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Exploring the genetic diversity of *Toxoplasma gondii* in Europe by molecular fine characterization

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List of abbreviations

MS	microsatellite
WGS	whole genome sequencing
NGS	next-generation sequencing
MLST	multilocus sequence typing
p. i.	post infection
HIV	Human Immunodeficiency Virus
PCR-RFLP	PCR-restriction fragment length polymorphism
HRM	high resolution melting
SNP	single nucleotide polymorphism

1 Introduction

Toxoplasma gondii is a protozoan parasite that can infect a large variety of warm-blooded species. It has been detected on all continents and can cause clinical disease in animals as well as in humans (Dámek et al. 2023, Dubey 2022, Stelzer et al. 2019, Schlüter et al. 2014, Robert-Gangneux and Dardé 2012). At the global level, *T. gondii* has a complex population structure (Galal et al. 2019). Many regions of the world are dominated by a few clonal lineages (Shwab et al. 2018, Shwab et al. 2014), while *T. gondii* populations discovered in other regions like South America are much more diverse (Galal et al. 2019, Lorenzi et al. 2016). Genotypes found in Central and South America can be associated with higher virulence and greater clinical relevance (Shwab et al. 2018, Khan et al. 2006), making it important to detect the introduction of such genotypes to new areas like Europe. This work focused on Europe where *T. gondii* type II is the predominant clonal genotype (Galal et al. 2022, Shwab et al. 2018, Lorenzi et al. 2016).

The predominance of *T. gondii* type II in Europe has been analyzed and described as part of global studies on population genetics of *T. gondii*, including only a few European studies (Galal et al. 2019, Shwab et al. 2014). However, no study has so far been conducted that focuses exclusively on Europe and summarizes all European studies on genotyping of *T. gondii*.

The first aim of this work was to summarize the available genotyping information on European *T. gondii* isolates and to map the distribution of strains circulating in Europe.

Moreover, it was aimed to compare the currently applied genotyping methods. A frequently used genotyping method for *T. gondii* is based on microsatellite (MS) markers (Ajzenberg et al. 2010). It represents the current reference standard for genotyping and fingerprinting. However, it is largely unknown, to which extent the MS typing results obtained by different laboratories are consistent. Comparable MS typing results are necessary to combine larger data sets on *T. gondii* genotypes originating from different laboratories. Therefore, the second aim of this work was to reach consistency in *T. gondii* MS typing by performing a ring trial among five European laboratories.

MS typing and whole genome sequencing (WGS) analysis revealed genetic variability within *T. gondii* type II (Galal et al. 2022, Shwab et al. 2018, Lorenzi et al. 2016). To explore this further, the third aim was to establish a next-generation sequencing (NGS)-based typing method with a high typing resolution for closely related type II strains. This new multilocus sequence typing (MLST) method should improve the understanding of transmission pathways of *T. gondii*, allow to analyze outbreaks, trace infection sources or detect recombination and

the introduction of genotypes into new areas. To this end, a large number of *T. gondii* isolates and clinical samples were collected that had tested positive for *T. gondii*. The results of the new NGS-based typing method were compared to MS typing, especially with regard to the ability to distinguish between different genotypes and the sensitivity of the methods.

Finally, the harmonized MS method was applied in a local European study for genotyping of *T. gondii.* For this purpose, *T. gondii* DNA extracted from tissue samples of red squirrels (*Sciurus vulgaris*) found dead in the Netherlands were studied in detail.

2 Literature

2.1 *Toxoplasma gondii*

Toxoplasma gondii is a zoonotic protozoan parasite of the phylum Apicomplexa. It occurs worldwide and can infect a large variety if not all warm-blooded species, but felids are the only definitive hosts of *T. gondii* (Dámek et al. 2023, Dubey 2022, Stelzer et al. 2019, Schlüter et al. 2014, Robert-Gangneux and Dardé 2012). The parasite is the causative agent of toxoplasmosis and one of the main causes of food-borne diseases. A WHO/FAO report (World Health Organization and Food and Agriculture Organization of the United Nations 2014) listed *T. gondii* as the fourth most important foodborne parasite in the world. In a European study, *T. gondii* was ranked second out of 24 important foodborne parasites (Bouwknegt et al. 2018).

The first description of *T. gondii* was made in 1908 by Nicolle and Manceaux, who discovered the parasite in an infected African rodent, the gundi (*Ctenodactylus gundi*), in Tunisia (Nicolle and Manceaux 1908). At the same time, *T. gondii* was identified in a rabbit in Brazil (Splendore 1908), hinting at the worldwide distribution of the parasite already at that time. *T. gondii* owes its name to its arched shape (Greek “toxon” = arch, “plasma” = structure) and to the animal in which it was discovered. In the 1920s and 1930s, several reports described the pathogenic potential of *T. gondii* infections in humans. Wolf et al. reported on *T. gondii* infections in infants (Wolf et al. 1939) and at about the same time, the first report of a fatal *T. gondii* infection was described in a young adult (Pinkerton and Weinman 1940). The obligate intracellular reproduction of *T. gondii* was observed during an isolation from infected tissue in 1939 (Sabin and Olitsky 1937). In 1970, the central role of cats in the *T. gondii* life cycle due to fecal excretion of oocysts was studied in detail (Dubey et al. 1970a).

2.2 Infectious stages of *T. gondii*

2.2.1 Tachyzoites

The term “tachyzoite” (Greek “tachys” = fast) was introduced by Frenkel (Frenkel 1973) because of the rapid multiplication of this infectious stage of *T. gondii*. Tachyzoites have a size of approximately 2 x 6 µm, an arched shape and a pointed anterior and a rounded posterior end. The parasite consists of various organelles such as conoid, micronemes, rhoptries, mitochondrion, apicoplast, Golgi complex, endoplasmic reticulum and dense granules (Black and Boothroyd 2000, Dubey et al. 1998) (Figure 1). The conoid is located at the apical end, through which the parasite releases its secretory proteins into the host cells during invasion (Figure 1). These proteins originate from the following secretory organelles: The rhoptries, which are located at the apical end and flow into the conoid, the micronemes, which are

likewise distributed at the apical end, and the dense granules, which occur ubiquitously in the cytoplasm (Black and Boothroyd 2000) (Figure 1). The apicoplast is a plastid that is responsible for metabolic processes, such as fatty acid, isoprenoid and heme synthesis (Sheiner et al. 2013). The nucleus is usually located in the central area of the tachyzoite, containing the nuclear genome, which has a size of about 65 Mb (Lorenzi et al. 2016).

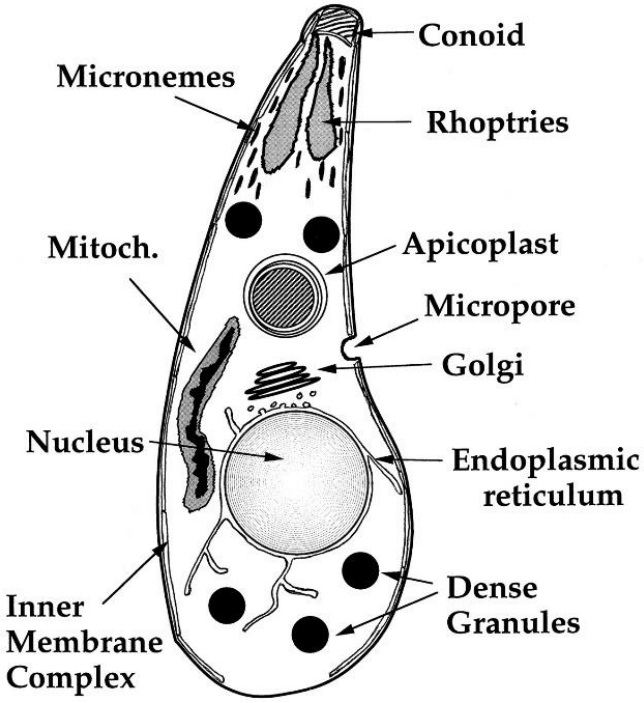

Figure 1: Structure of a *Toxoplasma gondii* tachyzoite, obtained from Black and Boothroyd (2000)

2.2.2 Bradyzoites and tissue cysts

The term “bradyzoite” (Greek “bradys” = slow) was also introduced by Frenkel (Frenkel 1973) because of the slow multiplication of this infectious stage of *T. gondii* within a tissue cyst. The tissue cysts are permanent forms in the life cycle of *T. gondii* and reach a diameter of 5 to 100 µm, depending on the age of the cysts and the type of tissue (Dubey et al. 1998). Tissue cysts develop within the host cells and each cyst can enclose hundreds of bradyzoites with a size of approximately 7 x 1.5 µm (Dubey et al. 1998). Bradyzoites differ in their structure only slightly from tachyzoites. They are more slender than tachyzoites and the nucleus is located more closely to the posterior end (Dubey et al. 1998). Furthermore, tissue cysts containing bradyzoites are less susceptible to destruction by proteolytic enzymes than tachyzoites (Jacobs et al. 1960), making them play an important role in the life cycle of *T. gondii*. Definitive

hosts ingest intermediate hosts containing tissue cysts (Dubey 2022, Dubey et al. 1970b) and after the destruction of the cyst wall in the small intestine of the definitive host, the released bradyzoites can penetrate the enterocytes (Dubey and Frenkel 1972) and thus infect the animal.

2.2.3 Oocysts

Non-sporulated oocysts have a diameter of 10 x 12 µm, exhibiting a subspherical to spherical shape. When observed through light microscopy, the oocyst wall consists of two colorless layers (Dubey et al. 1998). After the excretion of the oocysts by felids, sporulation takes place within 1 to 5 days, influenced by aeration and temperature conditions. Sporulated oocysts are slightly larger than non-sporulated oocysts with a size of 11 x 13 µm and a subspherical to ellipsoidal shape (Dubey et al. 1998). Each sporulated oocyst contains two ellipsoidal sporocysts with a size of 6 x 8 µm and each sporocyst contains four sporozoites with a size of 2 x 6-8 µm (Dubey et al. 1998). Ultrastructurally, sporozoites, tachyzoites and bradyzoites are similar, but there are some differences for example in the number of cell organelles and in the number and size of inclusion bodies (Dubey et al. 1998).

2.3 Life cycle and transmission pathways of *T. gondii*

T. gondii has a biphasic life cycle. Felids, including domestic cats and wild felids, are the only definitive hosts of *T. gondii* (Miller et al. 1972, Frenkel et al. 1970, Hutchison 1965) and virtually all warm-blooded species seem to be intermediate hosts (Dubey 2022).

2.3.1 Life cycle in definitive hosts

The sexual reproduction of *T. gondii* occurs only in the intestinal epithelium of the definitive host (Dubey et al. 1970b) (Figure 2). Prior to this, the definitive host ingests tissue cysts through the oral consumption of infected intermediate hosts such as mice (Dubey 2022, Dubey et al. 1970b). As tissue cysts are resistant to gastric digestion (Jacobs et al. 1960), they reach the small intestine, where the cyst wall is dissolved by proteolytic enzymes, which allows the released bradyzoites to penetrate the epithelial cells (enterocytes) (Dubey and Frenkel 1972). This is followed by the development of bradyzoites into tachyzoites, which disseminate after rupture of the infected cell throughout the body via the bloodstream and lead to the formation of lesions and tissue cysts in various organs (Dubey and Frenkel 1972). In parallel, bradyzoites in the intestinal epithelium develop into five morphologically distinct asexual schizont types (Type A to E) (Dubey and Frenkel 1972). Several generations may occur within each type, which differentiate by schizogony to merozoites (Dubey and Frenkel 1972).

After this asexual replication, the sexual cycle begins, still intracellularly in the intestinal epithelium. The gametogony occurs within 3-15 days after ingestion of tissue cysts (Dubey and Frenkel 1972). Microgametes and macrogametes, which are generated from merozoites, fuse, which results in a diploid zygote surrounded by an environmentally resistant oocyst wall (Dubey and Frenkel 1972). Infected epithelial cells rupture and release the oocysts into the intestinal lumen, so they can be excreted into the environment via the feces of the infected felid. With each fecal excretion, millions of oocysts can be released in a short period of a few days (Dubey 2001, Dubey and Frenkel 1972). While the oral ingestion of tissue cysts is the most effective way of infection, the ingestion of tachyzoites or sporulated oocysts can also result in oocyst excretion, though in differing quantities and periods (Dubey 2005, 1996). In the environment, sporulation (meiosis) occurs within 1 to 5 days, depending on environmental conditions, resulting in the formation of eight haploid sporozoites, four in each sporocyst (Dubey et al. 1970a). Depending on environmental conditions, sporulated oocysts can remain infectious for several years (Dubey et al. 1970a).

If a felid is co-infected with multiple genetically distinct *T. gondii* strains, oocysts released into the environment can theoretically contain recombinant sporozoites (Herrmann et al. 2010). An overview of experimental crosses of different clonal strains resulting in the excretion of many genetically and phenotypically distinct descendants has been prepared by Behnke and colleagues (Behnke et al. 2016). However, the vast majority of oocysts-shedding cats seem to excrete oocysts containing genetically identical clones (Herrmann et al. 2010, Schares et al. 2008, Pena et al. 2006), due to the parasites ability of self-fertilization in cats infected with only one, genetically homogeneous strain (Pfefferkorn and Pfefferkorn 1980).

2.3.2 Life cycle in intermediate hosts

Virtually all warm-blooded species, including humans, may be intermediate hosts of *T. gondii* (Dubey 2022) (Figure 2). The asexual reproduction of *T. gondii* in intermediate hosts can be differentiated into two phases: The acute phase, characterized by rapid multiplication of tachyzoites, and the chronic phase with slow multiplication of bradyzoites within tissue cysts (Frenkel 1973). Intermediate hosts can become infected by ingestion of oocysts containing sporozoites. Oral uptake of tissue cysts can also lead to infection of the intermediate host, but bradyzoites are less infectious to mice than sporozoites (Dubey 1997). After the oocyst wall is dissolved after the gastric passage, sporozoites penetrate enterocytes of the intestinal epithelium. This occurs as early as 30 minutes after oral ingestion of oocysts (Dubey et al. 1997). At six hours post infection (p. i.), sporozoites are mainly located in the lamina propria, where they multiply in a variety of cells. Most sporozoites have converted to tachyzoites within

parasitophorous vacuoles in the infected cells after 12-18 hours p. i. (Dubey et al. 1997). *T. gondii* sporozoites and tachyzoites can infect nearly all cell types of the lamina propria, including endothelial cells, smooth muscle cells, fibroblasts, and cells of the hematopoietic system, except red blood cells (Dubey et al. 1997). By infecting migratory cell types such as macrophages, tachyzoites can be spread through the whole body via the bloodstream and the lymphatic system (Seipel et al. 2010, Lambert et al. 2006). Three days p. i., tachyzoites can be found in all types of tissue of the intermediate host, where they replicate intracellularly within parasitophorous vacuoles (Dubey et al. 1997).

The differentiation of tachyzoites into bradyzoites marks the beginning of the chronic phase of infection. The associated development of tissue cysts starts around the third day p. i. (Dubey et al. 1997). Tissue cysts develop intracellularly in the cytoplasm of the host cells, predominantly in brain and skeletal muscle (Dubey et al. 1998) and they are believed to persist throughout the whole life of the intermediate host (Dubey 2022). However, occasional rupture or reactivation of tissue cysts can release parasite stages into the extracellular space (Ferguson et al. 1989). The process is well controlled in immunocompetent intermediate hosts, like humans and rodents (Montoya and Liesenfeld 2004, Beaman et al. 1994), but in immunocompromised individuals, including human patients, reactivation and conversion of bradyzoites back into tachyzoites can occur, triggering acute toxoplasmosis (Montoya and Liesenfeld 2004).

The life cycle of *T. gondii* is completed when a felid as a definitive host ingests an intermediate host harboring tissue cysts. In addition, felids can become infected by ingesting sporulated oocysts. Infected felids may serve *T. gondii* also as intermediate hosts, since tachyzoites and tissue cysts can develop in these animals.

Besides the described transmission routes, transplacental transmission of *T. gondii* is possible in definitive hosts as well as in several intermediate hosts, including humans (Dubey 2022, Beverley 1959).

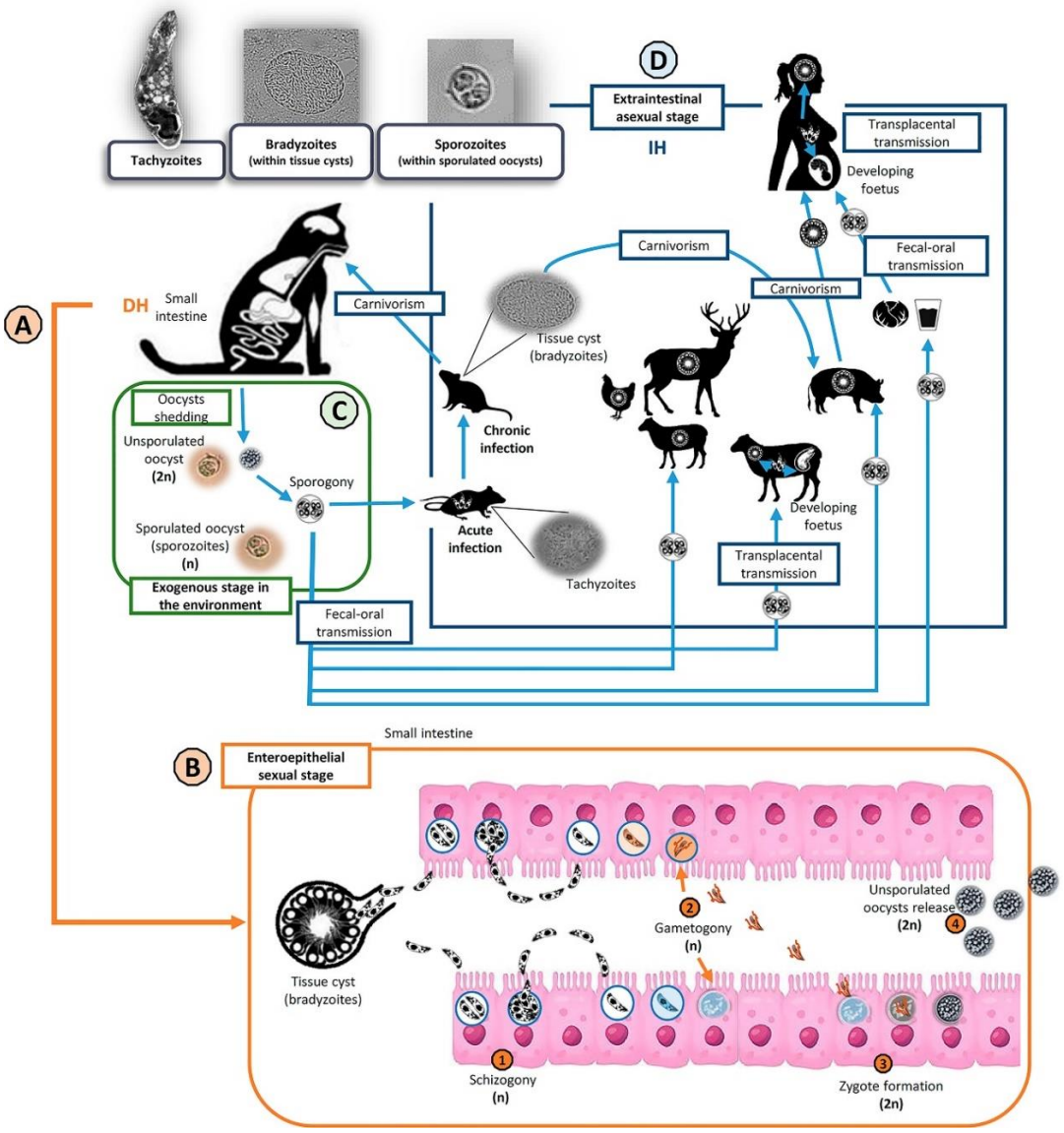

Figure 2: *Toxoplasma gondii* life cycle and transmission routes.
Invasive stages in the life cycle of *T. gondii* (top left): tachyzoites, bradyzoites (inside tissue cysts) and sporozoites (within sporulated oocysts). Members of the family Felidae (domestic and wild cats) are the only known definitive hosts (DH) of *T. gondii* (A). Cats become infected mainly after ingestion of viable tissue cyst. Then, the entero-epithelial sexual part of the cycle occurs in the small intestine of DH (B). After tissue cyst wall digestion and bradyzoites release (haploids, n), several asexual replication cycles by schizogony take place before gametogony begins. After fertilization of haploid macrogametes by haploid microgametes, resulting in the formation of diploid (2n) zygotes, an oocyst wall is developed around the parasite and epithelial cells lysis permits the release of the non-sporulated oocysts to the lumen. During the exogenous stage of the cycle (C), felids shed non-sporulated non-infectious oocysts in their feces, but sporogony occurs in the environment within 5 days given suitable conditions of aeration, humidity, and temperature. Sporogony involves meiosis (postzygotic) and sporulation. Sporulated oocysts are infectious for both intermediate and definitive hosts. The extraintestinal asexual part of the cycle (D) can occur in a wide variety of warm-blooded animals as intermediate hosts (IH). IH can mainly get infected via fecal-oral transmission (ingestion of sporulated oocysts), carnivorism (ingestion of tissue cysts) or transplacental transmission (congenital infection). Usually, the infection remains asymptomatic and becomes chronic (tissue cyst formation), except in pregnant and immunocompromised hosts, where it may have serious clinical implications (e.g. ocular toxoplasmosis, pneumonia, encephalitis). Obtained from Fernández-Escobar et al. (2022)

2.4 Toxoplasmosis

Infection with *T. gondii* leads to toxoplasmosis. The infection sources and the clinical symptoms are diverse and vary depending on the infected species and the time of infection, e.g. pregnancy. Clinical signs of toxoplasmosis in cats can be very variable, depending for example on the age or the immune status of the infected animal (Dubey 2022). There seems to be a higher risk for severe toxoplasmosis in kittens and immunosuppressed cats. In principle, cats of any age or breed can die of toxoplasmosis (Dubey 2022). General symptoms described in the literature are fever, anorexia, dyspnea caused by pneumonia or abdominal pain due to hepatitis or pancreatitis. In addition, cats can develop encephalitis with neurological symptoms like stupor, incoordination or circling and symptoms of ocular toxoplasmosis like iritis, retinal hemorrhages, mydriasis or anisocoria are also described (Dubey and Carpenter 1993). Cats infected during pregnancy can develop placentitis, and congenitally infected kittens can develop severe toxoplasmosis (Powell and Lappin 2001, Dubey et al. 1995, Sato et al. 1993).

Herbivorous intermediate hosts can become infected horizontally by ingesting oocysts or vertically by transplacental infection, leading to abortion or congenital toxoplasmosis (Dubey 2022). Severe cases of toxoplasmosis seem to be rare in adult livestock animals, but clinical signs such as fever or diarrhea are described as symptoms of acute toxoplasmosis (Dubey 2022). By far more relevant in livestock are abortion outbreaks and stillbirths caused by transplacental transmission of *T. gondii.* In New Zealand, Australia, the United Kingdom, Norway, and the United States *T. gondii* has been recognized as one of the main causes of infective ovine abortion (Dubey 2022). Therefore, a live vaccine (Toxovax) has been developed and is commercially available in the United Kingdom and New Zealand for reducing losses to the sheep industry (Buxton and Innes 1995, Buxton et al. 1993).

T. gondii infections have been extensively studied in laboratory mice, but reports on clinical toxoplasmosis in naturally infected rodents, including rats and mice, seem to be lacking (Dubey 2022). On the other hand, some studies reported on clinical toxoplasmosis in other small mammals like hares and squirrels (Kik et al. 2015, Jokelainen et al. 2011). Necropsies and further investigations of naturally infected as well as experimentally infected animals provide evidence that small mammals can also die in consequence of an infection with *T. gondii* (Dubey 2022, Kik et al. 2015, Jokelainen et al. 2011).

Humans may acquire postnatal infections mainly through the consumption of raw or undercooked meat containing tissue cysts (Jones and Dubey 2012, Munoz-Zanzi et al. 2010, Cook et al. 2000). Common sources of postnatal infection also include the consumption of

inadequately washed fruits and vegetables (Jones and Dubey 2012, Jones et al. 2006), which may harbor oocysts, oocyst-contaminated drinking water (Jones and Dubey 2010, de Moura et al. 2006, Bowie et al. 1997), and, last but not least, exposure to cat litter boxes. In rare cases humans can become postnatally infected via blood transfusion or organ transplantation.

In an immune-competent person, an infection with *T. gondii* is typically rapidly controlled by the immune system leading to either no or mild symptoms and clinical signs such as fatigue, headache, swollen lymph nodes and slight fever (Dubey 2022, Montoya and Liesenfeld 2004). During the differentiation of sporozoites into tachyzoites in the host's intestinal epithelium, a strong proliferation can lead to ileitis (Schreiner and Liesenfeld 2009). As these symptoms are common in other diseases, e.g. influenza and mononucleosis, acute toxoplasmosis is often not recognized. Besides these non-specific symptoms, an infection with *T. gondii* can also lead to ocular toxoplasmosis, manifested as retinochoroiditis (Dubey 2022).

In immunocompromised individuals such as patients suffering from Human Immunodeficiency Virus (HIV) infection or persons taking immunosuppressants after organ transplantation (Montoya and Liesenfeld 2004), a reactivation of the infection may occur. Phase conversion of latent bradyzoites, primarily found in the brain or striated muscle tissue (Dubey et al. 1998), into uncontrolled replicating tachyzoites can result in severe clinical symptoms like encephalitis (Montoya and Liesenfeld 2004) and can cause the death of the patient (Dubey 2022).

