanding of how these factors interplay with infection risk adds to our broader comprehension of the epidemiological landscape and emphasizes the need for targeted preventive strategies, especially for dogs exhibiting these risk-associated behaviours and conditions. Recent research has revealed the potential neural localization of bradyzoite cysts in intermediate hosts, which can lead to behavioural modifications. The second study of this chapter exploring the correlation between serological evidence of exposure to *T. gondii* and pathological anxiety in dogs, indicate that large size dogs were more likely to develop

anxiety when testing positive for *T. gondii*, and that raw meat consumption is a valid risk factor for seropositivity.

- The consumption of raw or undercooked meat is widely acknowledged as a significant risk factor for human *T. gondii* infection. Recent risk assessments carried out in Europe have particularly highlighted the role of beef in the context of human infection risk. Interestingly, cattle, conversely, exhibit clinical resistance to *T. gondii* infection, and direct diagnostic tools rarely directly detect the parasite in bovine muscle tissues. Chapter six of this thesis is exclusively dedicated to the distinctive bovine host. It specifically focuses on a serological investigation aimed at observing the natural infection dynamics of *T. gondii*. In this study, for the first time an assessment of antibody kinetics for *T. gondii* was conducted at three different time points during the production cycle of fattening cattle, thereby underscoring the dynamic nature of *T. gondii* infection in cattle. This dynamic nature is characterized by evidence of both new infections and a decline in antibody levels over the production cycle. Additionally, the results of blood analysis suggest an activation of the innate immune response in cattle that have been exposed to *T. gondii*. Moreover, within the context of this research, the second section of this chapter provides the first description of a co-infection involving *T. gondii* and *Sarcocystis hominis* (both zoonotic parasites) in a case of Bovine Eosinophilic Myositis (BEM), that caused the condemnation of a bovine carcass. Notably, a novel matrix, such as the meat juice pellet, was effectively employed for the simultaneous detection of both parasites from the Sarcocystidae family.

- In the final chapter of the thesis (7), we have employed a combination of three distinct genotyping methods to create the first comprehensive genotyping profiles for *T. gondii* in Italy. Our results highlight the predominance of Type II strains, particularly in cases of ovine abortion and in fatal toxoplasmosis among captive *Lemur catta*. This multifaceted approach not only advances our understanding of the genetic diversity within *T. gondii* but also offers valuable insights into its patterns of transmission. This comprehensive analysis is of paramount importance for the effective management of the impact of this pathogen on both human and animal health in Italy.