Moreover, like in other hosts, vertical, i.e. transplacental, transmission can lead to prenatal infection, especially when a pregnant woman is initially infected with *T. gondii*. Depending on the time of infection during pregnancy, miscarriage or prenatal-acquired toxoplasmosis in the child can be the result (Dunn et al. 1999). The latter often manifests in neuropathies, such as hydrocephalus or ocular toxoplasmosis (Berrébi et al. 2010, McAuley et al. 1994).

Infection with *T. gondii* can be diagnosed using indirect or direct methods. Indirect detection methods include serological tests that detect *T. gondii* antibodies in the serum of the host (Weiss and Dubey 2009). Direct detection of *T. gondii* DNA or of infectious stages of the parasite in bodily fluids and tissues is employed particularly in the diagnosis of congenital toxoplasmosis (Grover et al. 1990), ocular toxoplasmosis (Montoya et al. 1999), or in immunocompromised patients (Lavrard et al. 1995, Johnson et al. 1993). Typically, tissue, cerebrospinal fluid, blood, or amniotic fluid are examined for *T. gondii* DNA (Montoya 2002).

2.5 Prevalence of *T. gondii* infection

T. gondii is widely prevalent due to its broad host range, but its prevalence varies across regions and between host species (Dubey 2022). The parasite is one of the main causes of food-borne diseases. A WHO/FAO report (World Health Organization and Food and Agriculture Organization of the United Nations 2014) listed *T. gondii* as the fourth most important foodborne parasite in the world.

In cats, *T. gondii* antibodies have been found worldwide, but the seroprevalence varies across countries and according to the lifestyle or behavior of the cat (Dubey 2022). If cats hunt for their food, the seroprevalence is higher than in domestic cats. In general, seropositivity to *T. gondii* increases with the age of the cat. Seroprevalences of up to 90.0% are described in South American countries and of about 50.0% in Europe (Dubey 2022). On the other hand, the prevalence of *T. gondii* detected in fecal samples of cats is only about 1.6% in Europe (Dámek et al. 2023), since oocyst shedding of infected cats generally lasts only up to a few weeks.

Antibodies to *T. gondii* have been found in livestock worldwide. In general, the seroprevalence increases with the age of the hosts and is higher in free-ranging animals than in those kept indoors (Dubey 2022). A recently published review summarized prevalence estimates of *T. gondii* in European livestock animals based on direct detection of the parasite in tissue samples (Dámek et al. 2023). The prevalence estimates of *T. gondii* in pigs was 20.8%, in sheep 13.5% and in cattle only 3.9%. As tissue cysts are not homogeneously distributed within infected animals, seroprevalence may be a more representative indicator of *T. gondii* infection. On the other hand, undercooked meat containing tissue cysts is an important infection source for humans, making the prevalence of *T. gondii* important to calculate the risk of infection of humans (Dámek et al. 2023).

Pappas et al. summarized the global status of *T. gondii* seroprevalence in women who were pregnant or of childbearing age (Pappas et al. 2009). The estimates ranged from about 1.0% in South Korea to nearly 80.0% in Brazil. The study described in general a trend towards lower values in the Western world as the seroprevalence was higher in South America than in North America and Europe (Pappas et al. 2009). European studies on *T. gondii* antibodies did not uniformly represent the general European seroprevalence status, as more studies were conducted in certain regions, while there was a lack of available data from several countries (Calero-Bernal et al. 2023, Pappas et al. 2009). An overall estimated seroprevalence of approximately 30.0% was described in pregnant women or women of childbearing age in Europe (Pappas et al. 2009).

2.6 Genetic diversity and population structure of *T. gondii*

Investigating the genetic diversity and population structure of pathogens is essential for gaining insights into their epidemiology and pathogenicity, and it may help to establish effective disease control strategies. At the global level, *T. gondii* has a complex population structure (Galal et al. 2019). During the 1990s researchers initially described a clonal population structure of *T. gondii* with only three archetypal clonal lineages (type I, type II and type III) (Howe and Sibley 1995, Dardé et al. 1992, Sibley and Boothroyd 1992). Among the strains studied by Howe and Sibley, less than 5.0% showed a genetic mix of the three clonal types (Howe and Sibley 1995). The clonal population structure may result from long-term separation of different lineages and indicates that reproduction in nature occurs primarily by asexual replication or by self-fertilization of the parasite (Howe and Sibley 1995). Su et al. (2003) suggested that the clonal lineages type I, II and III emerged within the last 10,000 years after a single genetic cross. A subsequent study proposed a genealogy of these three archetypal clonal lineages, which indicates that types I and III originated from a cross between an ancestral type II strain and one of two further ancestral strains, called α or β (Boyle et al. 2006). Nevertheless, recombinant strains are also described in literature, which shows that natural sexual recombination occurs (Herrmann et al. 2010, Howe and Sibley 1995). There is no doubt that genetic recombination is likely to be a key aspect in the evolution of *T. gondii* (Boyle et al. 2006).

After the initial discovery of a clonal population structure of *T. gondii* in Europe and North America (Howe and Sibley 1995, Dardé et al. 1992), it was speculated that this also represents the global population structure of the parasite. Subsequent studies, performed with multilocus genotyping methods and based on geographically distant samples from different hosts, revealed a much higher genetic diversity than previously expected, especially in South America (Khan et al. 2006, Ajzenberg et al. 2004). More recent phylogenetic studies identified worldwide 16 well-defined haplogroups, belonging to six major clades (A-F) (Lorenzi et al. 2016, Su et al. 2012).

Within the clonal population structure described for Europe, clonal type II is the predominant genotype (Shwab et al. 2018, Shwab et al. 2014). However, some studies indicate that there might be a gradient in the prevalence of this genotype from north to south, as type II is described to be more prevalent in Northern and Western Europe. In Southern and Eastern Europe, type III seems to be more frequently detected (Kuruca et al. 2019, Messaritakis et al. 2008, Dubey et al. 2006), although type II is still predominant. This has to be further confirmed as previous studies have only focused on a few countries, implying that some European areas

are underrepresented (Shwab et al. 2018, Ajzenberg et al. 2015, Verma et al. 2015). In a German study, clones that were a mixture of type II and type III were detected in an oocyst sample shed by a single cat, suggesting that naturally recombinant isolates circulate in Europe (Herrmann et al. 2010). The clonal type I is rarely isolated from animals and humans in Europe. This is also the case for non-archetypal genotypes (Shwab et al. 2014). Taken together, there are mainly two clonal lineages of *T. gondii* circulating in Europe, with type II being predominant, followed by type III.

In Africa, a clonal population structure of *T. gondii* with a few dominant lineages is also reported by several genetic studies (Galal et al. 2019, Galal et al. 2018, Shwab et al. 2014). On the one hand, the archetypal clonal lineages type II and type III are widely distributed in Africa, with type II being described as the predominant lineage in North and East Africa and appearing to be more marginal in West and Central Africa (Galal et al. 2018). On the other hand, there are non-archetypal clonal lineages like Africa 1 and Africa 3 circulating in Africa (Galal et al. 2018). It appears that the genetic diversity of *T. gondii* is mainly clonal in the domestic environment in Africa, while there seems to be a higher diversity in the more pristine tropical areas (Galal et al. 2018).

Existing studies also indicate that *T. gondii* has a high degree of genetic uniformity in Asia. In a study of Chaichan et al., more than 80.0% of 390 analyzed samples belonged to the clonal lineages type I, II, III and Chinese 1 (Chaichan et al. 2017). Chinese 1 is described to be by far the most commonly found genotype (Shwab et al. 2014). However, a large part of the Asian continent remains underexplored. It has been hypothesized that a large diversity of host species in tropical areas of Asia might be associated with a higher genetic diversity of *T. Gondii,* i.e. the situation in Asia may resemble that observed in Africa (Chaichan et al. 2017).

Genetic studies of *T. gondii* in North America also revealed a mainly clonal population structure (Shwab et al. 2014). Like in Europe, type II and type III are predominant while type I is rarely detected (Shwab et al. 2014). Besides the three initially described clonal genotypes, a fourth clonal type (type 12) has been discovered in North America (Khan et al. 2011), which was mainly found in wild animals (Dubey et al. 2011). In general, the genetic diversity seems to be somewhat higher as compared to Europe (Shwab et al. 2014).

Several studies describe a highly diverse population structure of *T. gondii* in Central and South America, where no genotype appears to be clearly dominant (Shwab et al. 2014). This stands in sharp contrast to the other continents. The highest genetic diversity was observed in the Amazonian rainforest (Mercier et al. 2011, Ajzenberg et al. 2004). A phylogenetic study suggested that the most recent common ancestor of modern *T. gondii* strains has emerged

1.5 My ago in the Amazonian rainforest (Bertranpetit et al. 2017). This postulated ancient event may have occurred within an advantageous environment and could thus have facilitated the diversification of *T. gondii* alleles and the accumulation of mutations through genetic drift (Ajzenberg et al. 2004). Another hypothesis is that sexual reproduction between different *T. gondii* strains is more common in South America, resulting in a high genetic diversity of *T. gondii* strains, as eight wild cat species coexist on this continent (Galal et al. 2019). In addition, the warm and humid climate in the tropical region of South America could be conducive to the survival of oocysts in this environment and their spread over long distances (Galal et al. 2019).

It can be assumed that *T. gondii* spread worldwide already a long time before the development of human agriculture. The domestic life cycle of *T. gondii* associated with human agricultural settlements may have led to the development of clonal populations, which dominate the Northern Hemisphere (Shwab et al. 2018). This could be explained by selective pressure and the founder effect. In this hypothesis, *T. gondii* genotypes adapted to agricultural environments may have expanded with the spread of agriculture, and possibly replaced the native parasite genotypes (Shwab et al. 2018).

2.7 Genetic diversity and virulence of *T. gondii*

Genetic characterization studies often aim to find a link between genetics and virulence characteristics of *T. gondii*. That could be useful for detecting genotypes associated with greater clinical relevance or specific clinical manifestations (Gilbert et al. 2008, Carme et al. 2002, Grigg et al. 2001).

Prior to the development of genotyping methods based on the characterization of specific genetic markers, *T. gondii* samples were grouped by their virulence to mice. To test the virulence of individual *T. gondii* strains, numerous laboratory studies have been conducted. Mice serve as a natural intermediate host in the life cycle of *T. gondii*, allowing insights into the parasite's biology in its natural environment. The clonal lineage type I is described to be highly virulent in laboratory mice (e.g. SWISS mice, C57BL/6), as inoculation with just one parasite is sufficient to cause acute toxoplasmosis resulting in the death of the infected laboratory mouse (100% cumulative mortality, $LD_{100} = 1$) (Sibley and Boothroyd 1992). On the other hand, type II is classified to be of intermediate virulence (99.0-30.0%, $LD_{50} \geq 1000$) and type III is defined as non-virulent (< 30.0%, $LD_{50} > 10^5$) (Su et al. 2002, Sibley and Boothroyd 1992). Furthermore, differences in virulence also depend on the mouse strain (Liesenfeld 2002), the inoculation dose, and the route of inoculation (Saraf et al. 2017). In contrast to most laboratory mouse strains, rats (Dubey and Frenkel 1998), particular wild mouse strains (Hassan et al.

2019, Lilue et al. 2013), and adult chickens (Dubey 2010) are described to be resistant to *T. gondii* type I (Hassan et al. 2019, Lilue et al. 2013, Sibley and Boothroyd 1992).

T. gondii type I and non-archetypal genotypes seem to be related to an incremented virulence in humans. In a study conducted in the USA, a tendency toward the virulent type I lineage or recombinant genotypes was described in isolates from patients with ocular toxoplasmosis (Grigg et al. 2001). A report from Europe described a case of severe congenital toxoplasmosis related to an infection with a non-archetypal *T. gondii* strain (Delhaes et al. 2010). Furthermore, a notably strong association between congenital *T. gondii* infections and the development of ocular toxoplasmosis has been described in Brazil compared to Europe, probably related to the epidemic population structure of *T. gondii* prevailing in this country (Gilbert et al. 2008). In addition, French Guiana deserves special attention, as a large number of non-archetypal *T. gondii* isolates have been detected there in association with severe clinical cases of toxoplasmosis (Blaizot et al. 2019, Demar et al. 2012, Carme et al. 2002).

2.8 Genotyping methodologies of *T. gondii*

To characterize *T. gondii* strains genetically, different genotyping methodologies have been developed, including the widely used PCR-restriction fragment length polymorphism (RFLP), MS typing and MLST methods. In addition, technologies such as high resolution melting (HRM) analysis are less commonly used (Liu et al. 2015). Initial studies on genetic diversity of *T. gondii* were based on single-locus typing methods, but as these methods underestimated genetic diversity, multilocus typing strategies were established.

2.8.1 PCR-RFLP

The PCR-RFLP analysis relies on the ability of restriction endonucleases to identify single nucleotide polymorphisms (SNPs) present in amplification products (Su et al. 2010). This genotyping technique was initially designed to differentiate archetypal clonal lineages based on their banding patterns and only single or few markers were used (Howe and Sibley 1995, Sibley and Boothroyd 1992). Over the years, a multiplex (multilocus) nested PCR-RFLP method was developed, which is easy to use and able to determine the lineage genotype of *T. gondii* also in clinical samples with a low parasite burden. With this multiplex PCR it is possible to amplify up to eleven widely used genotyping markers, consisting of SAG1, SAG2 (both 5'- and 3'- ends), alt. SAG2, SAG3, BTUB, GRA6, C22-8, C29-2, L358, PK1 distributed over eight chromosomes of the nuclear genome of *T. gondii* and Apico located on the apicoplast genome (Su et al. 2010). The PCR-RFLP method can only distinguish between different lineage genotypes, but is not able to detect intra-lineage variability ("fingerprinting").

On the other hand, the advantages of this method are that it is cost-efficient and easy to apply since no automated sequencer is needed.

2.8.2 MS typing

MS sequences are tandem repeats of short (1 to 6 bp) DNA motifs that are ubiquitous in eukaryotic genomes. MS typing is based on the fact that there may be characteristic differences in the length of alleles within the same locus of different strains due to strand slippage, occurring during DNA replication (Liu et al. 2015). Thus, MS typing is carried out by determining the exact size of amplification products for different marker regions. Ajzenberg et al. established a multilocus MS typing method for *T. gondii,* which allows to amplify up to 15 markers in a multiplex PCR (Ajzenberg et al. 2010). Primers used for the PCR are labeled with three different fluorophores and the dye-labeled amplification products are separated by size using capillary electrophoresis. The 15 markers, located on eleven different chromosomes of the *T. gondii* genome*,* can be divided into eight genotyping markers (TUB2, W35, TgM-A, B18, B17, M33, IV.1, and XI.1), which are able to distinguish different lineages, and seven fingerprinting markers (M48, M102, N60, N82, AA, N61, and N83), able to detect intra-lineage genotype variability of archetypal and non-archetypal lineages (Ajzenberg et al. 2010). Like the PCR-RFLP method, MS typing is cost-efficient, but requires a capillary sequencer. The suitability for fingerprint is an important advantage compared to the PCR-RFLP method, but so far PCR-RFLP typing is more widely used.

2.8.3 MLST

The MLST analysis is based on DNA sequence polymorphisms detected in genomic regions, including SNPs, insertions and deletions (Liu et al. 2015). PCR-RFLP and MS typing are preferably used for genetic characterization of *T. gondii* in epidemiological studies, since both are simple and cost-efficient compared to MLST analysis. If the amount of *T. gondii* DNA is not limited, MLST is preferred due to its high resolution (Su et al. 2010). MLST analysis displays the whole variability of a sequenced region and studies using this approach provided an important gain of knowledge regarding the population structure of *T. gondii* (Khan et al. 2011, Khan et al. 2007). This demonstrates the importance of this method for studying the population genetics and phylogeny of the parasite. MLST analysis could be based on introns (e.g. EF1, HP2, UPRT1, UPRT2 and UPRT7) and coding regions (e.g. BSR4, BTUB, GRA6, GRA7, MIC2, ROP18, SAG1 and SAG3) (Bertranpetit et al. 2017, Prestrud et al. 2008, Khan et al. 2007). In addition, a recent study developed a method to generate RFLP profiles from DNA sequence data, via in silico digestion of known PCR-RFLP marker sequences, which allows

the integration of sequence data to the conventional RFLP genotyping method (Castro et al. 2020).

2.8.4 Whole genome sequence analysis

WGS can display the complete genome of *T. gondii* and therefore provides the most detailed information about genetic variability. Different NGS platforms like Illumina or Ion Torrent may be used to generate the data. The genome of *T. gondii* has a size of approximately 65 Mb (Lorenzi et al. 2016). Initially it was described to be composed of 14 chromosomes (Lorenzi et al. 2016), but more recent studies suggest 13 chromosomes (Galal et al. 2022, Berna et al. 2021, Xia et al. 2021). Due to the size of the genome, WGS is by far the most expensive method for genotyping of *T. gondii* and may therefore not be suitable for laboratories with limited resources (Vilares et al. 2020). In addition, analysis of the WGS data is a bioinformatically challenging task, also due to the size of the *T. gondii* genome. Furthermore, highly concentrated DNA is required, but if the amount of DNA and resources are not limited, WGS should be preferred for detailed analyses of population structure and genetic diversity of *T. gondii*.

Lorenzi et al. examined the population structure of *T. gondii* by analyzing SNPs that were defined by comparison of 61 *T. gondii* genomes to the genome of the type II reference strain ME49 (Lorenzi et al. 2016). This analysis identified a population structure of *T. gondii*, which consists of six major clades that show strong geographic separation, as described in previous studies (Su et al. 2012, Khan et al. 2007). It was observed that members of a common clade often share large conserved haploblocks across their genomes, providing evidence for a shared ancestry (Lorenzi et al. 2016). The authors hypothesized that the shared inheritance of the conserved haploblocks is associated with shared clusters of highly related genes that may influence transmission, host range and pathogenicity of *T. gondii* (Lorenzi et al. 2016). Furthermore, the study suggested the that genetic crosses between *T. gondii* strains of different clades can occur (Lorenzi et al. 2016).

Galal et al. analyzed a set of 156 genomes of *T. gondii* isolates and compared them to the genome of the type I reference strain RH (Galal et al. 2022). The authors described the presence of five clonal lineages of *T. gondii* distributed across more than one continent (type I, type II, type III, Africa 1, and Africa 4) (Galal et al. 2022). Furthermore, four clonal lineages were identified that were only present in South America. Two of them corresponded to the previously described MS genotypes Caribbean 1 and Caribbean 2. Yet, most of the analyzed *T. gondii* strains from South America were non-clonal (Galal et al. 2022). Moreover, an ancestry analysis was performed in that study. The results of this analysis suggest that the

intercontinental lineages (type I, type II, type III, Africa 1, and Africa 4) and New World non-hybrid wild populations (called Amazonian and Pan-American) were putative ancestral *T. gondii* populations (Galal et al. 2022). All other genomes analyzed in the study were defined as potentially hybrid as they shared chromosome regions with the defined ancestral strains (Galal et al. 2022). In Europe, Africa and Asia, most *T. gondii* strains belonged to one of the intercontinental clonal lineages. *T. gondii* strains originating from South or Central America belonged to the well-defined distinct wild *T. gondii* populations or were probably the result of hybridizations between different ancestral strains (Galal et al. 2022). Furthermore, the authors identified a unique haplotype on chromosome 1a, which was common to all intercontinental *T. gondii* lineages and hybrid populations derived from these lineages (Galal et al. 2022). This *T. gondii* haplotype probably accompanied the wildcat (*Felis silvestris*) already thousands of years ago, found its way to domestic cats and followed its spread in the Old World and later in the New World (Galal et al. 2022). The authors hypothesized that this haplotype plays a role in the sexual reproduction of *T. gondii* in domestic cats (Galal et al. 2022).

3 Research publications

3.1 *Toxoplasma gondii* genotyping: A closer look into Europe

Authors: Mercedes Fernández-Escobar, Gereon Schares, Pavlo Maksimov, Maike Joeres, Luis M. Ortega-Mora, Rafael Calero-Bernal

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3.1.1 Declaration of author contributions

Contribution of Maike Joeres: Literature research and collection of data about microsatellite typing, reviewing/editing the manuscript, co-authoring the manuscript.

Contributions of other authors: All authors have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Toxoplasma gondii Genotyping: A Closer Look Into Europe

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Toxoplasma gondii is a major zoonotic agent which may cause harmful effects mainly in pregnant and immunocompromised hosts. Despite many efforts on its genetic characterization, an entirely clear picture of the population structure in Europe has not been achieved yet. The present study aimed to summarize the available genotyping information and to map the distribution of circulating strains. There is consensus on type II *T. gondii* genotypes prevailing in Europe, but the absence of harmonization in the use of typing methods limits detailed knowledge. Standardized, high-end typing tools and integrative strategies are needed to fill the gaps and complete an accurate image of the *T. gondii* genetic population in Europe.

Keywords: *Toxoplasma gondii*, Europe, genotypes, typing methodologies, population structure

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INTRODUCTION

Toxoplasma gondii, the etiologic agent of toxoplasmosis, is an apicomplexan obligate intracellular protist of major medical and veterinary relevance. The complex life cycle of *T. gondii* is defined as facultative heteroxenous, with virtually all warm-blooded animals as intermediate hosts (including humans, domestic and wild mammals and birds), and members of the Felidae family acting as definitive hosts (Dubey, 2021a) (**Figure 1**). Toxoplasmosis is a zoonosis of global distribution (Robert-Gangneux and Dardé, 2012; Dubey, 2021a) and represents an excellent example of the One Health concept, since *T. gondii* is present and circulates through all compartments defined in this paradigm (Aguirre et al., 2019; Djurković-Djaković et al., 2019). Due to its wide host range the parasite is of importance not only in public health, but also in livestock industry and wildlife management programs. A FAO/WHO report considered *T. gondii* as the fourth most important foodborne parasite in the world (FAO and WHO, 2014). In addition, globalization and trade could contribute to the inter-regional and intercontinental spread of new parasite strains (Bertranpetit et al., 2017; Galal et al., 2019).

In humans, this parasite infects up to a third of the total global population (Bigna et al., 2020; Rostami et al., 2020). The infection is usually asymptomatic and results in chronicity; however, a primary infection in pregnant women could cause congenital transmission and consequent serious damage to the fetus (Jones et al., 2001). In immunocompromised individuals, severe neurologic and pulmonary clinical signs are frequently observed consequences of a re-activated or new infection (Wang et al., 2017; Robert-Gangneux et al., 2018). Finally, ocular toxoplasmosis is an increasingly

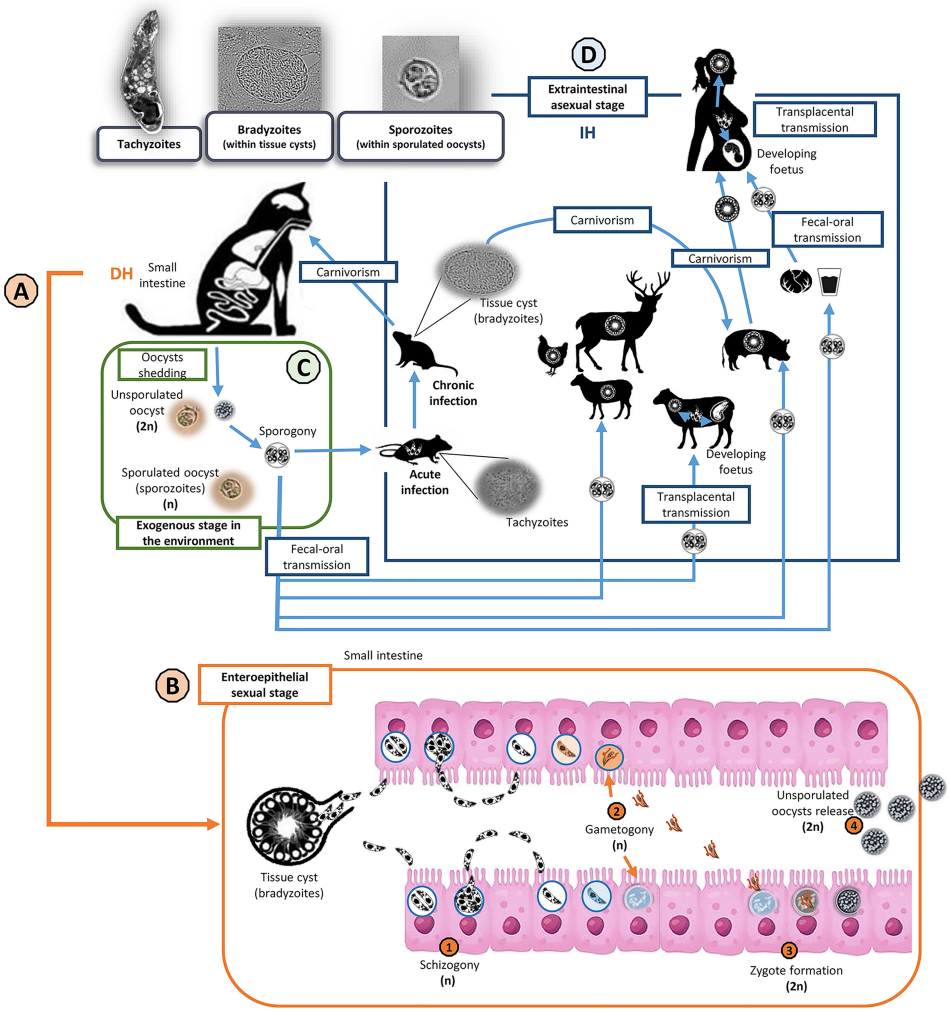

FIGURE 1 | *Toxoplasma gondii* life cycle and transmission routes. Invasive stages in the life cycle of *T. gondii* (top left): tachyzoites, bradyzoites (inside tissue cysts) and sporozoites (within sporulated oocysts). Members of the family Felidae (domestic and wild cats) are the only known definitive hosts (DH) of *T. gondii* **(A)**. Cats become infected mainly after ingestion of viable tissue cyst. Then, the enteroepithelial sexual stage of the cycle takes part in the small intestine of DH **(B)**. After tissue cyst wall digestion and bradyzoites release (haploids, n), several asexual replication cycles by schizogony take place before gametogony begins. After fertilization of haploid macrogametes by haploid microgametes, resulting in diploid (2n) zygotes formation, an oocyst wall is developed around the parasite and epithelial cells lysis permits the release of the unsporulated oocysts to the lumen. During the exogenous stage of the cycle **(C)**, felids shed unsporulated non-infectious oocysts in their feces, but sporogony occurs in the environment within 5 days given suitable conditions of aeration, humidity, and temperature. Sporogony involves meiosis (postzygotic) and sporulation. Sporulated oocysts are infectious for both intermediate and definitive hosts. The extraintestinal asexual stage of the cycle **(D)** could occur in a wide variety of warm-blooded animals, as intermediate hosts (IH). IH can mainly get infected *via* fecal-oral transmission (ingestion of sporulated oocysts), carnivorism (ingestion of tissue cysts) or transplacental transmission (congenital infection). Usually, the infection courses asymptomatic and becomes chronic (tissue cyst formation), except in pregnant and immunocompromised host, when it could have serious clinical implications.

recognized clinical issue in some parts of the world, also in immunocompetent patients (Shobab et al., 2013; Maenz et al., 2014).

In livestock, *T. gondii* infection is associated with significant economic losses linked to reproductive failure in several domestic species such as sheep and goats (Stelzer et al., 2019; Dubey et al., 2020a; Dubey et al., 2020b). Infection by *T. gondii* in livestock is also a risk to public health when animals destined for human consumption are involved (Opsteegh et al., 2016). Moreover, the parasite is a cause of concern in wildlife and zoo

animals since *T. gondii* may cause lethal infection in particular species (Dubey, 2021a).

Although important oocyst-associated human toxoplasmosis outbreaks have been documented in the past few years (Pinto-Ferreira et al., 2019; Dardé et al., 2020; Dubey, 2021b), the relevance of the environmental route remains poorly investigated. *Toxoplasma gondii* oocysts have been detected in a wide spectrum of matrices worldwide, including fresh produce, water, soil or even bivalves (*e.g.*, mussels and oysters), which can accumulate *T. gondii* oocysts by water filtration (Shapiro et al., 2019; Marquis et al., 2019; Almeria and Dubey, 2021).

Strategies to reduce the disease burden of toxoplasmosis should be based on close collaboration between both medical practitioners and veterinarians under the One Health umbrella. The relative contributions of the different transmissible stages, sources, and transmission pathways (**Figure 1**) remain partly unknown. This lack of information on the attribution to specific infection sources has hampered the development of effective intervention strategies. That fact could be partly due to the absence of a systematic surveillance system for this zoonotic foodborne pathogen (van der Giessen et al., 2021). In addition, there are major geographical differences in the epidemiology of the infection as well as in food consumption habits around the world, which affect the importance of different transmission routes and specific food products for the occurrence of the infection (Galal et al., 2019).

In Europe, *T. gondii* is considered an important foodborne parasite that ranked high according to the multiple-criteria decision analyses (MCDA) (Bouwknegt et al., 2018) and disease-burden estimations for toxoplasmosis (Havelaar et al., 2015). Congenital toxoplasmosis is notifiable in 29 of 35 European countries surveyed, with routine testing of pregnant women in some countries such as Austria, Belgium, and France; nevertheless, underreporting is a major problem in most countries. ln animals, risk-based surveillance system of EU livestock needs to be improved to reduce human meat-borne infections; there is a lack of standardization and validation of diagnostic techniques as well as significant limitation in the number of animals tested and the information associated with them (*e.g.*, age and breeding system) (van der Giessen et al., 2021; EFSA and ECDPC, 2021).

Concerning the genetic diversity of *T. gondii* circulating in Europe, type II strains and, to a lesser extent, type III strains, are the dominating populations, both in domestic and wild environments (Khan et al., 2007; Lorenzi et al., 2016). However, the current globalization of trade seems to be causing risk situations that pose new research and public health challenges (Galal et al., 2019). For instance, cases of severe human toxoplasmosis have been reported in France due to the consumption of imported South and North American horsemeat contaminated with non-archetypal strains of the parasite (Elbez-Rubinstein et al., 2009; Pomares et al., 2011).

Because of the importance of a genetic characterization of *T. gondii* strains for epidemiological and clinical studies, this work is aimed to summarize present knowledge on the genetic population structure of *T. gondii* in Europe and the distribution of genotypes within the different compartments comprised in the One Health concept (*i.e.*, human, domestic and wild animals, and environment).

TOXOPLASMA GONDII, A COMPLEX ORGANISM WITH COMPLEX GENETICS

The *Toxoplasma gondii* Life Cycle, an Avenue for a Rich Genetic Diversity

A global distribution and a complex life cycle, including a sexual phase that makes genetic recombination events possible, have led to a wide genetic and phenotypic diversity within *T. gondii* populations circulating worldwide (**Figure 1**).

Almost all life cycle stages of *T. gondii* are haploid, with the exception of a short diploid phase from the zygote formation in the small intestine of felines (Martorelli et al., 2019) to sporulation in the environment, when haploid sporozoites are the result of a postzygotic meiosis (Dubey, 2021a) (**Figure 1**). Unlike for many apicomplexan parasites, the sexual phase is not mandatory in the case of *Toxoplasma* and *Neospora* genera and zoites can propagate by asexual replication indefinitely (Beck et al., 2009) (**Figure 1**).

During the 1990s, restriction fragment length polymorphism (RFLP) among other methods allowed researchers to establish the existence of three clonal lineages distinguished according to their virulence for mice. Type I isolates were 100% lethal to mice, irrespectively of the dose, while types II and III were moderately or non-virulent in a dose-dependent manner (Dardé et al., 1992; Sibley and Boothroyd, 1992; Howe and Sibley, 1995; Howe et al., 1996). Since then, global population structure and genetic variability of *T. gondii* has been extensively investigated. The rapid development of multilocus-sequencing methods, and the description of a wide panel of new PCR-RFLP and microsatellite (MS) markers led to solid observations on the predominance of three clonal/archetypal types or lineages in Europe and North America, but new concepts of "recombinant" and "atypical/non-canonical" strains appeared on the scene (Ajzenberg et al., 2002; Ajzenberg et al., 2005; Khan et al., 2005; Su et al., 2006; Khan et al., 2007). Later, pioneering long term activities in sampling *T. gondii* isolates world wide (*e.g.*, [Lehmann et al., 2006; Dubey et al., 2020c]), the establishment of a Toxoplasma Biological Resource Centre located in France (Rocaboy et al., 2020), or the release of the specific genomic database ToxoDB (http://ToxoDB.org), provided an excellent and continuing foundation for further population genetic analyses.

An Eye on *Toxoplasma* Genome-Wide Aspects

The total haploid genome of *T. gondii* contains 13 chromosomes, with a total genome size of about 65 million base pairs (Mbp) and more than 8300 protein coding genes identified (Lorenzi et al., 2016; Xia et al., 2021). The genome-wide polymorphism rate between the three archetypal clonal lineages has been estimated to be approximately 1%, characterized by an extensive bi-allelism

falling into type I, II and III single nucleotide polymorphisms (SNP) (Grigg et al., 2001; Khan et al., 2005; Boyle et al., 2006; Sibley and Ajioka, 2008). The origin of this clonality has been suggested to be due to a recent emergence from a common ancestor within the last 10,000 years during the domestication process of cats and various livestock species (Su et al., 2003). In addition, an extensive bypassing of the sexual cycle may have led to a continuous asexual propagation, resulting in rare possibilities for meiotic crosses between the highly similar parental strains (Sibley and Ajioka, 2008) only observed occasionally in naturally infected cats (Herrmann et al., 2012a). Nevertheless, this hypothesis is not applicable to the South American subcontinent, where a notably higher prevalence (and burden) of the infection, a larger spectrum of susceptible intermediate host species along with an increased diversity of wild felids might have promoted more frequent recombination events resulting in a contrasting, extremely diverse and largely non-archetypal population (Shwab et al., 2014; Bertranpetit et al., 2017).

Global *Toxoplasma gondii* Population Genetic Structure

Until date there have been several comprehensive attempts to unravel the population structure of the parasite aided by great advances in molecular typing techniques. In an extensive and in-depth study based on phylogenetic analysis of above 950 typed isolates worldwide, 15 well-defined haplogroups were identified (Su et al., 2012), which were subsequently expanded to 16 and assorted into 6 major clades (clade A-F) based on whole genome sequencing analyses (Lorenzi et al., 2016).

The three clonal types dominating Europe and North America (corresponding to haplogroups (HG) 1, 2 and 3) were joined by a fourth clonal lineage (HG12) largely confined to North America, where it is more common in wild animals. In contrast, much greater genetic diversity is observed in South America, where the population seems to consist of a few major clonal complexes and abundant less related isolates (Khan et al., 2007; Pena et al., 2008; Khan et al., 2011; Jiang et al., 2018).

It has been suggested that African and Asian *T. gondii* populations could be a mixture between both above situations, with abundance of isolates belonging to type I, II, and III clonal lineages, coexisting with a considerable number of other recombinant or atypical genotypes, but exhibiting a less divergent character than in South America; however, both continents remain poorly explored, especially in tropical regions (Chaichan et al., 2017; Galal et al., 2018). With regard to the geographical origin of the species, paradoxically there are conflicting theories. On the one hand, a combination of molecular phylogenetic and phenotypic analyses suggested a North American common ancestor that entered South America and diversified there after reestablishment of the Panamanian land bridge (Khan et al., 2007; Minot et al., 2012). Nevertheless, subsequent phylogenetic and geostatistical approaches led to hypothesize a South American origin of *T. gondii* and its initial spread through North America, Asia, Europe and finally Africa, through different migration routes, linked to the co-evolution of Felidae family members and humans (Bertranpetit et al., 2017).

TOXOPLASMA GONDII GENOTYPING TOOLS IN EUROPE: IS THERE A CONSENSUS?

Available genotyping methodologies, PCR-RFLP, PCR-sequencing, MS-typing among others, have been irregularly applied in different areas, over different matrices and in a different manner by distinct research groups. The present section aims to examine the use of common methodologies within the European context. PubMed database was searched combining the terms "Toxoplasma gondii", "genotyping", "typing", "type" and each different possible host designations or categories (*e.g.*, human, goat, fox, marine mammals, etc.) or environmental matrices (*e.g.*, water, soil, fresh produce, etc.) considered. Both *T. gondii* strain genotyping studies involving isolated viable parasites or DNA positive specimens/clinical samples from Europe were included. Nevertheless, data from overseas territories in other continents and zoo-kept animals were not covered, in order to better limit the origin of infections to continental Europe. Finally, 101 and 43 studies including PCR-RFLP/PCR-Sequencing or MS typing, respectively, were selected (see **Supplementary Tables S1, S2**). Despite the large number of studies aiming at a genetic characterization of European *T. gondii* strains, the data are limited due to several factors. After analysis of the extracted data, it seems to be apparent that there is a notable variance in the identity and number of markers used among the studies (**Figures 2A–C**). The selected studies comprised the use of up to 15 different PCR-RFLP (**Figure 2A**), PCR-Seq or MS (**Figure 2B**) markers. The use of an insufficient number of molecular markers may represent a problem because a large part of diversity might be missed or genotypically different parasites not efficiently distinguished. This is especially worrying in the case of PCR-RFLP and PCR-sequencing, since an important proportion (40%, 40/101) of these studies implemented a single-locus typing method, therefore involving major limitations for reliable strain classification (**Figure 2C**). The most frequently used marker was *SAG2* (5' and 3' ends of the gene) probably because it was among the first PCR-RFLP markers described, setting a milestone on *T. gondii* genetic studies (**Figure 2A**) (Sibley and Boothroyd, 1992; Howe and Sibley, 1995). On the other hand, comparison between studies is hardly possible if assays are based on infrequently used genes, such as *ROP1* (Haque et al., 1999; Turčeková et al., 2013), or on markers, like the *B1* gene, mostly applied in a certain type of environmental specimens (*i.e.*, water, soil, air, vegetables, or fruit) (**Figure 2A**) (Burg et al., 1989; Sroka et al., 2008; Sroka et al., 2009; Sroka et al., 2010). Regarding MS typing procedures, the number of markers has not been observed as a problematic issue since the use of five "genotyping" markers or the complete panel of eight "genotyping" plus seven "fingerprinting" MS markers is quite widely used (**Figure 2B**).

Furthermore, it is observed that regardless of the typing technique used, the collected information (from 21 different countries) is highly unbalanced between countries, and there is a lack of data for large areas of the European continent (**Figure 2D**). There are cases in which the same samples have been analyzed by different techniques (PCR-RFLP, MS and/or

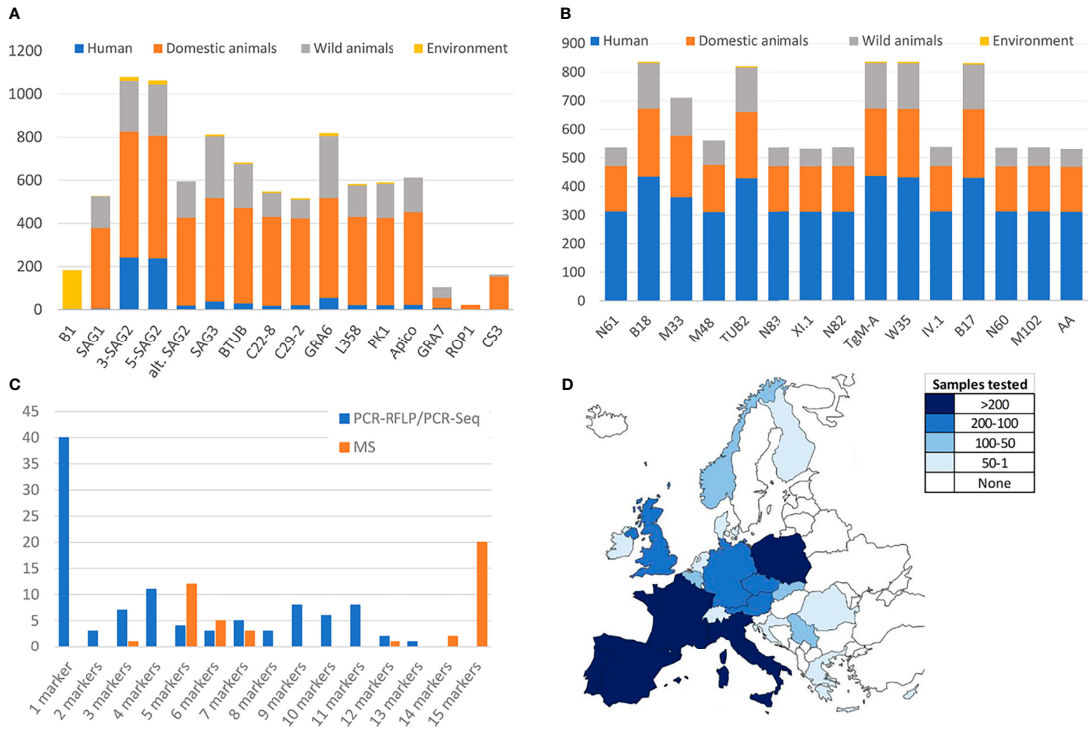

FIGURE 2 | Analyses of the methodologies used within European *Toxoplasma gondii* genotyping studies. **(A)** Number of samples typed by each PCR-RFLP marker in genotyping European studies. **(B)** Number of samples typed by each MS marker in genotyping European studies. **(C)** Proportion of studies implementing different number of PCR-RFLP/PCR-Seq or MS genotyping markers. **(D)** Geographic distribution of genotyped samples across Europe. Only studies meeting the cut-off criterion (at least 4 PCR-RFLP/PCR-Seq or 5 MS markers applied) have been considered.

PCR-sequencing), with matching results although with of course different resolution power (Prestrud et al., 2008; Stajner et al., 2013; Verma et al., 2015).

Aiming to find the right balance between reliability and robustness, and taking into account the number of studies implementing a different number of PCR-RFLP/PCR-Seq or MS genotyping markers (**Figure 2C**), a minimum of four and five genomic regions analyzed was established as a "cut-off", respectively. To this end, 51 (with typing results on n=804 samples) and 42 studies (n=831 samples typed) including PCR-RFLP/PCR-Seq or MS typing, respectively, were considered to represent a robust pan-European overview (**Table 1** and **Figure 3**).

GENERAL PICTURE OF THE GENETIC POPULATION IN EUROPE

PCR-RFLP and MS typing are the most widely used methods, but except for predominant lineages and some unique strains, equivalence between assigned genotypes by each technique remains at some extent confusing; thus, remarks will be given separately. The classification of an isolate into archetypal, recombinant or atypical, or even distinguishing between a recombinant strain and a mixed infection (co-infection) is a sensitive issue. In most cases this requires the availability of viable parasites in a sample that could be separated into different co-existing clonal populations, *e.g.*, by limiting dilution cloning (Herrmann et al., 2010). The unambiguous identification of mixed infections is difficult in only DNA positive materials and largely depends on the number and the discriminating power of markers used for genotyping. Therefore, from a critical viewpoint, mixed infections, as well as infections with recombinant (mixture of type I, II or III alleles as a consequence of recombination events) and atypical (including unique polymorphisms at any loci) strains should be treated as a whole (MRA category), differentiating them from the widely prevalent archetypal clonal strains (*e.g.*, types I, II and III). Based on the One Health concept, we sorted genotypic information according to samples or isolates origin into four "compartments", namely humans, domestic animals, wildlife, and environment (**Table 1** and **Figure 3**).

TABLE 1 | Prevalence of the *Toxoplasma gondii* genetic types observed in isolates and DNA positive specimens/clinical samples in Europe according to the four compartments within the One Health concept (human, domestic animals, wildlife, and environment) and based on PCR-RFLP/PCR-sequencing or MS data.

	Humans		Domestic animals[1]		Wildlife[2]		Environment[3]		TOTAL	
	RFLP/Seq (%)	MS (%)	RFLP/Seq (%)	MS (%)	RFLP/Seq (%)	MS (%)	RFLP/Seq (%)	MS (%)	RFLP/Seq (%)	MS (%)
Type I	0	11	10	4	2	1	2	4	14	20
	(0)	(2.6)	(2)	(1.7)	(0.8)	(0.6)	(22.2)	(80)	(1.7)	(2.4)
Type II[4]	29	370	431	217	172	142	7	0	639	729
	(87.9)	(86.4)	(86)	(91.2)	(65.9)	(88.8)	(77.8)	(0)	(79.5)	(87.7)
Type III	1	13	31	16	33	6	0	0	65	35
	(3)	(3)	(6.2)	(6.7)	(12.6)	(3.8)	(0)	(0)	(8.1)	(4.2)
MRA	3	27	29	1	54	10	0	1	86	39
	(9.1)	(6.3)	(5.8)	(0.4)	(20.7)	(6.2)	(0)	(20)	(10.7)	(4.7)
Likely importation/migration related genotypes	–	7	–	0	–	1	–	0	-	8
		(1.6)		(0)		(0.6)		(0)		(1)
TOTAL	33	428	501	238	261	160	9	5	804	831

Percentages are given in brackets. MRA: Mixed infections and recombinant or atypical genotypes; Likely importation/migration related genotypes (Africa1, Caribbean2, Caribbean3); -: PCR-RFLP method is not valid for intra-genotype differentiation. Compartments: (1) livestock (poultry, cattle, small ruminants, equines, pigs) and pets (carnivores); (2) rodents, marine mammals, wild ungulates (Cervidae, Bovidae, swine), mesocarnivores, wild cats, and wild avian species; (3) water, soil, air, fresh produce, ticks, and bivalves; (4) PCR-RFLP profiles suggesting a type II PRU variant (type II alleles combined with type I allele at Apico marker) were included within Type II category.

Toxoplasma gondii Genetic Diversity Based on PCR-RFLP or PCR-Sequencing Methodologies

Concerning strain types detected in humans, only three countries are represented (Germany, Poland and Serbia) in five studies with a total of 33 samples typed (Djurković-Djaković et al., 2006; Nowakowska et al., 2006; Stajner et al., 2013; Marković et al., 2014; Herrmann et al., 2014). Among them, almost 90% (29/33) corresponded with type II strains, only one type III was detected, and MRA infections were described in three cases. The presumed predominance of type II in Europe is evident but non-conclusive since data could be representative only of central Europe.

Most European (geno)typed samples have been collected from infected domestic (pets and livestock) and wild animals. Regarding domestic animals, the range of countries represented is wider but not enough, with molecular studies from Austria, Czech Republic, Denmark, France, Germany, Ireland, Italy, Poland, Portugal, Serbia, Spain, Switzerland, and The Netherlands (22 studies with a total of 501 samples) (**Table 1** and **Supplementary Table S1**). Likewise, studies could be sorted according to the host, including data from sheep, goat, cattle, pig, horse, chicken, dog, and cat, standing out chicken and pig species in terms of sampling effort, with 102 and 76 samples typed, respectively. Type II strains were reported in 86% (431/501) of samples, together with 6.2% (31/501) of type III, 2% of type I (10/501) and approximately 6% (29/501) of MRA infections (**Table 1** and **Figure 3**). Concerning wild animals, European studies include data from Croatia, Czech Republic, Denmark, Germany, Italy, Norway, Poland, Serbia, Spain, and the UK, with a total of 261 samples collected in 25 different studies. It involves data from a wide variety of hosts such as rodents, marine mammals, wild cats, wild swine, mesocarnivores, wild ruminant ungulates, and wild avian species. Within the group of wild animals, mesocarnivores were those with the highest number of studies (n=8) and samples analyzed (n=144). Approximately 66% of strains circulating in wildlife were reported to be type II (172/261), 20.7% MRA (54/261), 12.6% type III (33/261), and 0.8% type I (2/261) (**Table 1** and **Figure 3**).

Regarding genotypes present in environmental samples, the situation is even more restricted, with only two studies having met the requirements accounting for a total of nine samples. Type II strains were reported in seven samples of vegetables in the Czech Republic (Slany et al., 2019) whereas type I alleles were observed in DNA extracted from two ticks (*Dermacentor reticulatus*) collected in field areas of Poland (**Table 1**) (Wojcik-Fatla et al., 2015).

As a whole, literature data on PCR-RFLP typing or PCR-sequencing suggest a clear predominance of type II strains circulating in Europe, that comprises of 79.5% (639/804) of the total samples collected in 51 different studies included (**Table 1**). Previous serotyping studies largely corroborated this type II predominance (Nowakowska et al., 2006; Morisset et al., 2008; Maksimov et al., 2012). Reports on type I strains are truly scarce, 1.7% (14/804) of samples, whereas type III strains seem to be responsible for 8.1% of total samples (65/804). Finally, MRA infections were reported for 10.7% (86/804) of the records. Despite the limitation on the data, it could be pointed out the higher burden of type III strains and MRA infections in the case of wildlife animal species in comparison with the rest of European compartments considered. In **Figure 2D**, geographic distribution of genotyped samples across Europe is represented. Germany, Italy, and Serbia are the countries with the highest number of PCR-RFLP/PCR-Seq based genotyping investigations.

Complementarily, we proposed the use of sequencing data from *Toxoplasma* molecular markers deposited in NCBI database (https://www.ncbi.nlm.nih.gov/nucleotide?cmd=search) to implement possible phylogenetic analyses. After a detailed screening and manual curation of nucleotide sequences available from *T. gondii* specific genetic markers (n=7776), only entries from Europe (n=464; 6%) were extracted (**Table 2** and **Supplementary Table S3**). Then, the only markers that had sufficient high-quality sequences from at least four different

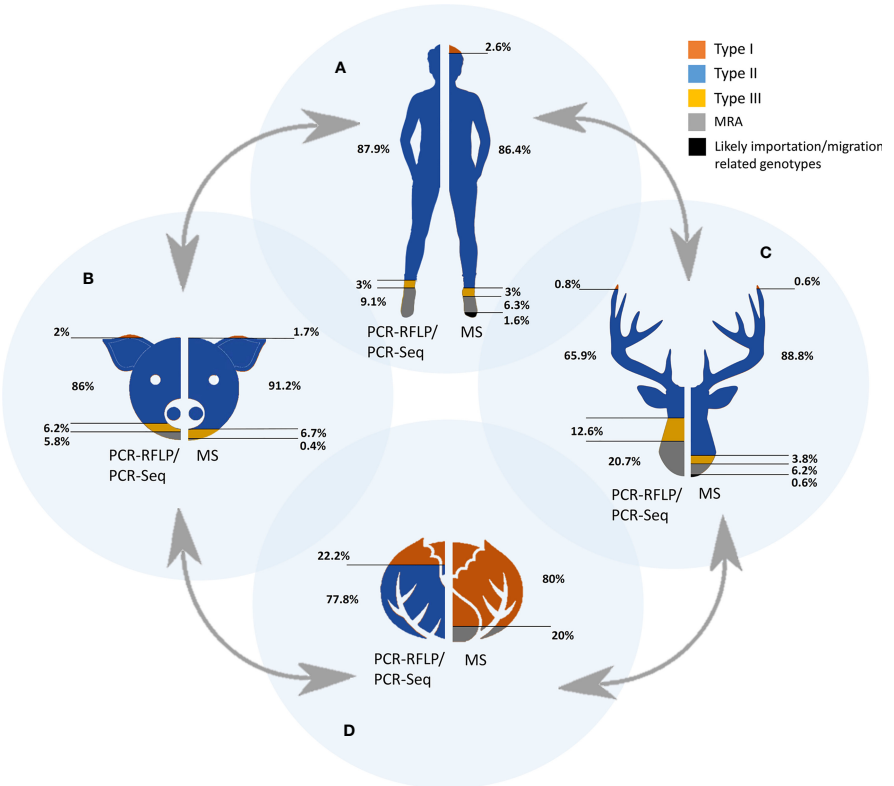

FIGURE 3 | Occurrence of *Toxoplasma gondii* genetic types by each of the One Health compartments. The four interconnected compartments comprised in the One Health concept are represented as humans **(A)**, domestic animals **(B)**, wild animals **(C)** and environment **(D)**. Colored areas represent the proportion of each genetic types detected either by PCR-RFLP/PCR-Seq or MS-typing. MRA: mixed infections, recombinant and atypical genotypes detected.

European countries to perform a robust phylogenetic analysis were loci *GRA6* (n=86) and *SAG3* (n=49). In addition, *B1* gene (n=76) was included as it is the marker that better represents the environmental compartment (**Supplementary Table S3**). Sequences were downloaded and assessed further (**Figure 4**). The composition of dendrograms obtained was not related to the geographical origin of sequenced *T. gondii* strains, and sequences were barely allocated to defined clusters along with respective type I, II and III canonical references. *GRA6* and *SAG3* sequences seem to provide a higher resolution than *B1* sequences, without discriminative power to split major lineages into separated clusters.

Toxoplasma gondii Genetic Diversity Based on MS Methodologies

Under the view of the available literature (**Supplementary Table S2**), the number of samples typed by less than 5 MS loci is negligible compared to the 831 samples typed in 42 different studies by using five or more MS markers (**Table 1**). Apart from type I, II, III or MRA infections, by MS typing it was also possible to identify specific genotypes such as *Africa1*, *Caribbean2*, *Caribbean3* even characterizing only five loci (*B18*, *TUB*, *Tg-MA*, *W35* and *B17*).

Unlike the previously mentioned methods, the MS-based methodology has been widely used in the genetic characterization of human samples, involving a total of 428 samples in 20 different studies. Despite the participation of a greater number of European countries, France clustered 77.3% of the human samples analyzed (Ajzenberg et al., 2009; Ajzenberg et al., 2015), followed by Portugal (11.7%) (Ajzenberg et al., 2009; Vilares et al., 2017), Denmark (4.7%) (Jokelainen et al., 2018), and Belgium (4.4%) (Gisbert Algaba et al., 2020); most of the other countries contributed with up to three single isolates (Austria, Germany, Romania, Serbia, The Netherlands, and UK). Concerning strain types detected in human population, 86.4% corresponded with type II strains, the types I and III were found in low proportions (2.6 and 3% respectively), and those of MRA infections corresponded to 6.3% of cases. In addition, six cases of human infection with *Africa1* strains and one case with *Caribbean2* were detected in France, Denmark, and Belgium (Ajzenberg et al., 2010; Fekkar et al., 2011; Su et al., 2012; Jokelainen et al., 2018) (**Table 1** and **Figure 3**). The

TABLE 2 | Summary on sequence data available in GenBank for European *Toxoplasma gondii* isolates and samples.

Marker or gene	Country								Total
	France	Italy	The Netherlands	Norway	Poland	Portugal	Spain	United Kingdom	
Apico		1							1
B1	1	6			69				76
BTUB		3			4			1	8
c22-8		5							5
c29-2		1							1
CS3							2		2
GRA6	12	35	2	1	19	12	3	2	86
GRA7	33					12	3		48
PK1		1							1
ROP8	1								1
SAG1	12	3			17				32
SAG2						118			118
3′-SAG2		1			3			1	5
5′-SAG2		4						1	5
alt. SAG2		22			3				25
SAG3	12	1			14	12	5	5	49
UPRT-intron1				1					1
Total	71	83	2	2	129	154	13	10	464

Available sequences were further analyzed when four or more countries were covered. B1 gene was also included (data from three countries) as it is the marker that better represents the environmental compartment. Sequences likely related to migration/importation, reference strains, or with sequencing errors were excluded.

predominance of type II in Europe is again clear but once more it should be borne in mind that extensive areas of the continent are still not represented.

The second most studied compartment was that of domestic animals, involving a total of 238 samples in 15 different investigations. Once again, France (36.9%) and Portugal (20.6%), together with Austria (27.3%), stood out in the number of genotyped samples. Data from Finland, Germany, Italy, Romania, Serbia, and The Netherlands are also available. In respect of the different hosts studied, most of the samples were collected from chicken (93) and sheep (91) (Verma et al., 2015; Bertranpetit et al., 2017; Shwab et al., 2018). In pets and livestock, type II strains were reported in 91.2% (217/238) of samples, along with a 6.7% (16/238) of type III and 1.7% of type I (4/238). Apart from that, only one sample presented a MRA profile (0.4%, 1/238). Concerning wildlife, European studies included data from Belgium, Czech Republic, England, Finland, France, Italy, Norway, Portugal, Serbia, and Spain, with a total of 160 samples collected in 15 different publications; a wide variety of hosts were included in such surveys, highlighting red foxes (*Vulpes vulpes*) (n=54) (Aubert et al., 2010; De Craeye et al., 2011) and wild boars (*Sus scrofa ferus*) (n=44) (Richomme et al., 2009; Gisbert Algaba et al., 2020). Among strains circulating in wild animals, 88.8% corresponded to type II (142/160), 6.2% (10/160) to MRA infections and 3.8% (6/160) to type III. Only one case of type I and another of a *Caribbean3* genotype were reported (0.6% each, 1/160) from a pigeon from Portugal and a wild boar from Italy, respectively (Vilares et al., 2014; Sgroi et al., 2020).

As occurred in previous section regarding PCR-RFLP and PCR-sequencing based studies, typing reports on environmental samples again are quite rare. One study reported genotyping results from Mediterranean mussels (*Mytilus galloprovincialis*) collected in southern Italy (Santoro et al., 2020), with four samples surprisingly belonging to type I and one sample typed as a recombinant or mixed profile. As this is the only study, including such a small sample size, general conclusions cannot be reached at this time.

On balance, the prevalence figures obtained from reviewing the available data on *T. gondii* strains genotyped by MS in Europe are quite similar to those obtained by PCR-RFLP and PCR-sequencing methods. The predominance of type II strains in Europe is again evident, involving 87.7% (729/831) of the total samples analyzed in 42 studies that meet the criteria of at least 5 genotyping markers characterized (**Table 1** and **Figure 3**). Type I strains remain infrequent, representing 2.4% (20/831) of samples. On the other hand, the prevalence of type III and non-assorted, recombinant strains or mixed infections were slightly lower compared to PCR-RFLP and PCR-sequencing methods with almost 4.2% (35/831) and 4.7% (39/831) of total records, respectively. Finally, MS-typing was able to resolve other non-canonical haplogroups, *i.e.*, *Caribbean1*, *Caribbean3* or *Africa1*, allowing to identify *T. gondii* strains possibly imported to Europe (1%, 8/831), either by human migration or trade. Overall, France, Portugal, Austria, and Belgium are the countries with the highest number of MS genotyping results; in contrast, there are large areas of the continent from which there is no information, especially northern European countries (**Figure 2D**).

A Global optimal (go)eBURST Full MST (goeBURST distance) analysis (Feil et al., 2004; Francisco et al., 2009) of all *T. gondii* DNA samples typed by 15 MS markers (n=487) using PHYLOViZ 2.0a (http://www.phyloviz.net/) was performed. At Locus Variant Level 4 the minimum spanning tree-like structure clearly separated type I, type II, type III and MRA genotyping results (**Figure 5A**). Within the type II group a high level of diversity was observed. There seems to be no clear regional pattern, separating type II samples from different parts of Europe (*e.g.*, the northern part, Denmark, Norway and the eastern part, Austria, Czech Republic, Romania), as shown in **Figure 5B**. This is only

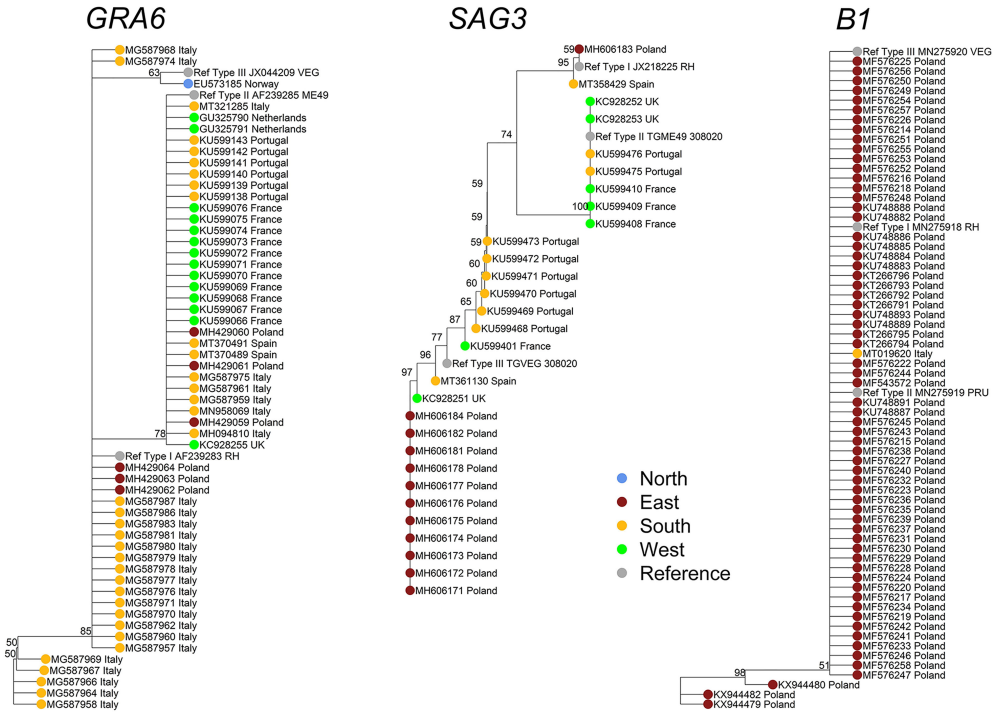

FIGURE 4 | Phylogenetic analyses of the *Toxoplasma gondii* population in Europe based on available *GRA6*, *SAG3*, and *B1*-derived sequences. To analyze the genetic population of *T. gondii* in Europe based on the nucleotide sequences of different *T. gondii*-specific markers, the respective entries were downloaded from the NCBI nucleotide database (https://www.ncbi.nlm.nih.gov/nucleotide?cmd=search) using the R package "rentrez". In the first step, using a search string (*e.g.*, "Toxoplasma[ORGN] AND GRA6[ALL]"), all available data on the respective markers were downloaded by the R function "rentrez::entrez_search" and "rentrez::entrez_summary". A total of 7776 entries were identified and downloaded worldwide. From these data, but after a detailed literature screening and manual curation of the dataset, only nucleotide entries from European isolates (n=501) were extracted and annotated accordingly (**Table S3**). However, for quality reasons as well as due to low number of available sequences per respective marker, several sequences and markers were excluded from the analysis (**Table 2**). To this end, 76 nucleotide sequences from *B1* gene, 86 from *GRA6* and 49 from *SAG3* loci were downloaded as an independent multifasta data file for each selected *T. gondii* typing marker. The download was performed using the R function "rentrez::entrez_fetch". Alignment of the respective nucleotide sequences with calculation of the Tamura-Nei genetic distance and generation of the Neighbor-Joining trees was performed using Geneious Prime® 2021.1.1 [build 2021-03-12 13:25 Java version 11.0.9 + 11 (64 bit)]. The trees were exported in "newick" format and then modified with the R packages "base", "ape", "dendextend" and "castor". To simplify the view of the trees, the resolution was reduced using the r function "castor::collapse_tree_at_resolution" by applying the cut-off in the "resolution" parameter for each tree. This parameter specifies the phylogenetic resolution at which the tree should be collapsed. This is the maximum distance a descending tip can be from a node such that the node collapses into a new tip. If set to 0 (default), only nodes whose descending tips are identical to the node will be collapsed; finally, 56, 29, and 65 sequences were analyzed for *GRA6*, *SAG3*, and *B1*, respectively. Thus, resolution cut-offs of 0.008 (GRA6 tree), 0.005 (SAG3 tree) and 0.004 (B1 tree) were applied respectively.

partially in accord with results reported in France for *T. gondii* strains involved in human toxoplasmosis where in rural regions *T. gondii* associated with cases of congenital toxoplasmosis were genetically different between the eastern and western part of the country based on MS typing results (Ajzenberg et al., 2015).

INTEGRATIVE ANALYSIS: EVIDENCE FROM A PAN-EUROPEAN PERSPECTIVE

The complex biology and epidemiology of *T. gondii* means that researchers face not only the detection of routes or sources of transmission, as in other emerging zoonotic diseases, but also the enormous variety of susceptible hosts that makes it an underestimated and silent concern, only visible in specifically vulnerable groups of populations (immunosuppressed or pregnant hosts). This review examines the distribution of various *T. gondii* genotypes throughout the European continent taking into account the different One Health compartments. As a whole, the predominance of clonal type II strains is evident, but exhaustive published data collection and analysis suggests the existence of an interesting proportion of divergent strains (MRA), slightly more concentrated in the wildlife compartment. Hence, the dichotomy "domestic *versus* wild" so manifest in the American continent is possibly present

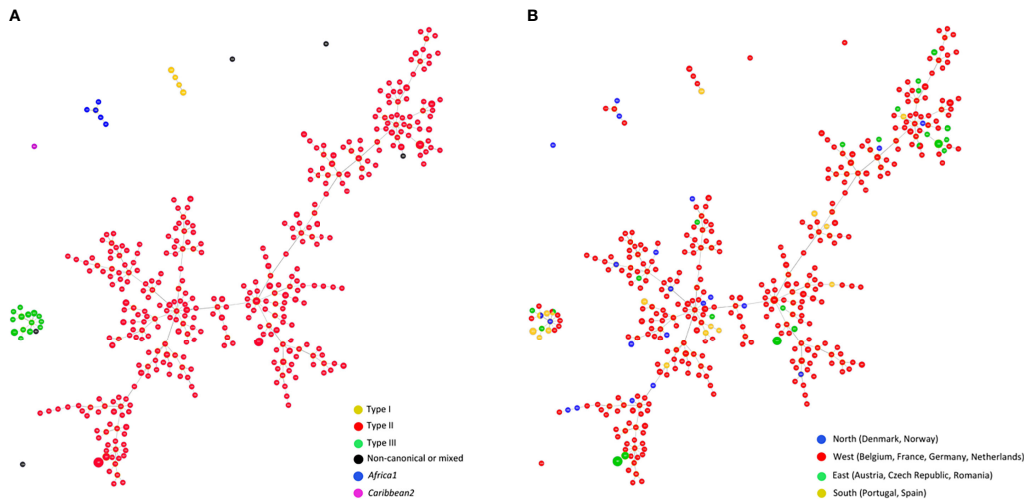

FIGURE 5 | Global optimal eBURST analysis using the Full-MST (goeBURST distance) option of European *Toxoplasma gondii* samples typed by 15 MS regions. In total, n = 487 isolates microsatellite typed at all 15 microsatellite markers were included, representing n=384 separate microsatellite types. Applying a Locus Variant Level 4, types clearly separated into four major groups; each circle represents an individual type; size of circles correlates with total number of samples with identical profile. **(A)** Distribution by genotypes of the population, distinguishing between Type I, II, III, non-canonical or mixed patterns, *Africa1*, and *Caribbean2* types. **(B)** Geographical distribution of the population distinguishing between samples from North, West, East or South Europe. Type I, type III and non-canonical *T. gondii* types, including also a group representing *Africa1* type, are clearly separated from Type II while no clear regional patterns can be observed in Type II *T. gondii* samples.

in Europe as well (Mercier et al., 2011; Jiang et al., 2018; Galal et al., 2019). Nonetheless, the potential genetic diversity of *T. gondii* in wildlife has been less studied than in domestic animals, with fewer samples available, with less effort/success on parasite isolation and consequently limited PCR amplification and a limited resolution of typing assays (Herrmann et al., 2012b; Verin et al., 2013; Bacci et al., 2015; Uzelac et al., 2019). If only studies in which the isolation of the parasite was achieved (mainly in mice or cell culture) and where a sufficient number of RFLP or MS genotyping markers were applied are taken into account, the genotypes described are mainly clonal type II. On the other hand, the selection of certain strains at the expense of others during isolation procedures has been demonstrated in literature (Verma et al., 2017; Fernández-Escobar et al., 2020a). Therefore, data obtained directly from clinical samples should not be ignored but need verification, and conclusions should be drawn with caution. In short, findings should be always interpreted cautiously, as well as with interest, since strains that circulate in wildlife are a source of infection for domestic animals and humans, and have been associated with greater pathogenicity at least in North and South America (Dubey et al., 2014). Virulence characterization data of European field *T. gondii* strains are worryingly scarce (Uzelac et al., 2020; Fernández-Escobar et al., 2021).

Clonal type III-related strains were also highlighted, mainly detected in animal hosts. Some authors claimed that type III alleles are more frequently detected in southern Europe compared to other parts of the continent (Kuruca et al., 2019; Uzelac et al., 2021), but the reality is that France, Italy, and Portugal are the countries that have published the most *T. gondii* genotyping studies, with a lower contribution from northern countries (**Figure 2D**), implying large areas without information. Type I alleles are particularly underrepresented in Europe. Most articles describing type I alleles during genotyping (Turčeková et al., 2013; Papini et al., 2015; Mancianti et al., 2015; Battisti et al., 2018; Santoro et al., 2020; Sroka et al., 2020) only involved direct genotyping from tissue samples DNA, with an often lower success in the amplification of typing markers. On the other hand, two studies (Verma et al., 2015; Moskwa et al., 2017) showed a complete clonal type I profile in two isolates obtained from an aborted bovine fetus in Portugal [firstly reported by (Canada et al., 2002)] and from an aborted fetus of European bison (*Bison bonasus bonasus L.*) in Poland, respectively. Clonal type I isolates fully typed by 15 MS markers have been also described infecting humans (Ajzenberg et al., 2010).

Standardization of typing methods is definitively necessary for the integration of genetic data. The BRC biobank (Biological Resource Center for Toxoplasma, www.toxocrb.com) was one of the approaches that comes closest to this objective, storing around 1500 strains from different hosts (humans or animals) and from different countries around the world, all genotyped by the widely applied 15 MS markers (Ajzenberg et al., 2010; Rocaboy et al., 2020). There are important limitations of traditional methodologies used for *T. gondii* typing, because only quite specific and restricted sites within a large *T. gondii* genome are assessed. Whole-genome sequencing (WGS) data analysis has emerged as the most suitable approach for a thorough analysis of the genetic diversity in *T. gondii*, its evolutionary history, and

population structure. Although WGS is difficult to apply as a routine technique for strain typing, the number of studies using this technology is growing rapidly, mainly due to its enormous potential and the continued costs reduction. WGS data are publicly available only for a few isolates from Europe (namely PRU, MAS, FOU, BOF, TgH26044, TgH21016, TgH20005, Cz-H3, among others) of which only two (PRU, Cz-H3) belong to the dominant clonal type II. The others, although isolated in Europe (*i.e.*, France, Belgium) are at least partially reminiscent of strains likely originating from other continents, like FOU and BOF (Africa) or MAS (South America) (Lorenzi et al., 2016). The European Type II isolate PRU (Pruginaud) was assorted to Clade D, a clade which was established based on WGS data and comprises, in addition to other type II strains, of North American HG12 strains and some atypical North or South American strains (Lorenzi et al., 2016). The generation of WGS data on further strains including European type II strains could help to better understand the real genetic diversity within the dominant European strains, to explore the possible exchange of sequence blocks between clonal lineages in Europe and probably to link genetic differences not covered by the traditional widely used typing methods with phenotypic differences (*e.g.*, virulence in mice) evidenced in literature between European isolates (Fernández-Escobar et al., 2020b; Fernández-Escobar et al., 2021).

OUTSTANDING QUESTIONS

Under the light of the data exposed, authors identified some key questions that should be addressed:

- To what extent are the different anthropogenic factors involved in shaping *T. gondii* population structure in Europe?
- Is there an unexplored *T. gondii* biodiversity in the wild in Europe?
- Are traditional typing methods (PCR-RFLP, MS-typing) going to be replaced by Next-Generation or Third-Generation Sequencing techniques?
- Will Whole Genome Sequencing of European *T. gondii* help to understand differences in virulence?

CONCLUSIONS

Despite many important efforts on *T. gondii* genotyping in Europe, the situation is still blurred and in need of extra and closer look. Still many questions remain unsolved and will constitute medium term challenges for researchers. Some important facts, like the lack of consensus over the methodologies and markers applied, the huge differences in samples' quality and concentration, the sampling disparities among regions and the fact that vast areas remain unexplored, as well as the scarcity of data from human cases and environment, are the main limitations to having a complete picture. In this respect, epidemiological surveillance systems must be strengthened at many levels, in humans and in livestock industry (for example on farms, slaughterhouses, and during veterinary inspection of hunted and home slaughtered animals). Therefore, close collaboration between the medical and veterinary sectors is crucial.

There is consensus on type II *T. gondii* prevailing in Europe, followed by type III, but the presence of a noticeable proportion of recombinant and atypical genotypes whose phylogenetic positioning remains obscure, deserves further investigation. Standardized, high-end typing tools and integrative strategies within the One Health approach are needed to fill the existing gaps and provide a clear picture of the *T. gondii* population in Europe.

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All authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2022.842595/full#supplementary-material

REFERENCES

Aguirre, A. A., Longcore, T., Barbieri, M., Dabritz, H., Hill, D., Klein, P. N., et al. (2019). The One Health Approach to Toxoplasmosis: Epidemiology, Control, and Prevention Strategies. *Ecohealth* 16, 378–390. doi: 10.1007/s10393-019-01405-7

Ajzenberg, D., Bañuls, A. L., Tibayrenc, M., and Dardé, M. L. (2002). Microsatellite Analysis of *Toxoplasma Gondii* Shows Considerable Polymorphism Structured Into Two Main Clonal Groups. *Int. J. Parasitol.* 32, 27–38. doi: 10.1016/s0020-7519(01)00301-0

Ajzenberg, D., Collinet, F., Aubert, D., Villena, I., Dardé, M. L. French ToxoBs network group, et al. (2015). The Rural-Urban Effect on Spatial Genetic Structure of Type II *Toxoplasma Gondii* Strains Involved in Human Congenital Toxoplasmosis, France 2002-2009. *Infect. Genet. Evol.* 36, 511–516. doi: 10.1016/j.meegid.2015.08.025

Ajzenberg, D., Collinet, F., Mercier, A., Vignoles, P., and Dardé, M. L. (2010). Genotyping of *Toxoplasma Gondii* Isolates With 15 Microsatellite Markers in a Single Multiplex PCR Assay. *J. Clin. Microbiol.* 48, 4641–4645. doi: 10.1128/JCM.01152-10

Ajzenberg, D., Dumètre, A., and Dardé, M. L. (2005). Multiplex PCR for Typing Strains of *Toxoplasma Gondii*. *J. Clin. Microbiol.* 43, 1940–1943. doi: 10.1128/JCM.43.4.1940-1943.2005

Ajzenberg, D., Yera, H., Marty, P., Paris, L., Dalle, F., Menotti, J., et al. (2009). Genotype of 88 *Toxoplasma Gondii* Isolates Associated With Toxoplasmosis in

Immunocompromised Patients and Correlation With Clinical Findings. *J. Infect. Dis.* 199, 1155–1167. doi: 10.1086/597477

Almeria, S., and Dubey, J. P. (2021). Foodborne Transmission of *Toxoplasma Gondii* Infection in the Last Decade. An Overview. *Res. Vet. Sci.* 135, 371–385. doi: 10.1016/j.rvsc.2020.10.019

Aubert, D., Ajzenberg, D., Richomme, C., Gilot-Fromont, E., Terrier, M. E., de Gevigney, C., et al. (2010). Molecular and Biological Characteristics of *Toxoplasma Gondii* Isolates From Wildlife in France. *Vet. Parasitol.* 171, 346–349. doi: 10.1016/j.vetpar.2010.03.033

Bacci, C., Vismarra, A., Mangia, C., Bonardi, S., Bruini, I., Genchi, M., et al. (2015). Detection of *Toxoplasma Gondii* in Free-Range, Organic Pigs in Italy Using Serological and Molecular Methods. *Int. J. Food Microbiol.* 202, 54–56. doi: 10.1016/j.ijfoodmicro.2015.03.002

Battisti, E., Zanet, S., Trisciuoglio, A., Bruno, S., and Ferroglio, E. (2018). Circulating Genotypes of *Toxoplasma Gondii* in Northwestern Italy. *Vet. Parasitol.* 253, 43–47. doi: 10.1016/j.vetpar.2018.02.023

Beck, H. P., Blake, D., Dardé, M. L., Felger, I., Pedraza-Díaz, S., Regidor-Cerrillo, J., et al. (2009). Molecular Approaches to Diversity of Populations of Apicomplexan Parasites. *Int. J. Parasitol.* 39, 175–189. doi: 10.1016/j.ijpara.2008.10.001

Bertranpetit, E., Jombart, T., Paradis, E., Pena, H., Dubey, J., Su, C., et al. (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

Bigna, J. J., Tochie, J. N., Tounouga, D. N., Bekolo, A. O., Ymele, N. S., Youda, E. L., et al. (2020). Global, Regional, and Country Seroprevalence of *Toxoplasma Gondii* in Pregnant Women: A Systematic Review, Modelling and Meta-Analysis. *Sci. Rep.* 10, 12102. doi: 10.1038/s41598-020-69078-9

Bouwknegt, M., Devleesschauwer, B., Graham, H., Robertson, L. J., van der Giessen, J. W.The Euro-Fbp Workshop Participants (2018). Prioritisation of Food-Borne Parasites in Europe 2016. *Euro Surveill.* 23, 17–00161. doi: 10.2807/1560-7917.ES.2018.23.9.17-00161

Boyle, J. P., Rajasekar, B., Saeij, J. P. J., Ajioka, J. W., Berriman, M., Paulsen, I., et al. (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

Burg, J. L., Grover, C. M., Pouletty, P., and Boothroyd, J. C. (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

Canada, N., Meireles, C. S., Rocha, A., da Costa, J. M., Erickson, M. W., and Dubey, J. P. (2002). Isolation of Viable *Toxoplasma Gondii* From Naturally Infected Aborted Bovine Fetuses. *J. Parasitol.* 88, 1247–1248. doi: 10.1645/0022-3395(2002)088[1247:IOVTGF]2.0.CO;2

Chaichan, P., Mercier, A., Galal, L., Mahittikorn, A., Ariey, F., Morand, S., et al. (2017). Geographical Distribution of *Toxoplasma Gondii* Genotypes in Asia: A Link With Neighboring Continents. *Infect. Genet. Evol.* 53, 227–238. doi: 10.1016/j.meegid.2017.06.002

Dardé, M. L., Bouteille, B., and Pestre-Alexandre, M. (1992). Isoenzyme Analysis of 35 *Toxoplasma Gondii* Isolates and the Biological and Epidemiological Implications. *J. Parasitol.* 78 (5), 786-794.

Dardé, M. L., Mercier, A., Su, C., Khan, A., and Grigg, M. E. (2020). "Chapter 3: Molecular Epidemiology and Population Structure of Toxoplasma Gondii," in *Toxoplasma Gondii: The Model Apicomplexan - Perspectives and Methods*, *3rd ed*. Eds. L. M. Weiss and K. Kim (New York, NY: Academic Press). doi: 10.1016/C2011-0-07157-0

De Craeye, S., Speybroeck, N., Ajzenberg, D., Dardé, M. L., Collinet, F., Tavernier, P., et al. (2011). *Toxoplasma Gondii* and *Neospora Caninum* in Wildlife: Common Parasites in Belgian Foxes and Cervidae? *Vet. Parasitol.* 178, 64–69. doi: 10.1016/j.vetpar.2010.12.016

Djurković-Djaković, O., Dupouy-Camet, J., van der Giessen, J., and Dubey, J. P. (2019). Toxoplasmosis: Overview From a One Health Perspective. *Food Waterborne Parasitol.* 15, e00054. doi: 10.1016/j.fawpar.2019.e00054

Djurković-Djaković, O., Klun, I., Khan, A., Nikolić, A., Knezević-Usaj, S., Bobić, B., et al. (2006). A Human Origin Type II Strain of *Toxoplasma Gondii* Causing Severe Encephalitis in Mice. *Microbes Infect.* 8, 2206–2212. doi: 10.1016/j.micinf.2006.04.016

Dubey, J. P. (2021a). *Toxoplasmosis of Animals and Humans. 3rd ed.* (Boca Raton, FL: CRC Press).

Dubey, J. P. (2021b). Outbreaks of Clinical Toxoplasmosis in Humans: Five Decades of Personal Experience, Perspectives and Lessons Learned. *Parasitol. Vectors.* 14, 263. doi: 10.1186/s13071-021-04769-4

Dubey, J. P., Murata, F. H. A., Cerqueira-Cézar, C. K., and Kwok, O. C. H. (2020a). 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, J. P., Murata, F. H. A., Cerqueira-Cézar, C. K., Kwok, O. C. H., and Su, C. (2020b). 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, J. P., Pena, H. F. J., Cerqueira-Cézar, C. K., Murata, F. H. A., Kwok, O. C. H., Yang, Y. R., et al. (2020c). Epidemiologic Significance of *Toxoplasma Gondii* Infections in Chickens (*Gallus Domesticus*): The Past Decade. *Parasitology* 147, 1263–1289. doi: 10.1017/S0031182020001134

Dubey, J. P., Van Why, K., Verma, S. K., Choudhary, S., Kwok, O. C., Khan, A., et al. (2014). Genotyping *Toxoplasma Gondii* From Wildlife in Pennsylvania and Identification of Natural Recombinants Virulent to Mice. *Vet. Parasitol.* 200, 74–84. doi: 10.1016/j.vetpar.2013.11.001

Elbez-Rubinstein, A., Ajzenberg, D., Dardé, M. L., Cohen, R., Dumètre, A., Yera, H., et al. (2009). Congenital Toxoplasmosis and Reinfection During Pregnancy: Case Report, Strain Characterization, Experimental Model of Reinfection, and Review. *J. Infect. Dis.* 199, 280–285. doi: 10.1086/595793

European Food Safety Authority and European Centre for Disease Prevention and Control (2021). The European Union One Health 2019 Zoonoses Report. *EFSA J.* 19, e06406. doi: 10.2903/j.efsa.2021.6406

FAO and WHO (2014). *Multicriteria-Based Ranking For Risk Management Of Food-Borne Parasites: Microbiological Risk Assessment Series No. 23*. (Rome, Italy: Food & Agriculture Organization) ISBN: 978-92-5-108200-3 .

Feil, E. J., Li, B. C., Aanensen, D. M., Hanage, W. P., and Spratt, B. G. (2004). eBURST: Inferring Patterns of Evolutionary Descent Among Clusters of Related Bacterial Genotypes From Multilocus Sequence Typing Data. *J. Bacteriol.* 186, 1518–1530. doi: 10.1128/JB.186.5.1518-1530.2004

Fekkar, A., Ajzenberg, D., Bodaghi, B., Touafek, F., Le Hoang, P., Delmas, J., et al. (2011). Direct Genotyping of *Toxoplasma Gondii* in Ocular Fluid Samples From 20 Patients With Ocular Toxoplasmosis: Predominance of Type II in France. *J. Clin. Microbiol.* 49, 1513–1517. doi: 10.1128/JCM.02196-10

Fernández-Escobar, M., Calero-Bernal, R., Benavides, J., Regidor-Cerrillo, J., Guerrero-Molina, M. C., Gutiérrez-Expósito, D., et al. (2020a). Isolation and Genetic Characterization of *Toxoplasma Gondii* in Spanish Sheep Flocks. *Parasitol. Vectors.* 13, 396. doi: 10.1186/s13071-020-04275-z

Fernández-Escobar, M., Calero-Bernal, R., Regidor-Cerrillo, J., Vallejo, R., Benavides, J., Collantes-Fernández, E., et al. (2020b). Isolation, Genotyping, and Mouse Virulence Characterization of *Toxoplasma Gondii* From Free Ranging Iberian Pigs. *Front. Vet. Sci.* 7, 604782. doi: 10.3389/fvets.2020.604782

Fernández-Escobar, M., Calero-Bernal, R., Regidor-Cerrillo, J., Vallejo, R., Benavides, J., Collantes-Fernández, E., et al. (2021). *In Vivo* and *In Vitro* Models Show Unexpected Degrees of Virulence Among *Toxoplasma Gondii* Type II and III Isolates From Sheep. *Vet. Res.* 52, 82. doi: 10.1186/s13567-021-00953-7

Francisco, A. P., Bugalho, M., Ramirez, M., and Carriço, J. A. (2009). Global Optimal eBURST Analysis of Multilocus Typing Data Using a Graphic Matroid Approach. *BMC Bioinf.* 10, 152. doi: 10.1186/1471-2105-10-152

Galal, L., Ajzenberg, D., Hamidović, A., Durieux, M. F., Dardé, M. L., and Mercier, A. (2018). *Toxoplasma* and Africa: One Parasite, Two Opposite Population Structures. *Trends Parasitol.* 34, 140–154. doi: 10.1016/j.pt.2017.10.010

Galal, L., Hamidović, A., Dardé, M. L., and Mercier, M. (2019). Diversity of *Toxoplasma Gondii* Strains at the Global Level and Its Determinants. *Food Waterborne Parasitol.* 15, e00052. doi: 10.1016/j.fawpar.2019.e00052

Gisbert Algaba, I., Verhaegen, B., Murat, J. B., Coucke, W., Mercier, A., Cox, E., et al. (2020). Molecular Study of *Toxoplasma Gondii* Isolates Originating From Humans and Organic Pigs in Belgium. *Foodborne Pathog. Dis.* 17, 316–321. doi: 10.1089/fpd.2019.2675

Grigg, M. E., Ganatra, J., Boothroyd, J. C., and Margolis, T. P. (2001). Unusual Abundance of Atypical Strains Associated With Human Ocular Toxoplasmosis. *J. Infect. Dis.* 184, 633–639. doi: 10.1086/322800

Haque, S., Hanna, S., Gharbi, S., Franck, J., Dumon, H., and Haque, A. (1999). Infection of Mice by a *Toxoplasma Gondii* Isolate From an AIDS Patient:

Virulence and Activation of Hosts' Immune Responses Are Independent of Parasite Genotype. *Parasite Immunol.* 21, 649–657. doi: 10.1046/j.1365-3024.1999.00273.x

Havelaar, A. H., Kirk, M. D., Torgerson, P. R., Gibb, H. J., Hald, T., Lake, R. J., et al. (2015). World Health Organization Global Estimates and Regional Comparisons of the Burden of Foodborne Disease in 2010. *PLoS Med.* 12, e1001923. doi: 10.1371/journal.pmed.1001923

Herrmann, D. C., Bärwald, A., Maksimov, A., Pantchev, N., Vrhovec, M. G., Conraths, F. J., et al. (2012a). *Toxoplasma Gondii* Sexual Cross in a Single Naturally Infected Feline Host: Generation of Highly Mouse-Virulent and Avirulent Clones, Genotypically Different From Clonal Types I, II and III. *Vet. Res.* 43, 39. doi: 10.1186/1297-9716-43-39

Herrmann, D. C., Maksimov, P., Hotop, A., Groß, U., Däubener, W., Liesenfeld, O., et al. (2014). Genotyping of Samples From German Patients With Ocular, Cerebral and Systemic Toxoplasmosis Reveals a Predominance of *Toxoplasma Gondii* Type II. *Int. J. Med. Microbiol.* 304, 911–916. doi: 10.1016/j.ijmm.2014.06.008

Herrmann, D. C., Maksimov, P., Maksimov, A., Sutor, A., Schwarz, S., Jaschke, W., et al. (2012b). *Toxoplasma Gondii* in Foxes and Rodents From the German Federal States of Brandenburg and Saxony-Anhalt: Seroprevalence and Genotypes. *Vet. Parasitol.* 185, 78–85. doi: 10.1016/j.vetpar.2011.10.030

Herrmann, D. C., Pantchev, N., Vrhovec, M. G., Barutzki, D., Wilking, H., Fröhlich, A., et al. (2010). Atypical *Toxoplasma Gondii* Genotypes Identified in Oocysts Shed by Cats in Germany. *Int. J. Parasitol.* 40, 285–292. doi: 10.1016/j.ijpara.2009.08.001

Howe, D. K., and Sibley, L. D. (1995). *Toxoplasma Gondii* Comprises Three Clonal Lineages: Correlation of Parasite Genotype With Human Disease. *J. Infect. Dis.* 172, 1561–1566. doi: 10.1093/infdis/172.6.1561

Howe, D. K., Summers, B. C., and Sibley, L. D. (1996). Acute Virulence in Mice Is Associated With Markers on Chromosome VIII in *Toxoplasma Gondii*. *Infect. Immun.* 64, 5193–5198. doi: 10.1128/iai.64.12.5193-5198.1996

Jiang, T., Shwab, E. K., Martin, R. M., Gerhold, R. W., Rosenthal, B. M., Dubey, J. P., et al. (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, 611–619. doi: 10.1016/j.ijpara.2018.01.008

Jokelainen, P., Murat, J. B., and Nielsen, H. V. (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, 579–586. doi: 10.1007/s10096-017-3152-z

Jones, J. L., Lopez, A., Wilson, M., Schulkin, J., and Gibbs, R. (2001). Congenital Toxoplasmosis: A Review. *Obstet. Gynecol. Surv.* 56, 296–305. doi: 10.1097/00006254-200105000-00025

Khan, A., Dubey, J. P., Su, C., Ajioka, J. W., Rosenthal, B. M., and Sibley, L. D. (2011). Genetic Analyses of Atypical *Toxoplasma Gondii* Strains Reveal a Fourth Clonal Lineage in North America. *Int. J. Parasitol.* 41, 645–655. doi: 10.1016/j.ijpara.2011.01.005

Khan, A., Fux, B., Su, C., Dubey, J. P., Dardé, M. L., Ajioka, J. W., et al. (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, A. J., Boyle, J., Cole, R., et al. (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

Kuruca, L., Uzelac, A., Klun, I., Lalošević, V., and Djurković-Djaković, O. (2019). *Toxoplasma Gondii* Genotypes Circulating in Domestic Pigs in Serbia. *Acta Vet. Hung.* 67, 204–211. doi: 10.1556/004.2019.022

Lehmann, T., Marcet, P. L., Graham, D. H., Dahl, E. R., and Dubey, J. P. (2006). Globalization and the Population Structure of *Toxoplasma Gondii*. *Proc. Natl. Acad. Sci. U. S. A.* 103, 11423–11428. doi: 10.1073/pnas.0601438103

Lorenzi, H., Khan, A., Behnke, M. S., Namasivayam, S., Swapna, L. S., Hadjithomas, M., et al. (2016). Local Admixture of Amplified and Diversified Secreted Pathogenesis Determinants Shapes Mosaic *Toxoplasma Gondii* Genomes. *Nat. Commun.* 7, 10147. doi: 10.1038/ncomms10147

Maenz, M., Schlüter, D., Liesenfeld, O., Schares, G., Gross, U., and Pleyer, U. (2014). Ocular Toxoplasmosis Past, Present and New Aspects of an Old Disease. *Prog. Retin. Eye Res.* 39, 77–106. doi: 10.1016/j.preteyeres.2013.12.005

Maksimov, P., Zerweck, J., Maksimov, A., Hotop, A., Gross, U., Spekker, K., et al. (2012). Analysis of Clonal Type-Specific Antibody Reactions in *Toxoplasma Gondii* Seropositive Humans From Germany by Peptide-Microarray. *PLoS One* 7 (3), e34212. doi: 10.1371/journal.pone.0034212

Mancianti, F., Nardoni, S., Mugnaini, L., Zambernardi, L., Guerrini, A., Gazzola, V., et al. (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

Marković, M., Ivović, V., Stajner, T., Djokić, V., Klun, I., Bobić, B., et al. (2014). Evidence for Genetic Diversity of *Toxoplasma Gondii* in Selected Intermediate Hosts in Serbia. *Comp. Immunol. Microbiol. Infect. Dis.* 37, 173–179. doi: 10.1016/j.cimid.2014.03.001

Marquis, N. D., Bishop, T. J., Record, N. R., Countway, P. D., and Fernández Robledo, J. A. (2019). Molecular Epizootiology of *Toxoplasma Gondii* and *Cryptosporidium Parvum* in the Eastern Oyster (*Crassostrea Virginica*) From Maine (USA). *Pathogens* 8, 125. doi: 10.3390/pathogens8030125

Martorelli, B., Wilson, S. K., Dubey, J. P., and Knoll, L. J. (2019). Intestinal Delta-6-Desaturase Activity Determines Host Range for *Toxoplasma Sexual* Reproduction. *PLoS Biol.* 17, e3000364. doi: 10.1371/journal.pbio.3000364

Mercier, A., Ajzenberg, D., Devillard, S., Demar, M. P., de Thoisy, B., Bonnabau, H., et al. (2011). Human Impact on Genetic Diversity of *Toxoplasma Gondii*: Example of the Anthropized Environment From French Guiana. *Infect. Genet. Evol.* 11, 1378–1387. doi: 10.1016/j.meegid.2011.05.003

Minot, S., Melo, M. B., Li, F., Lu, D., Niedelman, W., Levine, S. S., et al. (2012). Admixture and Recombination Among *Toxoplasma Gondii* Lineages Explain Global Genome Diversity. *Proc. Natl. Acad. Sci. U S A.* 109, 13458–13463. doi: 10.1073/pnas.1117047109

Morisset, S., Peyron, F., Lobry, J. R., Garweg, J., Ferrandiz, J., Musset, K., et al. (2008). Serotyping of *Toxoplasma Gondii*: Striking Homogeneous Pattern Between Symptomatic and Asymptomatic Infections Within Europe and South America. *Microbes Infect.* 10 (7), 742–747. doi: 10.1016/j.micinf.2008.04.001

Moskwa, B., Bień, J., Kornacka, A., Cybulska, A., Goździk, K., Krzysiak, M. K., et al. (2017). First *Toxoplasma Gondii* Isolate From an Aborted Foetus of European Bison (*Bison Bonasus Bonasus L.*). *Parasitol. Res.* 116, 2457–2461. doi: 10.1007/s00436-017-5549-0

Nowakowska, D., Colón, I., Remington, J. S., Grigg, M., Golab, E., Wilczynski, J., et al. (2006). Genotyping of *Toxoplasma Gondii* by Multiplex PCR and Peptide-Based Serological Testing of Samples From Infants in Poland Diagnosed With Congenital Toxoplasmosis. *J. Clin. Microbiol.* 44, 1382–1389. doi: 10.1128/JCM.44.4.1382-1389.2006

Opsteegh, M., Maas, M., Schares, G., and van der 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 2016:EN-996), 1–294. doi: 10.2903/sp.efsa.2016.EN-996

Papini, R. A., Buzzone, G., Nardoni, S., Rocchigiani, G., and Mancianti, F. (2015). Seroprevalence and Genotyping of *Toxoplasma Gondii* in Horses Slaughtered for Human Consumption in Italy. *J. Equine Veterinary Sci.* 35, 657–661. doi: 10.1016/j.jevs.2015.06.012

Pena, H. F., Gennari, S. M., Dubey, J. P., and 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

Pinto-Ferreira, F., Caldart, E. T., Pasquali, A. K. S., Mitsuka-Breganó, R., Freire, R. L., and Navarro, I. T. (2019). Patterns of Transmission and Sources of Infection in Outbreaks of Human Toxoplasmosis. *Emerg. Infect. Dis.* 25, 2177–2182. doi: 10.3201/eid2512.181565

Pomares, C., Ajzenberg, D., Bornard, L., Bernardin, G., Hasseine, L., Dardé, M. L., et al. (2011). Toxoplasmosis and Horse Meat, France. *Emerg. Infect. Dis.* 17, 1327–1328. doi: 10.3201/eid1707.101642

Prestrud, K. W., Dubey, J. P., Asbakk, K., Fuglei, E., and Su, C. (2008). First Isolate of *Toxoplasma Gondii* From Arctic Fox (*Vulpes Lagopus*) From Svalbard. *Vet. Parasitol.* 151 (2-4), 110–114. doi: 10.1016/j.vetpar.2007.11.011

Richomme, C., Aubert, D., Gilot-Fromont, E., Ajzenberg, D., Mercier, A., Ducrot, C., et al. (2009). Genetic Characterization of *Toxoplasma Gondii* From Wild Boar (*Sus Scrofa*) in France. *Vet. Parasitol.* 164, 296–300. doi: 10.1016/j.vetpar.2009.06.014

Robert-Gangneux, F., and Dardé, M. L. (2012). Epidemiology of and Diagnostic Strategies for Toxoplasmosis. *Clin. Microbiol. Rev.* 25, 264–296. doi: 10.1128/CMR.05013-11

Robert-Gangneux, F., Meroni, V., Dupont, D., Botterel, F., Aguado Garcia, J. M., Brenier-Pinchart, M. P., et al. (2018). Toxoplasmosis in Transplant Recipients, Europe 2010-2014. *Emerg. Infect. Dis.* 24, 1497–1504. doi: 10.3201/eid2408.180045

Rocaboy, C., Dardé, M.-L., Aubert, D., Mercier, A., Ortis, N., Passebosc-Faure, K., et al. (2020). Biological Resource Center for *Toxoplasma*. *Open J. Bioresources* 7, 7. doi: 10.5334/ojb.61

Rostami, A., Riahi, S. M., Gamble, H. R., Fakhri, Y., Nourollahpour Shiadeh, M., Danesh, M., et al. (2020). Global Prevalence of Latent Toxoplasmosis in Pregnant Women: A Systematic Review and Meta-Analysis. *Clin. Microbiol. Infect.* 26, 673–683. doi: 10.1016/j.cmi.2020.01.008

Santoro, M., Viscardi, M., Boccia, F., Borriello, G., Lucibelli, M. G., Auriemma, C., et al. (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

Sgroi, G., Viscardi, M., Santoro, M., Borriello, G., D'Alessio, N., Boccia, F., et al. (2020). Genotyping of *Toxoplasma Gondii* in Wild Boar (*Sus Scrofa*) in Southern Italy: Epidemiological Survey and Associated Risk for Consumers. *Zoonoses Public. Health* 67, 805–813. doi: 10.1111/zph.12762

Shapiro, K., Bahia-Oliveira, L., Dixon, B., Dumètre, A., de Wit, L. A., VanWormer, E., et al. (2019). Environmental Transmission of *Toxoplasma Gondii*: Oocysts in Water, Soil and Food. *Food Waterborne Parasitol.* 15, e00049. doi: 10.1016/j.fawpar.2019.e00049

Shobab, L., Pleyer, U., Johnsen, J., Metzner, S., James, E. R., Torun, N., et al. (2013). *Toxoplasma* Serotype Is Associated With Development of Ocular Toxoplasmosis. *J. Infect. Dis.* 208 (9), 1520–1528. doi: 10.1093/infdis/jit313

Shwab, E. K., Saraf, P., Zhu, X.-Q., Zhou, D.-H., McFerrin, B. M., Ajzenberg, D., et al. (2018). Human Impact on the Diversity and Virulence of the Ubiquitous Zoonotic Parasite *Toxoplasma Gondii*. *Proc. Natl. Acad. Sci. U. S. A.* 115, E6956–E6963. doi: 10.1073/pnas.1722202115

Shwab, E. K., Zhu, X.-Q., Majumdar, D., Pena, H. F. J., Gennari, S. M., Dubey, J. P., et al. (2014). Geographical Patterns of *Toxoplasma Gondii* Genetic Diversity Revealed by Multilocus PCR-RFLP Genotyping. *Parasitology* 141, 453–461. doi: 10.1017/S0031182013001844

Sibley, L. D., and Ajioka, J. W. (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, L. D., and Boothroyd, J. C. (1992). Virulent Strains of *Toxoplasma Gondii* Comprise a Single Clonal Lineage. *Nature* 359, 82. doi: 10.1038/359082a0

Slany, M., Dziedzinska, R., Babak, V., Kralik, P., Moravkova, M., and Slana, I. (2019). *Toxoplasma Gondii* in Vegetables From Fields and Farm Storage Facilities in the Czech Republic. *FEMS Microbiol. Lett.* 366 (14), fnz170. doi: 10.1093/femsle/fnz170

Sroka, J., Karamon, J., Wójcik-Fatla, A., Piotrowska, W., Dutkiewicz, J., Bilska-Zając, E., et al. (2020). *Toxoplasma Gondii* Infection in Slaughtered Pigs and Cattle in Poland: Seroprevalence, Molecular Detection and Characterization of Parasites in Meat. *Parasitol. Vectors* 13, 223. doi: 10.1186/s13071-020-04106-1

Sroka, J., Szymanska, J., and Wójcik-Fatla, A. (2009). The Occurrence of *Toxoplasma Gondii* and *Borrelia Burgdorferi* Sensu Lato in *Ixodes Ricinus* Ticks From East Poland With the Use of PCR. *Ann. Agric. Environ. Med.* 16, 313–319.

Sroka, J., Wójcik-Fatla, A., Szymanska, J., Dutkiewicz, J., Zajac, V., and 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.

Sroka, J., Wójcik-Fatla, A., Zwoliński, J., Zajac, V., Sawczuk, M., and Dutkiewicz, J. (2008). Preliminary Study on the Occurrence of *Toxoplasma Gondii* in *Ixodes Ricinus* Ticks From North-Western Poland With the Use of PCR. *Ann. Agric. Environ. Med.* 15, 333–338.

Stajner, T., Vasiljević, Z., Vujić, D., Marković, M., Ristić, G., Mićić, D., et al. (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, L. M., Maksimov, P., Gethmann, J., et al. (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., Zhang, X., and Dubey, J. P. (2006). Genotyping of *Toxoplasma Gondii* by Multilocus PCR-RFLP Markers: A High Resolution and Simple Method for Identification of Parasites. *Int. J. Parasitol.* 36, 841–848. doi: 10.1016/j.ijpara.2006.03.003

Su, C., Evans, D., Cole, R. H., Kissinger, J. C., Ajioka, J. W., and Sibley, L. D. (2003). Recent Expansion of *Toxoplasma* Through Enhanced Oral Transmission. *Science* 299, 414–416. doi: 10.1126/science.1078035

Su, C., Zhang, X., and Dubey, J. P. (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, 5844–5849. doi: 10.1073/pnas.1203190109

Turčeková, L., Antolová, D., Reiterová, K., and Spišák, F. (2013). Occurrence and Genetic Characterization of *Toxoplasma Gondii* in Naturally Infected Pigs. *Acta Parasitol.* 58, 361–366. doi: 10.2478/s11686-013-0154-6

Uzelac, A., Klun, I., Ćirković, V., Bauman, N., Bobić, B., Štajner, T., et al. (2021). *Toxoplasma Gondii* Genotypes Circulating in Serbia-Insight Into the Population Structure and Diversity of the Species in Southeastern Europe, a Region of Intercontinental Strain Exchange. *Microorganisms* 9 (12), 2526. doi: 10.3390/microorganisms9122526

Uzelac, A., Klun, I., Ćirković, V., and Djurković-Djaković, O. (2020). *In Vivo* and *In Vitro* Virulence Analysis of Four Genetically Distinct *Toxoplasma Gondii* Lineage III Isolates. *Microorganisms* 8, 1702. doi: 10.3390/microorganisms8111702

Uzelac, A., Klun, I., Ćirović, D., Penezić, A., Ćirković, V., and Djurković-Djaković, O. (2019). Detection and Genotyping of *Toxoplasma Gondii* in Wild Canids in Serbia. *Parasitol. Int.* 73, 101973. doi: 10.1016/j.parint.2019.101973

van der Giessen, J., Deksne, G., Gómez-Morales, M. A., Troell, K., Gomes, J., Sotiraki, S., et al. (2021). Surveillance of Foodborne Parasitic Diseases in Europe in a One Health Approach. *Parasite Epidemiol. Control.* 13, e00205. doi: 10.1016/j.parepi.2021.e00205

Verin, R., Mugnaini, L., Nardoni, S., Papini, R. A., Ariti, G., Poli, A., et al. (2013). Serologic, Molecular, and Pathologic Survey of *Toxoplasma Gondii* Infection in Free-Ranging Red Foxes (*Vulpes Vulpes*) in Central Italy. *J. Wildl. Dis.* 49, 545–551. doi: 10.7589/2011-07-204

Verma, S. K., Ajzenberg, D., Rivera-Sanchez, A., Su, C., and Dubey, J. P. (2015). Genetic Characterization of *Toxoplasma Gondii* Isolates From Portugal, Austria and Israel Reveals Higher Genetic Variability Within the Type II Lineage. *Parasitology* 142, 948–957. doi: 10.1017/S0031182015000050

Verma, S. K., Sweeny, A. R., Lovallo, M. J., Calero-Bernal, R., Kwok, O. C., Jiang, T., et al. (2017). Seroprevalence, Isolation and Co-Infection of Multiple *Toxoplasma Gondii* Strains in Individual Bobcats (*Lynx Rufus*) From Mississippi, USA. *Int. J. Parasitol.* 47, 297–303. doi: 10.1016/j.ijpara.2016.12.007

Vilares, A., Gargaté, M. J., Ferreira, I., Martins, S., and Gomes, J. P. (2017). Molecular and Virulence Characterization of *Toxoplasma Gondii* Strains Isolated From Humans in Portugal. *Parasitol. Res.* 116, 979–985. doi: 10.1007/s00436-017-5374-5

Vilares, A., Gargaté, M. J., Ferreira, I., Martins, S., Júlio, C., Waap, H., et al. (2014). Isolation and Molecular Characterization of *Toxoplasma Gondii* Isolated From Pigeons and Stray Cats in Lisbon, Portugal. *Vet. Parasitol.* 205, 506–511. doi: 10.1016/j.vetpar.2014.08.006

Wang, Z. D., Wang, S. C., Liu, H. H., Ma, H. Y., Li, Z. Y., Wei, F., et al. (2017). Prevalence and Burden of *Toxoplasma Gondii* Infection in HIV-Infected People: A Systematic Review and Meta-Analysis. *Lancet HIV* 4 (4), e177–e188. doi: 10.1016/S2352-3018(17)30005-X

Wojcik-Fatla, A., Sroka, J., Zajac, V., Sawczyn, A., Cisak, E., and Dutkiewicz, J. (2015). *Toxoplasma Gondii* (Nicolle Et Manceaux 1908) Detected in *Dermacentor Reticulatus* (Fabricius) (Ixodidae). *Folia. Parasitol. (Praha)* 62, 2015.055. doi: 10.14411/fp.2015.055

Xia, J., Venkat, A., Bainbridge, R. E., Reese, M. L., Le Roch, K. G., Ay, F., et al. (2021). Third-Generation 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

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3.2 A ring trial to harmonize *Toxoplasma gondii* microsatellite typing: comparative analysis of results and recommendations for optimization

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Contribution of Maike Joeres: Study conception and preparation of the ring trial samples, data collection and analysis, preparation of the guidelines and writing the manuscript.

Contributions of other authors: All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by GC, KPF, NP, MFE, CMH, LOA, RCB, LG, CL, PM, MLD, LMO, PJ, AM, and GS. The first draft of the manuscript was written by GS, MJ, and GC and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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ORIGINAL ARTICLE

A ring trial to harmonize *Toxoplasma gondii* microsatellite typing: comparative analysis of results and recommendations for optimization

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Abstract
A ring trial among five European laboratories was organized to reach consistency in microsatellite (MS) typing of the zoonotic parasite *Toxoplasma gondii*. Three sample sets were circulated and analyzed by each laboratory following a previously published method that is based on fragment length polymorphism of 15 MS markers. The first sample set compared typing results in general and focused on effects of DNA concentration; the second sample set focused on the polymorphic fingerprinting markers that can differentiate *T. gondii* strains within the same archetypal lineage; and the third set focused on non-archetypal genotypes. Methodological variations between laboratories, including the software programs used to determine MS fragment length, were collated using a questionnaire. Overall, lineage-level typing results reached a high level of agreement, especially in samples with the highest DNA concentrations. However, laboratory-specific differences were observed for particular markers. Major median differences in fragment length, of up to 6 base pairs, were related to the fluorophore used to label fragment-specific primers. In addition, primer pairs with identical sequences obtained from different suppliers resulted in fragments of differing length. Furthermore, differences in the way the sequencing profiles were assessed and interpreted may have led to deviating results in fragment length determination. Harmonization of MS typing, for example, by using the same fluorophores or by numerical adjustments applied to the fragment-lengths determined, could improve the uniformity of the results across laboratories. This is the first interlaboratory comparison, providing guidelines (added as a supplement) for the optimization of this technique.

Keywords DNA quantification · Interlaboratory comparison · Toxoplasmosis · Genotyping · Subtyping

Introduction

Toxoplasma gondii is a zoonotic protozoan parasite that uses domestic cats and other felids as definitive hosts and causes clinical disease in both humans and animals [1–4]. It was recently ranked second out of 24 important foodborne parasites in Europe [5–7].

Globally, *T. gondii* has a complex population structure [8]. While populations of this parasite in many regions of the world belong to few clonal lineages [9], those observed in South America are much more diverse [8, 10].

A frequently used genotyping technique targets microsatellite (MS) sequences [11]. MS sequences are ubiquitous and polymorphic in the genomes of virtually all organisms [12]. For *T. gondii* typing, usually a set of up to 15 markers located on 11 different chromosomes of the *T. gondii* genome is used, 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). Fingerprinting markers are more polymorphic and were shown to resolve different isolates, applicable to both archetypal (type I, II, or III) and non-archetypal lineages [11].

MS sequences need to be amplified by multiplex or singleplex PCR using primer pairs, with one of the primers per pair labeled by a fluorophore. Subsequently, amplicons are separated on a capillary sequencer, including a size standard in each run, which allows determination of the lengths of the amplified MS fragments. Usually, three different fluorophores are used, which allows examination of a larger

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number of amplified fragments simultaneously in a single run on the capillary sequencer [11].

Another frequently used technique to type *T. gondii* is PCR-restriction fragment length polymorphism (PCR-RFLP), which can resolve *T. gondii* genotypes, but is—in contrast to MS typing—less suitable for differentiating parasites of the same lineage. PCR-RFLP *T. gondii* typing involves multiplex or singleplex PCR to amplify up to 11 markers, which are distributed over eight chromosomes, and the apicoplast [13].

Multilocus MS typing is used by many laboratories around the world [9, 14–17]. It is largely unknown, however, to which extent the lineage typing and fingerprinting results obtained by different laboratories are comparable. This is a challenge as a One Health approach, e.g., combining larger data sets on *T. gondii* genotypes from different sectors and across countries, which is needed to better understand the molecular epidemiology and transmission pathways of *T. gondii.*

To evaluate consistency in *T. gondii* MS typing, a ring trial was established among five European laboratories. Laboratories had different levels of experience with this typing technique, had slightly modified the original protocol, and used—at least in part—different laboratory equipment, reagents, and software. This ring trial led to the identification of major reasons for differences in MS typing. The results were used to establish harmonized guidelines for laboratories on implementing MS typing of *T. gondii*.

Materials and methods

Participating laboratories

Five European laboratories (A–E) participated. One laboratory (B) had previously established and published a MS typing method and served as the reference laboratory [11]. Another laboratory (C) had introduced the technique 4 years ago, one laboratory (E) 2 years ago, and the two remaining laboratories (A and D) very recently. Laboratory E organized sets of samples, shipment, and collection of results.

Origin of samples

The ring trial was divided into three consecutive parts. The first part was planned to assess the capacity to type archetypal lineages of *T. gondii*, types I, II, and III, and evaluate the effect of DNA concentration on the accuracy of results. The samples comprised DNA aliquots collected from reference strains belonging to the three lineages types I, II, and III with RH [18], ME49 [19], and NED [20], respectively, in different dilutions. In addition, a sample from a type II × III recombinant strain (D200273; DNA of the *T. gondii* isolate TGA32090; provided by the Biological Resource Centre (BRC) Toxoplasma [http://www.toxocrb.com/]) was included (Table 1). Samples with the two highest DNA concentrations were provided only once, while the three lowest concentrations were provided two to four times in the panel.

The second part was planned to assess the ability to discriminate different *T. gondii* type II strains using fingerprinting markers (Table 2). The DNA samples corresponded to 10 different *T. gondii* type II isolates, as confirmed by PCR-RFLP, and were provided in duplicate in the panel. Concentrations of DNA were adjusted so that they were similar to the 10^{-1} dilution of the first part.

Finally, the last part was established to confirm that the laboratories were able to identify non-archetypal genotypes by MS typing. The panel consisted of DNAs from non-archetypal *T. gondii* strains ($n = 7$) and two archetypal strains ($n = 2$), provided in duplicate (Table 2). Concentrations of DNA were similar to those of the 10^{-1} dilution of the first part.

For each part, *T. gondii* DNAs were diluted in bovine carrier DNA with a concentration of 100 ng/µL. Two samples (first part) or one sample (second and third parts) of bovine carrier DNA alone was included as negative controls. The trial was blinded for all participants, including the

Table 1 Composition of the sample set of the first part of the ring trial on microsatellite typing of *Toxoplasma gondii*

Concentration	Sample dilution in bovine carrier DNA				Number of replicas
	RH (10 ng/µL)	ME49 (10 ng/µL)	NED (10 ng/µL)	D200273[a] (10 ng/µL)	
Dilution 1	10^{-1}	10^{-1}	10^{-1}	10^{-1}	1
Dilution 2	10^{-2}	10^{-2}	10^{-2}	10^{-2}	1
Dilution 3	10^{-3}	10^{-3}	10^{-3}	10^{-3}	2
Dilution 4	10^{-4}	10^{-4}	10^{-4}	10^{-4}	4
Dilution 5	10^{-5}	10^{-5}	10^{-5}	10^{-5}	4
Negative control	Bovine carrier DNA (100 ng/µL)				2

[a]D200273: DNA of the *T. gondii* isolate TGA32090; provided by the Biological Resource Centre (BRC) Toxoplasma

Table 2 Composition of the sample sets of the second and third parts of the ring trial on microsatellite typing of *Toxoplasma gondii*

Sample no. in second part and third part of the ring trial	DNA identifier of providing laboratory	Ct value at laboratory E	BRC [a] identifier	Sample ID, alternative name	Country of origin	Host	References	Type, ToxoDB# as determined by PCR-RFLP in laboratory E	Number of replicas
2-1	D200109	18.22	NA	V15-2, E-EU-ELP-5	Czech Republic	Tiger	This study	Type II, #3	2
2-2	D200111	18.29	NA	V17-1, GER-EJP-10	Germany	Wild boar	[21]	Type II, #3	2
2-3	D200113	18.43	NA	P17/2479, GER-EJP-7	Germany	Cat	This study	Type II, #3	2
2-4	D200114	17.28	NA	P17/2480, GER-EJP-6	Germany	Cat	This study	Type II, #3	2
2-5	D200118	19.50	NA	V16-4, W-EU-EJP-1	Austria	Cat	This study	Type II, #1	2
2-6	D200119	20.19	NA	V16-5, GER-EJP-8	Germany	Cat	This study	Type II, #3	2
2-7	D200121	19.02	NA	V16-7, W-EU-EJP-2	Austria	Cat	This study	Type II, #1	2
2-8	D200127	17.52	NA	V30-3, GER-EJP-3	Germany	Chicken	This study	Type II, #1	2
2-9	D200129	18.27	NA	V87-2, E-EU-EJP-3	Czech Republic	Wildcat	This study	Type II, #3	2
2-10	D212556	19.54	NA	V10-1, GER-EJP-1	Germany	Deer	[21]	Type II, #3	2
2-11	Negative control, Bovine carrier DNA	NA	Not applicable	Not applicable	Not applicable	Not applicable	Not applicable	Not applicable	1
3-1	D221394	20.27	TgA119002	BENIN02, P19S1AJ6	Benin	Chicken	[22]	Africa 1	2
3-2	D221396	19.94	TgA105034	GABON03; GAB1-FEL-CAT001	Gabon	Cat	[23]	III	2
3-3	D221397	19.19	TgA105033	GABON02, GAB1-2007-CAP-AEG004	Gabon	Goat	[23]	III variant	2
3-4	D221398	18.40	TgH19006A	FRENCH-GUIANA15, GUYS006-BAY	French Guiana	Human	[24]	Amazonian	2
3-5	D221399	19.32	TgH18013A	FRENCH-GUIANA11, GUY013-2004-LAB	French Guiana	Human	[24]	Amazonian	2
3-6	D221400	18.52	TgH40002A	GUADE-LOUPE02, PAP002-2010-GOM	Guadeloupe	Human	[23]	Caribbean 2	2
3-7	D221401	17.73	TgA18009	FRENCHGUI-ANA01, GUY-CAN-FAM-009 (CH29)	French Guiana	Dog	[23]	Caribbean 1	2
3-8	D221402	18.38	TgH16012A	MARTINIQUE03, FDF012-MAN	Martinique	Human	[23]	Caribbean 3	2

Table 2 (continued)

Sample no. in second part and third part of the ring trial	DNA identifier of providing laboratory	Ct value at laboratory E	BRC [a] identifier	Sample ID, alternative name	Country of origin	Host	References	Type, ToxoDB# as determined by PCR-RFLP in laboratory E	Number of replicas
3-9	D221403	19.19	TgA117041	SENEGAL17, 160622Gdom02	Senegal	Chicken	[23]	II [b]	2
3-10	Negative control, bovine carrier DNA	NA	Not applicable	Not applicable	Not applicable	Not applicable	Not applicable	Not applicable	1

[a]Biological Resource Centre (BRC) Toxoplasma. [b]Designated as type II in literature although W35 showed a variation from type II pattern (244 bp instead of 242)

organizing laboratory (E); only in the third part, the operators knew about the non-archetypal nature of some of the isolates included, but were unaware of the identity and order of the samples.

Irrespective of laboratory-specific protocols, each laboratory was asked to use 5 µL template DNA per reaction in the multiplex typing PCR.

After completing each part, interlaboratory divergences were assessed and discussed among the participants. All laboratories tried to improve their protocols and procedures for the subsequent part. The aim was to improve the individual typing results of each of the participating laboratories and to harmonize MS typing by using and extending the internal guidelines of laboratory B.

Questionnaire to asses divergence from original method

A questionnaire was distributed to collate the technical and methodological details in each laboratory. During online meetings, further details on individual protocols, such as use of particular rules to analyze sequencing profiles, were recorded.

Statistics

After reception of the data, all results were computed in tables for each part and studied separately (Supplementary File Table 1). For each sample, the coded number of the organizing laboratory and of the external laboratory was registered in an EXCEL file along with the operator, the software used, the typing results, the Ct value obtained by initial real-time PCR, the sample volume used for the reactions, the identified genetic type, and the number of typing markers identified. To compare the results among laboratories, the R software (R version 4.1.2, https://cran.r-project.org/) was used for linear regression and specifically, the R packages "binom," "ggpubr," "ggplot2," and "cowplot" for calculating confidence intervals and preparing graphical representations of the results.

Results

Questionnaire results

All participants used the MS typing technique as reported previously [11]. However, questionnaire data and subsequent communication during online meetings revealed a number of deviations from the original protocol, even in the laboratory where the method had been initially established (laboratory B). Interestingly, one of the laboratories (A) had replaced the fluorophore HEX_{Fl} with VIC_{Fl}, another (D) had replaced NED_{Fl} with $TAMRA_{Fl}$, and two laboratories (B, E) had replaced NED_{Fl} with $Atto550_{Fl}$, for three or two of the MS marker regions, respectively (Table 3).

Further differences were related to the types of sequencing devices, the size standards, and the software used to assess the fragment length of amplified MS regions (Table 4).

PCR results to quantify *T. gondii* DNA in samples

In the first part, linear regression analysis of the Ct values reported by each laboratory on serially diluted DNAs of reference isolates revealed R^2 values between 0.577 and 0.710 for individual laboratories (Fig. 1; Table 5). Comparison of the individual regression line equations revealed that the Ct values reported by the laboratories differed. Laboratories D and E reported the lowest Ct values and laboratories A and

Table 3 Sets of fluorophores used by the laboratories (A-E) participating in a ring trial on microsatellite typing of *Toxoplasma gondii*

Group of markers	Laboratory				
	A	B	C	D	E
N61, B18, M33, M48, TUB2, N83, XI.1	6-FAM$_{Fl}$	6-FAM$_{Fl}$	6-FAM$_{Fl}$	6-FAM$_{Fl}$	6-FAM$_{Fl}$
N82, TgM-A, W35, IV.1, B17	VIC$_{Fl}$	HEX$_{Fl}$	HEX$_{Fl}$	HEX$_{Fl}$	HEX$_{Fl}$
N60, M102	NED$_{Fl}$	Atto550$_{Fl}$	NED$_{Fl}$	TAMRA$_{Fl}$	Atto550$_{Fl}$
AA	NED$_{Fl}$	NED$_{Fl}$	NED$_{Fl}$	TAMRA$_{Fl}$	Atto550$_{Fl}$

B the highest for the same given strain (Table 5). Overall, the pairwise comparisons between Ct values reported by the different laboratories revealed R^2 values between 0.94 and 0.84. The proportions of recognized positive samples ranged from 85.4% (laboratory D) to 100% (laboratory B) (Table 5). All laboratories reported negative results for negative control samples.

Typing archetypal *T. gondii* and impact of DNA concentration

The identification of the genetic type of samples was based on lineage typing markers (TUB2, W35, TgM-A, B18, B17, M33, IV.1, and XI.1). The proportion of correct identifications of the canonical types I, II, and III and a type II × III recombinant decreased depending on the dilution of the samples. If a participant had added a question mark to the result or provided an ambiguous typing result, NA was recorded. There were no differences in the typing results between the two software tools used by laboratory A and the two operators from laboratory E.

At the highest DNA concentrations, the 1st and 2nd dilutions, 71% (20/28) and 75% (21/28) of the results provided by all participants were correct. At the two following concentrations (3rd and 4th dilution), 52% (29/56) and 32% (36/112) of the typing outcome reported were correct. In contrast, the 5th dilution analysis revealed no (0/112) correct identification (Fig. 2). Overall, not only the proportion of incorrect typing results increased with higher dilution of the *T. gondii* DNA, but also the proportion of undetermined types, i.e., from 18% (5/28) or 14% (4/28) for the 1st and 2nd dilution to 41% (23/56), 62% (69/112), or 88% (98/112) for the 3rd, 4th, and 5th dilutions, respectively (Fig. 2).

If only the results for dilutions 1, 2, and 3 were included, small differences of up to minimum or maximum deviations of 2 bp relative to the results provided by the reference laboratory (B) were often recorded. In 27 cases, minimum or maximum values exceeding 2 bp were observed (Table 6). Most (63%; 17/27) deviations occurred in results reported by laboratory A. Some of the deviations were extreme and ranged up to 28 or 30 bp (laboratories A and C; Table 6).

Median differences in the typing results relative to reference laboratory B were not equally distributed among the laboratories. Overall, the majority (70%; 16/23) of the major differences (i.e., median differences > 1 bp) occurred in particular markers, for which participants employed primers labeled with different fluorophores compared to reference laboratory B. In lineage typing, major differences were only observed between laboratory A and the reference laboratory (100%; 4/4). In the affected marker regions, laboratory A had used VIC$_{Fl}$ instead of HEX$_{Fl}$ to label fragments (Table 6). In fingerprinting analysis, all laboratories reported differences of > 1 bp relative to laboratory B. Of 20 differences, 12 occurred in cases with differences in labeling (Table 6). If only median differences of > 2 bp were counted, 78% (7/9) of the differences occurred in cases with differences in fluorophore labeling (NED$_{Fl}$ instead of Atto550$_{Fl}$ and vice versa, and TAMRA$_{Fl}$ instead of NED$_{Fl}$; details on primer labeling in Table 3).

Based on the results of the first part of the ring trial, it was observed that the fluorophore attached to primers for amplification of MS markers may have had an impact on the fragment sizes determined by capillary sequencing (Table 7). Thus, the literature on this topic was reviewed and differences in publications on the MS typing of RH, ME49, and NED strains were observed on 20 occasions (Table 7). In the vast majority of these cases ($n = 17$), the laboratory had used an alternative to the originally reported fluorophore, i.e., HEX$_{Fl}$ was replaced with VIC$_{Fl}$, NED$_{Fl}$ with Atto550$_{Fl}$, or NED$_{Fl}$ with TAMRA$_{Fl}$ (Table 7). Also, the reference laboratory B had recently started to replace NED$_{Fl}$ with Atto550$_{Fl}$ for the amplification of the N60$_{Fl}$ and M102$_{Fl}$ markers (Table 7).

Fingerprinting *T. gondii* type II

Divergences in fragment length determination

Fingerprinting markers (M48, M102, N60, N82, AA, N61, and N83) can be used to differentiate strains within the same lineage. In the second part of the ring trial, laboratories B and E reported identical fingerprinting results in all regions of the duplicates of 10 different strains, i.e., on 70 occasions (10 strains, seven fingerprinting regions). While laboratory A reported non-existing differences in two (3%), laboratories C and D reported non-existing differences in seven (10%) or 18 (25%) of 70 occasions, respectively (Table 8).

Table 4 Technical or methodological details of the microsatellite typing technique as retrieved by questionnaire and further personal communications from laboratories participating in a ring trial on microsatellite typing of *Toxoplasma gondii*

Details	Laboratory, operator, or software					
	A-1[a]	A-2[a]	B	C	D	E-1 or E-2[b]
Real-time PCR protocol used to quantify DNA	Adapted from [25]	Adapted from [25]	[26, 27]	[28]	[29]	Adapted from [30]
Target of real-time PCR used to quantify DNA	REP529, GenBank accession no. AF146527	REP529, GenBank accession no. AF146527	REP529, GenBank accession no. AF146527	REP529, GenBank accession no. AF146527	REP529, GenBank accession no. AF146527	REP529, GenBank accession no. AF146527
Template volumes used in real-time PCR	5 µL	5 µL	5 µL	5 µL	3 µL	10 µL
PCR MS typing (Part I)	15 MS multiplex	15 MS multiplex	15 MS multiplex + singleplexes	15 MS multiplex	8 MS multiplex (typing markers) + 7 MS multiplex (fingerprinting)	15 MS multiplex
Supplier/provider of typing primers	IN	IN	AB	AB	EU	ME (EU in comparative experiments)
Template volume used for MS multiplex PCR	5 µL	5 µL	5 µL	5 µL	5 µL	5 µL
Volume of amplification product used for fragment length analysis	1 µL	1 µL	1 µL	1 µL	1 µL	1 µL
Multiplex PCR kit used for typing	QIAGEN Multiplex PCR Kit (cat. no. 206143)	QIAGEN Multiplex PCR Kit (cat. no. 206143)	QIAGEN Multiplex PCR Kit (cat. no. 206143)	QIAGEN Multiplex PCR Kit (cat. no. 206143)	QIAGEN Multiplex PCR Kit (cat. no. 206143)	QIAGEN Multiplex PCR Kit (cat. no. 206143)
Capillary sequencer	3730 DNA Analyzer (ABI)	3730 DNA Analyzer (ABI)	3130xl Genetic Analyzer (ABI)	SeqStudio Genetic Analyzer (ABI)	3730 Genetic Analyzer (ABI)	Hitachi 3500 Genetic Analyzer (ABI)
Size standard	LIZ 500 (TF)	LIZ 500 (TF)	ROX 500 (ABI)	ROX 500 (ABI)	LIZ 500 (ABI)	ROX 500 (ABI)
MS typing software	Peak Scanner 2.0 (no bins established for allele identification)	Osiris (first part of ring trial only, no bins established for allele identification)	Gene-Mapper (bins established for allele identification)	Peak Scanner online (semi-automatic MS EXCEL template to ease allele identification)	Geneious Prime (bins established for allele identification)	Geneious Prime (bins established for allele identification)

[a]In laboratory A, two different software tools were used, i.e., Peak Scanner 2.0 (A-1) or Osiris (A-2). [b]In laboratory E, two operators assessed raw data, i.e., electrophoresis profiles

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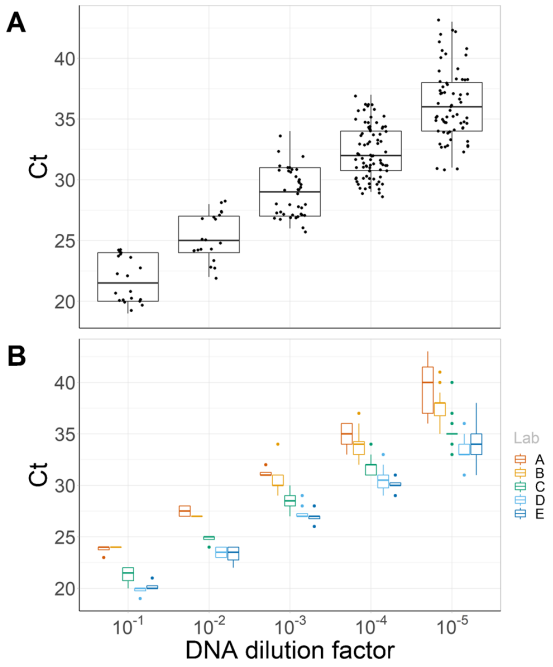

Fig. 1 Real-time PCR results to quantify specific DNA in the samples of the first part of the ring trial on *Toxoplasma gondii* microsatellite typing according to the dilution of samples. **A** Median, 25–75% quantile (box), minimum and maximum (whiskers) of Ct values reported by all participants of the ring trial. **B** Median, 25–75% quantile (box), minimum and maximum (whiskers) of Ct values stratified for laboratories participating in the ring trial (Lab)

Interlaboratory divergence in fragment length determination

In the second part of the ring trial, most of the differences recorded relative to reference laboratory B did not exceed minimum or maximum deviations of >2 bp. Unlike in the first part of the ring trial, minimum or maximum values exceeding 2 bp were observed in less cases (n = 10; Table 9). Most (60%; 6/10) of these deviations occurred in results reported by laboratory A. However, deviations were far less extreme as compared to the first part and ranged up to 6 bp in one laboratory (laboratory E; Table 9).

Typing non-archetypal *T. gondii* strains

Divergence in fragment length determination in duplicated samples

In the third part, not only fingerprinting, but also typing markers varied between the isolates. Since the samples had been provided in duplicate, it was possible to assess the extent, to which duplicates were correctly recognized. Compared to the second part of the ring trial, the ability to recognize samples with identical profiles increased for all laboratories except laboratory C (i.e., n = 7 in second part but n = 10 in third part). The results for one marker (IV.1) were not available for analysis in the case of one isolate (FRENCHGUIANA15) in laboratories A, C, and E, because they failed to amplify this marker (Table 10).

Interlaboratory divergence in fragment length determination

In the third part, most of the differences recorded relative to reference laboratory B did not exceed minimum or maximum deviations of > 2 bp. Compared to the other parts of the ring trial, minimum or maximum values exceeding 2 bp were observed in a small number of cases (n = 7; Table 11). The majority (71%; 5/7) of such deviations occurred in the results of laboratories that had chosen fluorophores that differed from those used by the reference laboratory (Table 11).

All laboratories correctly identified archetypal *T. gondii* type III or type II variants, although laboratories C and D did not report the variation in this isolate (Table 12). All laboratories, except laboratory C, recognized all non-archetypal strains as such. Laboratory C misclassified Africa 1 as type I, and the Caribbean 1, 2, and 3 as type III, and for two Amazonian isolates the result "Unclassified" was provided. All remaining laboratories, except laboratory C, correctly identified Caribbean 1, 2, and 3, determined the Amazonian isolate as Unclassified or as Amazonian, and the type III-like isolate as Unclassified (laboratory D), type III variant (laboratories B and E), or South American 4-like (laboratory A).

Effects due to use of different fluorophore labeling and different suppliers for primers

To confirm that the different fluorophores used caused differences in the apparent sizes of amplified PCR products, comparative experiments were performed using the DNAs from RH,

Table 5 Summarized results of the linear correlation between Ct value and DNA concentration in the samples of the first part of the ring trial on microsatellite typing for *Toxoplasma gondii*

Laboratory	R^2	Regression line equation	Proportion of positive samples recognized
A	0.606	$y = -1.142\ln(x) + 21.554$	97.9%
B	0.653	$y = -1.035\ln(x) + 21.818$	100%
C	0.710	$y = -1.075\ln(x) + 19.083$	95.8%
D	0.577	$y = -0.974\ln(x) + 18.629$	85.4%
E	0.596	$y = -1.01\ln(x) + 18.196$	93.8%

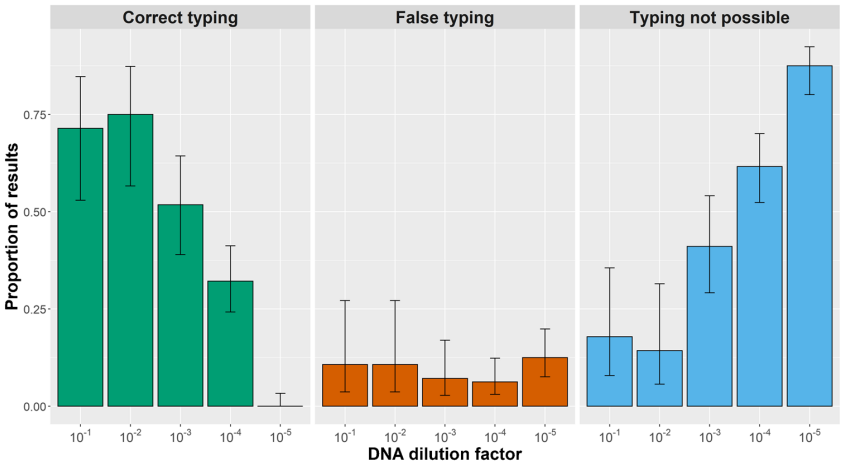

Fig. 2 Relationship between typing results and DNA concentration: Proportion and 95% confidence intervals of correct (green) and false (orange) typing or typing not possible (blue) in the samples of the first part of the ring trial on *Toxoplasma gondii* microsatellite typing according to the dilution of samples for all laboratories and operators (i.e., type I, II, III, or II × III recombinant). Note: Number of replicas per strain DNA varied according to sample dilution (please refer to Table 1)

ME49, and NED reference strains and the primer pairs corresponding to the marker regions N60, M102, and AA provided by the different laboratories, labeled with NED_{Fl}, $TAMRA_{Fl}$ or $Atto550_{Fl}$. Capillary sequencing as well as the assessment of profiles was done in laboratory E. The results obtained in laboratory E using reagents provided by reference laboratory B were identical with those previously obtained by laboratory B.

Different fluorophore labeling

NED_{Fl}-labeled N60 fragments were 4 to 5 bp shorter and M102 fragments 2 bp shorter as compared to the $Atto550_{Fl}$-labeled reference (Table 13). $TAMRA_{Fl}$-labeled N60 was 2 bp shorter compared to $Atto550_{Fl}$-labeled reference, while the M102 fragments had the same length.

Table 6 Results of the first part of the ring trial on microsatellite typing for *Toxoplasma gondii* in relation to results provided by laboratory B as a reference: Median [minimum; maximum] differences in length observed for each marker compared to the results provided by the reference laboratory (laboratory B). Median differences exceeding 1 bp are typed in bold. Minimum and maximum values exceeding 2 bp are underlined and in italics. Note: The use of Atto550 for N60 and M102 by reference laboratory B was a deviation from the original method. The analysis was restricted to sample dilutions 1, 2, and 3

Marker	Median differences [minimum; maximum] in fragment size relative to the results of laboratory B stratified by laboratory operator or software					
	A-1[a]	A-2[a]	C	D	E-1[b]	E-2[b]
Lineage typing marker						
B18	0 [− 1;0]	0 [− 1;0]	0 [0;4]	0 [0;0]	0 [0;0]	0 [0;0]
M33	0 [0;2]	0 [0;2]	1 [0;1]	0 [0;0]	0 [0;0]	0 [0;0]
TUB2	0 [0;0]	0 [0;0]	0 [0;0]	0 [0;0]	0 [0;0]	0 [0;0]
XI.1	0 [0;1]	0 [0;1]	0 [0;1]	0 [0;0]	0 [0;0]	0 [0;0]
TgM-A	0 [0;1][c]	0 [*− 12*;1][c]	0 [− 1;0]	0 [0;0]	0 [0;0]	0 [0;0]
W35	**3** [0;*3*][c]	**3** [0;*3*][c]	0 [0;0]	0 [0;0]	0 [0;0]	0 [0;0]
IV.1	**2** [*− 4*;2][c]	**2** [*− 4*;2][c]	0 [0;1]	0 [0;2]	0 [0;0]	0 [0;0]
B17	0 [*− 4*;*28*][c]	0 [− 1;*28*][c]	0 [0;1]	0 [− 2;0]	0 [0;0]	0 [0;0]
Fingerprinting marker						
N82	**2** [0;2][c]	**2** [1;*9*][a]	**2** [0;*15*]	0 [0;2]	0 [0;0]	0 [0;0]
N61	**4** [1;*10*]	**3** [1;*10*]	0 [0;*30*]	0 [− 2;0]	0 [0;0]	0 [0;0]
M48	1 [*− 3*;2]	1 [− 2;2]	1 [*− 3*;3]	0 [0;2]	0 [0;0]	0 [0;0]
N83	− 1 [− 1;0]	− 1 [− 1;0]	0 [0;2]	0 [0;0]	0 [0;0]	0 [0;0]
N60	**− 5** [*− 5*;0][d]	**− 4** [*− 5*;0][d]	**− 5** [*− 5*;0][d]	1 [− 1;1][e]	1 [1;1]	1 [1;1]
M102	**− 2** [*− 7*;0][d]	**− 2** [*− 7*;0][d]	**− 2** [*− 3*;0][d]	**2** [*− 4*;*4*][e]	**2** [0;2]	**2** [0;2]
AA	**2** [0;*5*]	**2** [0;*5*]	**2** [0;2]	1 [0;*3*][f]	**3.5** [*3*;*5*][g]	**3.5** [*3*;*5*][g]

[a]In laboratory A, two different software tools were used, i.e., Peak Scanner 2.0 (A-1) or Osiris (A-2). [b]In laboratory E, two operators (E-1, E-2) assessed raw data, i.e., electrophoresis profiles; Differences to reference laboratory: [c]VIC_{Fl} instead of HEX_{Fl}; [d]NED_{Fl} instead of $Atto550_{Fl}$; [e]$TAMRA_{Fl}$ instead Atto550; [f]$TAMRA_{Fl}$ instead of NED_{Fl}; [g]$Atto550_{Fl}$ instead of NED_{Fl}

Table 7 Results of the first part of the ring trial on microsatellite typing for *Toxoplasma gondii* in relation to literature data: Median differences in length observed for each marker of reference strains compared to the results provided in literature, i.e., for RH [31] or ME49 and NED [32]. Only markers are listed, for which laboratories used other fluorophores than those reported in the original reference. In the case of median differences exceeding 1 bp, entries are typed in bold. Fluorophores, different from the original description, are also indicated in bold. The analysis was restricted to sample dilutions 1, 2, and 3

Marker	Median differences in fragment size relative to literature, number of observations, fluorophore stratified by laboratory operator or software						
	A-1[a]	A-2[a]	B	C	D	E-1[b]	E-2[b]
N82	**1.6**, *n*=12, **VIC**$_{Fl}$	**1.6**, *n*=11, **VIC**$_{Fl}$	0, *n*=12, HEX$_{Fl}$	**2**, *n*=10, HEX$_{Fl}$	0, *n*=11, HEX$_{Fl}$	0, *n*=12, HEX$_{Fl}$	0, *n*=12, HEX$_{Fl}$
TgM-A	0.4, *n*=11, **VIC**$_{Fl}$	0.5, *n*=10, **VIC**$_{Fl}$	0, *n*=12, HEX$_{Fl}$	0, *n*=10, HEX$_{Fl}$	0, *n*=11, HEX$_{Fl}$	0, *n*=12, HEX$_{Fl}$	0, *n*=12, HEX$_{Fl}$
W35	**2.9**, *n*=9, **VIC**$_{Fl}$	**2.8**, *n*=9, **VIC**$_{Fl}$	0, *n*=9, HEX$_{Fl}$	0, *n*=8, HEX$_{Fl}$	0, *n*=11, HEX$_{Fl}$	0, *n*=12, HEX$_{Fl}$	0, *n*=12, HEX$_{Fl}$
IV.1	**2,** *n*=9, **VIC**$_{Fl}$	**2**, *n*=10, **VIC**$_{Fl}$	0, *n*=12, HEX$_{Fl}$	0, *n*=7, HEX$_{Fl}$	0, *n*=11, HEX$_{Fl}$	0, *n*=12, HEX$_{Fl}$	0, *n*=12, HEX$_{Fl}$
B17	− 0.3, *n*=9, **VIC**$_{Fl}$	− 0.35, *n*=10, **VIC**$_{Fl}$	0, *n*=12, HEX$_{Fl}$	0, *n*=8, HEX$_{Fl}$	0, *n*=11, HEX$_{Fl}$	0, *n*=12, HEX$_{Fl}$	0, *n*=12, HEX$_{Fl}$
N60	− 0.2, *n*=11 NED$_{Fl}$	− 0.3, *n*=11, NED$_{Fl}$	**4**, *n*=12, **Atto550**$_{Fl}$	− 1, *n*=10, NED$_{Fl}$	**5**, *n*=12, **TAMRA**$_{Fl}$	**5**, *n*=12, **Atto550**$_{Fl}$	**5**, *n*=12, **Atto550**$_{Fl}$
M102	− 0.5, *n*=9, NED$_{Fl}$	− 0.3, *n*=9, NED$_{Fl}$	**2**, *n*=11, **Atto550**$_{Fl}$	0, *n*=9, NED$_{Fl}$	**4**, *n*=12, **TAMRA**$_{Fl}$	**4**, *n*=12, **Atto550**$_{Fl}$	**4**, *n*=12, **Atto550**$_{Fl}$
AA	**1.8**, *n*=11, NED$_{Fl}$	**1.6**, *n*=11, NED$_{Fl}$	0, *n*=11, NED$_{Fl}$	**2**, *n*=7, NED$_{Fl}$	**3**, *n*=12, **TAMRA$_{Fl}$**	**3**, *n*=12, **Atto550**$_{Fl}$	**3**, *n*=12, **Atto550**$_{Fl}$

[a]In laboratory A, two different software tools were used, i.e., Peak Scanner 2.0 (A-1) or Osiris (A-2). [b]In laboratory E, two operators (E-1, E-2) assessed raw data, i.e., electrophoresis profiles

Different suppliers for primers

The Atto550$_{Fl}$-labeled N60 fragments were identical to the reference Atto550$_{Fl}$-labeled fragments, if primer pairs supplied by company EU were used by laboratory E. In contrast, if Atto550$_{Fl}$-labeled primer pairs bought from company ME were used by laboratory E, fragments were 2 bp longer than the reference Atto550$_{Fl}$-labeled fragments (Table 13). In the case of the AA marker, TAMRA$_{Fl}$ and Atto550$_{Fl}$ (EU) fragments were 2 bp longer and Atto550$_{Fl}$ (ME) fragments were 4 bp longer than reference NED$_{Fl}$ fragments.

Discussion

Typing of *T. gondii* strains is important to study the global population structure of the parasite. Genomic diversity of *T. gondii* may influence the epidemiology of the parasite, affecting, for example, definitive host and intermediate host adaptation [23, 33, 34]. In addition, some *T. gondii* genotypes are reported to have a higher virulence for particular hosts than other genotypes [35, 36]. Such differences in virulence may exist between different host species, but also at the intra-host-species level [37, 38].

Table 8 Failure in identifying duplicates in samples of the second part of the ring trial on microsatellite typing for *Toxoplasma gondii* per laboratory

DNA number of providing laboratory	Success in identifying duplicates stratified by laboratory operator or software				
	A-1[a]	B	C	D	E-1 or E-2[b]
D200109	AA^{-}	-	N61^{-}	AA$^{□}$, N83$^{□}$	-
D200111	-	-	-	AA$^{□}$, N83$^{□}$	-
D200113	-	-	N60$^{□}$, N61^{-}	M48$^{□}$, N61$^{□}$, N82$^{□}$	-
D200114	-	-	-	N82$^{□}$, AA$^{□}$, N83$^{□}$	-
D200118	-	-	N60^{-}, N61$^{□}$	M48$^{□}$, AA$^{□}$, N83$^{□}$	-
D200119	AA$^{□}$	-	N61^{-}	N61$^{□}$	-
D200121	-	-	-	N82$^{□}$	-
D200127	-	-	N61^{-}	-	-
D200129	-	-	-	N82$^{□}$	-
D212556	-	-	-	M48$^{□}$, N82$^{□}$	-
Negative control	-	-	-	-	-

[a]In laboratory A, Peak Scanner 2.0 (A-1) was used as software tool. [b]In laboratory E, two operators (E-1, E-2) assessed raw data, i.e., electrophoresis profiles; $^{-}$: 1 bp difference; $^{□}$: 2 bp difference

Table 9 Second part of the ring trial on microsatellite typing for *Toxoplasma gondii*: Median [minimum; maximum] differences in length observed for each marker compared to the reference laboratory. Median differences exceeding 1 bp are typed in bold. Minimum and maximum values exceeding 2 bp are underlined and in italics. Note: The use of $Atto550_{Fl}$ for N60 and M102 by reference laboratory B was a deviation from the original method

Marker	Median differences [minimum; maximum] in fragment size relative to results of laboratory B stratified by laboratory operator or software				
	A-1[a]	C	D	E-1[b]	E-2[b]
Lineage typing marker					
B18	0 [0;0]	0 [0;0]	0 [0;0]	0 [0;0]	0 [0;0]
M33	0 [− 1;0]	0 [0;0]	0 [0;0]	0 [0;0]	0 [0;0]
TUB2	1 [0;1]	0 [0;0]	0 [0;0]	0 [0;0]	0 [0;0]
XI.1	1 [0;1]	0 [0;1]	0 [0;0]	0 [0;0]	0 [0;0]
TgM-A	1 [1;1]	0 [0;0]	0 [0;0]	0 [0;0]	0 [0;0]
W35	**3** [*3;3*][c]	0 [0;1]	0 [0;0]	0 [0;0]	0 [0;0]
IV.1	**2** [2;2][c]	0 [0;1]	0 [0;0]	0 [0;0]	0 [0;0]
B17	0 [0;0][c]	0 [0;1]	0 [0;0]	0 [0;0]	0 [0;0]
Fingerprinting marker					
N82	**1.5** [1;2][c]	**2** [2;2]	**2** [0;2]	0 [0;0]	0 [0;0]
N61	1 [1;1]	1 [0;2]	0 [− 2;2]	0 [0;0]	0 [0;0]
M48	**2** [2;2]	1 [1;2]	**2** [0;2]	0 [− 1;1]	0 [− 1;1]
N83	0 [− 1;0]	0 [0;0]	0 [− 2;0]	0 [0;0]	0 [0;0]
N60	**− 5** [*− 5;− 3*][d]	**− 5** [*− 5;− 3*][d]	− 1 [− 1;0][e]	1 [1;2]	0 [0;2]
M102	**− 3** [*− 3;− 3*][d]	**− 2** [− 2; − 2][d]	**2** [2;2][e]	**2** [2;2]	**2** [0;2]
AA	**2** [− 1;2]	**2** [1;*3*]	**2** [0;2][f]	**3** [*3;5*][g]	**4** [*4;6*][g]

[a]In laboratory A, Peak Scanner 2.0 (A-1) was used as software tool. [b]In laboratory E, two operators (E-1, E-2) assessed raw data, i.e., electrophoresis profiles; Differences to reference laboratory: [c]VIC_{Fl} instead of HEX_{Fl}; [d]NED_{Fl} instead of $Atto550_{Fl}$; [e]$TAMRA_{Fl}$ instead Atto550; [f]$TAMRA_{Fl}$ instead of NED_{Fl}; [g]$Atto550_{Fl}$ instead of NED_{Fl}

Table 10 Failure in identifying duplicates in samples of the third part of the ring trial on microsatellite typing for *Toxoplasma gondii* per laboratory

DNA designation of providing laboratory	Success in identifying duplicates stratified by laboratory operator or software				
	A-1[a]	B	C	D	E-1[b]
BENIN02	-	-	$N61^{-}$, $N83^{□}$	-	-
FRENCHGUIANA01	-	-	-	-	-
GABON02	-	-	$XI.1^{-}$	-	-
GUADELOUPE02	-	-	-	-	-
MARTINIQUE03	-	-	-	-	-
SENEGAL17	-	-	$W35^{-}$, $IV.1^{-}$, $N61^{-}$	-	-
GABON03	-	-	$IV.1^{-}$, $N61^{□}$	-	-
FRENCHGUIANA11	-	-	$IV.1^{-}$	-	-
FRENCHGUIANA15	$IV.1^{NA}$	-	$N61^{-}$, $IV.1^{NA}$	$M102^{□}$, $N82^{□}$	$IV.1^{NA}$
Negative control	-	-	-	-	-

[a]In laboratory A, Peak Scanner 2.0 (A-1) was used as software tool. [b]In laboratory E, the operator E-1 assessed raw data, i.e., electrophoresis profiles; $^{-}$: 1 bp difference; $^{□}$: 2 bp difference; NA: no result for IV.1

Multilocus MS typing was established more than one decade ago [11] and has proven to be suitable to discriminate *T. gondii* strains at the level of lineages globally [8, 15, 39] as well as on the intra-lineage level [17, 40]. Essentially, laboratories currently use this technique to study strains and clinical samples from different geographical areas. As a consequence, differences in typing results between laboratories could introduce bias in population genetic studies comparing MS genotypes from different geographical locations. This is also true within the same geographical region such as in Europe with several laboratories using MS genotyping [9].

Our study revealed numerous differences in MS typing protocols, although all participants of the ring trial, including the reference laboratory, referred to the original description of the MS typing methodology [11]. Laboratories used different real-time PCR procedures to quantify *T. gondii* DNA prior to typing, different fluorophores (Table 3), capillary sequencers, size standards, and different software tools to assess fragment length of amplified marker regions (Table 4). Differences in the allele identification, supported by various software tools, were noted. Only with particular software, not available to all participants, was it possible to ease and automatize allele identification (Table 4). Not all participating laboratories used the same software and some were not able to automatize allele identification in the respective software. Some of the applied tools (i.e., Gene-Mapper and Geneious Prime) allowed the definition

Table 11 Third part of the ring trial on microsatellite typing for *Toxoplasma gondii*: Median [minimum; maximum] differences in length observed for each marker compared to the results reported by the reference laboratory. Median differences exceeding 1 bp are typed in bold. Minimum and maximum values exceeding 2 bp are underlined and in italics. Note: The use of $Atto550_{Fl}$ for $N60_{Fl}$ and $M102_{Fl}$ by reference laboratory B was a deviation from the original method

Marker	Median [minimum; maximum] differences per laboratory, software or operator			
	A-1[a]	C	D	E-1[b]
Lineage typing marker				
B18	0 [0;0]	0 [0;0]	0 [0;0]	0 [0;0]
M33	0 [0;0]	0 [0;0]	0 [− 2;0]	0 [− 2;0]
TUB2	0 [0;0]	0 [− 2;0]	0 [0;0]	0 [0;0]
XI.1	0 [0;0]	0 [0;0.5]	0 [0;0]	0 [0;0]
TgM-A	0 [0;0]	0 [0;1]	0 [0;0]	0 [0;0]
W35	0 [0;0][c]	0 [0;0.5]	0 [0;0]	0 [0;0]
IV.1	0 [0;0][c]	0 [0;0.5]	0 [0;0]	0 [0;0]
B17	0 [− 2;0][c]	0 [− 1;0]	**− 2** [− 2; − 2]	0 [− 2;0]
Fingerprinting marker				
N82	**2** [2;2][c]	**2** [2;2]	**2** [1;2]	0 [0;0]
N61	0 [0;0]	0.5 [0;1.5]	2 [2;2]	0 [0;0]
M48	0 [0;0]	1 [1;2]	0 [0;0]	0 [0;0]
N83	0 [0;0]	0 [0;1]	0 [0;0]	0 [0;0]
N60	**− 4** [*− 5;− 4*][d]	**− 4** [*− 5;− 4*][d]	**− 2** [− 2;*− 3*][e]	**2** [2;*3*]
M102	**− 4** [*− 4;− 4*][d]	**− 2** [− 2;− 1][d]	− 0 [− 1;0][e]	**2** [2;2]
AA	0 [0;0]	**2** [2;*3*]	2 [2;2][f]	**4** [*4;4*][g]

[a]In laboratory A, Peak Scanner 2.0 (A-1) was used as software tool. [b]In laboratory E, the operator E-1 assessed raw data, i.e., electrophoresis profiles; Differences to reference laboratory: [c]VIC_{Fl} instead of HEX_{Fl}; [d]NED_{Fl} instead of $Atto550_{Fl}$; [e]$TAMRA_{Fl}$ instead $Atto550_{Fl}$; [f]$TAMRA_{Fl}$ instead of NED_{Fl}; [g]$Atto550_{Fl}$ instead of NED_{Fl}

of loci and bins to ease allele identification. The inclusion of characterized reference DNAs can help to define loci and subsequently the respective bins. Furthermore, participating laboratories had different levels of experience in *T. gondii* MS typing, which ranged from several years to a few weeks.

The first part of this ring trial focused on lineage typing and the effect of *T. gondii* DNA concentrations on typing. As usually done for field samples, each laboratory tried to quantify *T. gondii* DNAs in the samples. Each laboratory used a different real-time PCR protocol (Table 4); however, overall high correlation coefficients between Ct values and DNA content in samples were observed. Nevertheless, Ct values differed by more than 3 Ct units in some cases (Table 5). This must be kept in mind for the interpretation of results reported in the literature. Nevertheless, laboratory-specific Ct values provide valuable information for optimizing DNA concentrations of samples (e.g., field samples) subsequently used for MS typing.

The results revealed that from dilution 3 (0.01 ng/µL *T. gondii* DNA) onwards, the proportion of samples increased, in which laboratories were no longer able to determine the lineage type. However, the proportion of reporting incorrect lineage typing results did not increase with decreasing DNA content. The results were consistent among the participating laboratories and relative to the results of the reference laboratory only up to dilution 2 (0.1 ng/µL *T. gondii* DNA). Thus, it seems to be important to estimate the level of *T. gondii* DNA concentration in samples prior to MS typing, and to use this information to select those samples, for which lineage typing (and subtyping) is most likely possible, or to optimize DNA concentration for typing (Fig. 3).

It should be noted that not only a limited concentration of DNA may negatively influence the accuracy of determining the correct fragment size, but also an excess of *T. gondii* DNA may cause problems. In the first part of this ring trial, it was noted that the proportion of correct lineage typing was lower in dilution 1 (1 ng/µL *T. gondii* DNA) than in dilution 2 (0.1 ng/µL *T. gondii* DNA) samples. It has been

Table 12 Typing results reported by the laboratories (A–E) compared to results reported in the literature: The set provided in the third part of the ring trial on microsatellite typing for *Toxoplasma gondii* comprised of two strains with an archetypal and seven strains with non-archetypal genotype

Sample ID	Type according to literature	Type determined by laboratory				
		A-1[a]	B	C	D	E-1[b]
BENIN02	Africa 1	Africa 1	Africa 1	I	Africa 1	Africa 1
GABON03	III	III	III	III	III	III
GABON02	III-like	South American 4-like	III variant TUB2	III	Unclassified	III variant TUB2
FRENCHGUIANA15	Amazonian	Unclassified	Amazonian	Unclassified	Amazonian	Unclassified
FRENCHGUIANA11	Amazonian	Unclassified	Amazonian	Unclassified	Amazonian	Unclassified
GUADELOUPE02	Caribbean 2	Caribbean 2	Caribbean 2	III	Caribbean 2	Caribbean 2
FRENCHGUIANA01	Caribbean 1	Caribbean 1	Caribbean 1	III	Caribbean 1	Caribbean 1
MARTINIQUE03	Caribbean 3	Caribbean 3	Caribbean 3	III	Caribbean 3	Caribbean 3
SENEGAL17	II[c]	II-like	II variant W35	II	II	II variant W35

[a]In laboratory A, Peak Scanner 2.0 (A-1) was used as software tool. [b]In laboratory E, the operator E-1 assessed raw data, i.e., electrophoresis profiles. [c]Designated as type II in literature although W35 showed a variation from type II pattern (244 bp instead of 242)

Table 13 Effect of different fluorophores and primer suppliers on microsatellite fragment sizes: Differences in microsatellite (MS) typing for *Toxoplasma gondii* between Atto550$_{Fl}$- and NED$_{Fl}$-labeled MS fragments N60, M102, and AA for reference *T. gondii* strains RH, ME49, and NED using reagents provided to laboratory E by laboratories participating of the ring trial on *T. gondii* microsatellite typing

Marker	Strain	Laboratory, commercial supplier of primer, primer labeling						
		Fragment size determined with reagents provided by reference laboratory B: supplier, fluorophore		Differences between fragment size determined in laboratory E using reagents of participating laboratories: laboratory, supplier, fluorophore				
		AB, Atto550$_{Fl}$	AB, NED$_{Fl}$	A, IN, NED$_{Fl}$	C, AB, NED$_{Fl}$	D, EU, TAMRA$_{Fl}$	E, ME, Atto550$_{Fl}$	E, EU, Atto550$_{Fl}$
N60	RH	149	NA	− 4	− 4	− 2	2	0
	ME49	147	NA	− 5	− 5	− 2	2	0
	NED	151	NA	− 4	− 4	− 2	2	0
M102	RH	168	NA	− 2	− 2	0	2	0
	ME49	176	NA	− 2	− 2	0	2	0
	NED	192	NA	− 2	− 2	0	2	0
AA	RH	NA	265	0	0	2	4	2
	ME49	NA	265	0	0	2	4	2
	NED	NA	267	0	0	2	4	2

noted previously that an overrepresentation of target DNA may cause so-called minus-A peaks during capillary electrophoresis [41]. Minus-A peaks can occur, if a number of amplified fragments lack a terminal adenine at the 3′ end, which is usually added by many DNA polymerases without the use of the template. We studied the occurrence of minus-A peaks for the markers M33 and M102. Both markers showed double peaks, where the intensity of the first peak (assumed to be a minus-A peak as detailed in the typing guidelines, provided as Supplementary File Text 1) increased with increasing DNA concentrations, while the intensity of the second peak (assumed to be the correct peak) decreased.

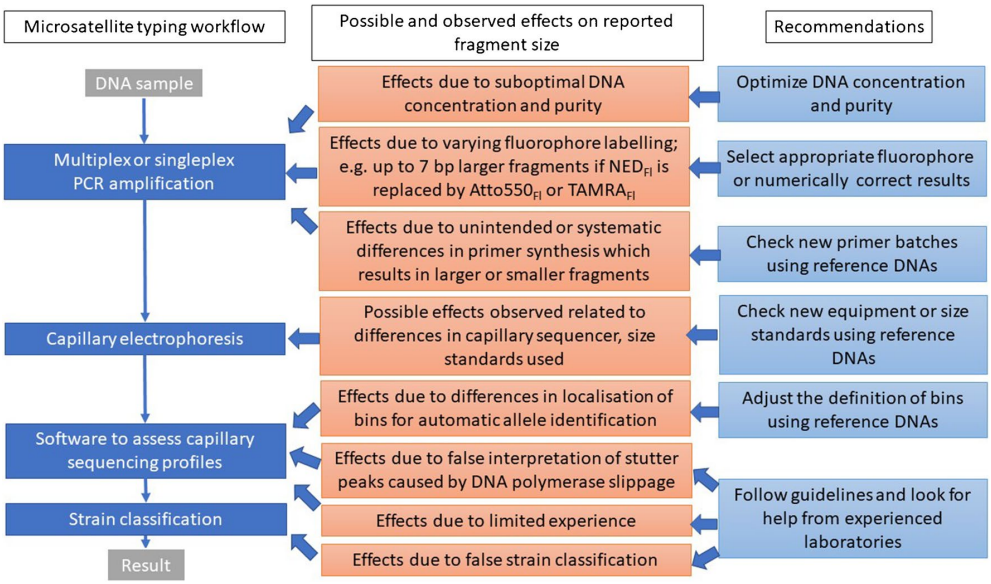

Fig. 3 Possible and observed effects on the *Toxoplasma gondii* microsatellite marker fragment size determination: Steps affected in the microsatellite typing workflow and recommendations for optimization

This can cause incorrect results, if the operator or the software normally choose the highest peak as the correct one.

Major differences, mainly affecting fingerprinting markers, were observed in results reported in all parts of the ring trial, especially among those laboratories that used different fluorophores for labeling forward primers (Tables 6, 7, 9, and 11). Comparative experiments performed exclusively in laboratory E, but using primers from the other participating laboratories, confirmed these observations (Table 13). Effects on apparent fragment sizes in capillary sequencing due to differences in fluorophore labeling, especially for fluorescein and rhodamine dyes, have been reported previously [42]. However, these effects and their root-causes remained largely understudied. The fluorophores used in our study, $Atto550_{Fl}$ and $TAMRA_{Fl}$, are rhodamine dyes, while NED_{Fl} belongs to the fluorescein dyes. The previous study reported that $TAMRA_{Fl}$-labeled fragments tended to be larger than NED_{Fl}-labeled fragments and that this effect depended on the fragment size, i.e., the smaller the fragment, the stronger the retardation in capillary electrophoresis of $TAMRA_{Fl}$ relative to NED_{Fl}-labeled fragments [42]. Results of comparative experiments with various reagents in laboratory E (Table 13) were mainly in accord with this observation for markers labeled with NED_{Fl} in the original protocol. The strongest effects of a 4–5-bp retardation in $Atto550_{Fl}$ relative to NED_{Fl}-labeled fragments were observed in the smallest fragment N60 (149–151 bp) and a 2-bp retardation in the larger fragments M102 (168–192 bp) and AA (265–267 bp). $TAMRA_{Fl}$-labeled fragments also appeared to be 2 bp larger relative to NED_{Fl}-labeled fragments, but size-dependent differences could not be determined.

In contrast to $TAMRA_{Fl}$ and $Atto550_{Fl}$, VIC_{Fl} (used in laboratory A instead of HEX_{Fl}) belongs to the fluorescein-like dyes, similar to HEX_{Fl}, so no effects on fragment size were expected. This was confirmed in our analysis.

It should be mentioned here that an additional retardation of 2 bp was noted when $Atto550_{Fl}$-labeled primer pairs, used to amplify N60, M102, or AA, had been purchased from the company ME and not from the companies AB or EU (Table 13). The reasons for the differences related to the primer supplier remain unknown. A potential error in the order of the primers was excluded and it should be noted that all primer pairs with different sequences from this supplier were affected. Most likely, the differences seem to be linked to primer production. Differences in the chemical reactions applied to label primers with fluorophores may be possible.

Thus, in general it seems to be important to validate new reagents by using defined reference DNAs, ideally included in each run of capillary sequencing (Fig. 3). In addition, comparative experiments with defined reference DNAs should become mandatory, if the method is newly established in a laboratory or even if previously used primers are replaced by new ones (Fig. 3). In our view, it is unlikely that different PCR kits or enzymes contribute to differences in the amplified fragments, but this was not assessed in our ring trial because all participants used the same multiplex PCR kit.

Results for MS typing were discussed among the participants in web-based meetings. Overall, an improvement of typing results relative to those of the reference laboratory was observed between the first and second parts of the ring trial, probably because participants gained experience and were given access to a laboratory internal guideline established in reference laboratory B. While one of the laboratories with little previous experience (laboratory A) obtained results that differed in determined fragment sizes relative to results provided by the reference laboratory by up to 28 bp (Table 6), including only dilution 1 and 2 results, this was no longer the case in further parts of the ring trial. In the second and third parts, deviations of a maximum of 5 bp were observed (Tables 9 and 11). This clearly shows the need for guidance, if *T. gondii* MS typing is newly established in a laboratory (Fig. 3).

So-called stutter peaks are frequently reported in MS typing (examples are displayed in the typing guidelines, provided as Supplementary File Text 1) and are caused by slippage of the DNA polymerase. They occur more often, when the number of MS repeats is >20, and less frequent, if the repeat number is <10 [41]. Specific guidelines (laboratory-specific guidelines similar to the guidelines provided in Supplementary File Text 1) may provide help to identify the correct fragment size (Fig. 3). However, stutter peaks remain a problem in MS typing, because it may not always be possible to determine the true variation in repeat numbers in the original DNA.

In the final part of the ring trial, DNA from strains not belonging to the archetypal lineages types I, II, and III was analyzed in the participating laboratories. Although the differences in the results were minor, especially for the typing markers (Table 12), none of the exotic strains was correctly classified by the semi-automatic system in place in one of the laboratories (laboratory C) (Table 2), due to limited references in the system version. Based on the results of laboratory C, correct typing would have been possible, if additional non-archetypal references would have been added to the system. This highlights the challenges of automatization for an organism with substantial genetic variation. One of the isolates was classified by some of the laboratories as type II, and by others as type II variant or type II-like, which shows that also for MS-based classification of lineages or the nomenclature of genotypes clear rules or guidelines are necessary (Fig. 3).

In conclusion, the results of this interlaboratory ring trial suggest that harmonization of MS typing appears to be possible, which might allow the combination of larger data sets on *T. gondii* genotypes. This is an important prerequisite

to study and unravel the molecular epidemiology of this parasite. The use of different fluorophores to label fragments during amplification was identified as a major source of divergence. After numerical adjustments of fragment size results, based on comparative analyses using defined reference DNAs, differences due to the use of other fluorophores no longer presented a problem and results were comparable to those previously reported in the literature. In addition, minor differences of 2 bp could be attributed to different primer suppliers. Further minor differences probably resulted from limited experience, less suitable software for assessing capillary electrophoresis profiles, and missing software options to automatize allele identification. These observations are not only important for typing *T. gondii*, but may also be relevant for other applications of MS typing (i.e., forensic identification and relatedness testing, cell line identification, or population studies).

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Data availability The datasets generated and/or analyzed during the current study are available as Supplementary File Data Table 1.

Declarations

Ethics approval Not applicable

Consent to participate Not applicable

Conflict of interest The authors declare no competing interests.

References

1. Stelzer S, Basso W, Silván JB, Ortega-Mora LM, Maksimov P, Gethmann J, Conraths FJ et al (2019) *Toxoplasma gondii* infection and toxoplasmosis in farm animals: risk factors and economic impact. Food Waterborne Parasitol 12:e00037. https://doi.org/10.1016/j.fawpar.2019.e00037
2. Schlüter D, Däubener W, Schares G, Gross U, Pleyer U, Lüder C (2014) Animals are key to human toxoplasmosis. Int J Med Microbiol 304:917–929. https://doi.org/10.1016/j.ijmm.2014.09.002
3. Robert-Gangneux F, Darde ML (2012) Epidemiology of and diagnostic strategies for toxoplasmosis. Clin Microbiol Rev 25:264–296. https://doi.org/10.1128/CMR.05013-11
4. Dubey JP (2022) Toxoplasmosis of animals and humans, 3rd edn. CRC Press, Boca Raton, USA
5. Bouwknegt M, Devleesschauwer B, Graham H, Robertson LJ, van der Giessen JW, The Euro-FBP Workshop P (2018) Prioritisation of food-borne parasites in Europe, 2016. Euro Surveill: Bulletin Europeen sur les Maladies Transmissibles Eur Commun Dis Bull 23. https://doi.org/10.2807/1560-7917.ES.2018.23.9.17-00161
6. Jones JL, Hanson DL, Dworkin MS, Alderton DL, Fleming JL, Kaplan JE, Ward J (1999) Surveillance for AIDS-defining opportunistic infections 1992-1997. Morb Mortal Wkly Rep CDC Surveilance Summ 48:1–22
7. Torgerson PR, Mastroiacovo P (2013) The global burden of congenital toxoplasmosis: a systematic review. Bull World Health Organ 91:501–508. https://doi.org/10.2471/BLT.12.111732
8. Galal L, Hamidović A, Dardé ML, Mercier M (2019) Diversity of *Toxoplasma gondii* strains at the global level and its determinants. Food Waterborne Parasitol 15:e00052. https://doi.org/10.1016/j.fawpar.2019.e00052
9. Fernández-Escobar M, Schares G, Maksimov P, Joeres M, Ortega-Mora LM, Calero-Bernal R (2022) *Toxoplasma gondii* genotyping: a closer look into Europe. Front Cell Infect Mi 12. https://doi.org/10.3389/fcimb.2022.842595
10. Lorenzi H, Khan A, Behnke MS, Namasivayam S, Swapna LS, Hadjithomas M, Karamycheva S et al (2016) Local admixture of amplified and diversified secreted pathogenesis determinants shapes mosaic *Toxoplasma gondii* genomes. Nat Commun 7:10147. https://doi.org/10.1038/ncomms10147
11. Ajzenberg D, Collinet F, Mercier A, Vignoles P, Darde 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
12. Ellegren H (2004) Microsatellites: simple sequences with complex evolution. Nat Rev Genet 5:435–445. https://doi.org/10.1038/nrg1348
13. 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 36:841–848. https://doi.org/10.1016/j.ijpara.2006.03.003

14. Su C, Khan A, Zhou P, Majumdar D, Ajzenberg D, Darde ML, Zhu XQ et al (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 109:5844–5849. https://doi.org/10.1073/pnas.1203190109
15. Chaichan P, Mercier A, Galal L, Mahittikorn A, Ariey F, Morand S, Boumediene F et al (2017) Geographical distribution of *Toxoplasma gondii* genotypes in Asia: a link with neighboring continents. Infect Genet Evol 53:227–238. https://doi.org/10.1016/j.meegid.2017.06.002
16. Galal L, Ajzenberg D, Hamidovic A, Durieux MF, Darde ML, Mercier A (2018) *Toxoplasma* and Africa: one parasite, two opposite population structures. Trends Parasitol 34:140–154. https://doi.org/10.1016/j.pt.2017.10.010
17. Shwab EK, Saraf P, Zhu XQ, Zhou DH, McFerrin BM, Ajzenberg D, Schares G et al (2018) Human impact on the diversity and virulence of the ubiquitous zoonotic parasite *Toxoplasma gondii*. Proc Natl Acad Sci U S A 115:E6956–E6E63. https://doi.org/10.1073/pnas.1722202115
18. Sabin A (1941) Toxoplasmic encephalitis in children. JAMA 116:801–814
19. Lunde MN, Jacobs L (1983) Antigenic differences between endozoites and cystozoites of *Toxoplasma gondii*. J Parasitol 69:806–808
20. Dardé ML, Bouteille B, Pestre-Alexandre M (1992) Isoenzyme analysis of 35 *Toxoplasma gondii* isolates and the biological and epidemiological implications. J Parasitol 78:786–794
21. Stollberg KC, Schares G, Mayer-Scholl A, Hrushetska I, Diescher S, Johne A, Richter MH et al (2021) Comparison of direct and indirect *Toxoplasma gondii* detection and genotyping in game: relationship and challenges. Microorganisms 9:1663. https://doi.org/10.3390/microorganisms9081663
22. 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
23. Galal L, Ariey F, Gouilh MA, Darde ML, Hamidovic A, Letourneur F, Prugnolle F et al (2022) A unique *Toxoplasma gondii* haplotype accompanied the global expansion of cats. Nat Commun 13:5778. https://doi.org/10.1038/s41467-022-33556-7
24. Mercier A, Ajzenberg D, Devillard S, Demar MP, de Thoisy B, Bonnabau H, Collinet F et al (2011) Human impact on genetic diversity of *Toxoplasma gondii*: example of the anthropized environment from French Guiana. Infect Genet Evol 11:1378–1387. https://doi.org/10.1016/j.meegid.2011.05.003
25. Slany M, Dziedzinska R, Babak V, Kralik P, Moravkova M, Slana I (2019) *Toxoplasma gondii* in vegetables from fields and farm storage facilities in the Czech Republic. FEMS Microbiol Lett 366. https://doi.org/10.1093/femsle/fnz170
26. Homan WL, Vercammen M, De Braekeleer J, Verschueren H (2000) Identification of a 200- to 300-fold repetitive 529 bp DNA fragment in *Toxoplasma gondii*, and its use for diagnostic and quantitative PCR. Int J Parasitol 30:69–75
27. Galal L, Schares G, Stragier C, Vignoles P, Brouat C, Cuny T, Dubois C et al (2019) Diversity of *Toxoplasma gondii* strains shaped by commensal communities of small mammals. Int J Parasitol 49:267–275. https://doi.org/10.1016/j.ijpara.2018.11.004
28. 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 37:579–586. https://doi.org/10.1007/s10096-017-3152-z
29. Opsteegh M, Langelaar M, Sprong H, den Hartog L, De Craeye S, Bokken G, Ajzenberg D et al (2010) Direct detection and genotyping of *Toxoplasma gondii* in meat samples using magnetic capture and PCR. Int J Food Microbiol 139:193–201. https://doi.org/10.1016/j.ijfoodmicro.2010.02.027
30. Talabani H, Asseraf M, Yera H, Delair E, Ancelle T, Thulliez P, Brezin AP et al (2009) Contributions of immunoblotting, real-time PCR, and the Goldmann-Witmer coefficient to diagnosis of atypical toxoplasmic retinochoroiditis. J Clin Microbiol 47:2131–2135. https://doi.org/10.1128/JCM.00128-09
31. Li M, Mo XW, Wang L, Chen H, Luo QL, Wen HQ, Wei W et al (2014) Phylogeny and virulence divergency analyses of *Toxoplasma gondii* isolates from China. Parasit Vectors 7:133. https://doi.org/10.1186/1756-3305-7-133
32. Ajzenberg D, Dumetre A, Darde ML (2005) Multiplex PCR for typing strains of *Toxoplasma gondii*. J Clin Microbiol 43:1940–1943. https://doi.org/10.1128/JCM.43.4.1940-1943.2005
33. Khan A, Ajzenberg D, Mercier A, Demar M, Simon S, Darde ML, Wang Q et al (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:e3182. https://doi.org/10.1371/journal.pntd.0003182
34. 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
35. Khan A, Jordan C, Muccioli C, Vallochi AL, Rizzo LV, Belfort R Jr, Vitor RW et al (2006) Genetic divergence of *Toxoplasma gondii* strains associated with ocular toxoplasmosis. Brazil Emerg Infect Dis 12:942–949. https://doi.org/10.3201/eid1206.060025
36. Behnke MS, Khan A, Wootton JC, Dubey JP, Tang K, Sibley LD (2011) Virulence differences in *Toxoplasma* mediated by amplification of a family of polymorphic pseudokinases. Proc Natl Acad Sci U S A 108:9631–9636. https://doi.org/10.1073/pnas.1015338108
37. 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 Mi 10:580425. https://doi.org/10.3389/fcimb.2020.580425
38. Calero-Bernal R, Fernández-Escobar M, Katzer F, Su C, Ortega-Mora LM (2022) Unifying virulence evaluation in *Toxoplasma gondii*: a timely task. Front Cell Infect Mi 12:868727. https://doi.org/10.3389/fcimb.2022.868727
39. Galal L, Sarr A, Cuny T, Brouat C, Coulibaly F, Sembène M, Diagne M et al (2019) The introduction of new hosts with human trade shapes the extant distribution of *Toxoplasma gondii* lineages. PLoS Negl Trop Dis 13:e0007435. https://doi.org/10.1371/journal.pntd.0007435
40. Ajzenberg D, Collinet F, Aubert D, Villena I, Darde ML, ToxoBs F, network g, Devillard S. (2015) The rural-urban effect on spatial genetic structure of type II *Toxoplasma gondii* strains involved in human congenital toxoplasmosis, France, 2002-2009. Infect Genet Evol 36:511–516. https://doi.org/10.1016/j.meegid.2015.08.025
41. de Valk HA, Meis JF, Klaassen CH (2007) Microsatellite based typing of *Aspergillus fumigatus*: strengths, pitfalls and solutions. J Microbiol Methods 69:268–272. https://doi.org/10.1016/j.mimet.2007.01.009
42. Hahn M, Wilhelm J, Pingoud A (2001) Influence of fluorophor dye labels on the migration behavior of polymerase chain reaction--amplified short tandem repeats during denaturing capillary electrophoresis. Electrophoresis 22:2691–2700. https://doi.org/10.1002/1522-2683(200108)22:13

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3.3 Genotyping of European *Toxoplasma gondii* strains by a new high-resolution next-generation sequencing-based method

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3.3.1 Declaration of author contributions

Contribution of Maike Joeres: Study conception and design, sample collection and DNA extraction, establishment and implementation of the next-generation sequencing-based method, data collection and analysis, writing the manuscript.

Contributions of other authors: PM, DH, RCB, MFE, PV, SMC, LMO, PJ and GS contributed to the study conception and design. RCB, MFE, BK, RB, MGV, KS, NB, SSO, JS, WP, PK, WB, AM, LG, MLD, AB, FS, CS, MP, NS, AL, RKD, RT, HW, EDB, PV, SMC, LMO, PJ, GS provided key biological materials or data. PM, PV, SMC and GS contributed to data collection and analysis with the support of DH and SCA. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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ORIGINAL ARTICLE

Genotyping of European *Toxoplasma gondii* strains by a new high-resolution next-generation sequencing-based method

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Abstract

Purpose A new high-resolution next-generation sequencing (NGS)-based method was established to type closely related European type II *Toxoplasma gondii* strains.

Methods *T. gondii* field isolates were collected from different parts of Europe and assessed by whole genome sequencing (WGS). In comparison to ME49 (a type II reference strain), highly polymorphic regions (HPRs) were identified, showing a considerable number of single nucleotide polymorphisms (SNPs). After confirmation by Sanger sequencing, 18 HPRs were used to design a primer panel for multiplex PCR to establish a multilocus Ion AmpliSeq typing method. *Toxoplasma gondii* isolates and *T. gondii* present in clinical samples were typed with the new method. The sensitivity of the method was tested with serially diluted reference DNA samples.

Results Among type II specimens, the method could differentiate the same number of haplotypes as the reference standard, microsatellite (MS) typing. Passages of the same isolates and specimens originating from abortion outbreaks were identified as identical. In addition, seven different genotypes, two atypical and two recombinant specimens were clearly distinguished from each other by the method. Furthermore, almost all SNPs detected by the Ion AmpliSeq method corresponded to those expected based on WGS. By testing serially diluted DNA samples, the method exhibited a similar analytical sensitivity as MS typing.

Conclusion The new method can distinguish different *T. gondii* genotypes and detect intra-genotype variability among European type II *T. gondii* strains. Furthermore, with WGS data additional target regions can be added to the method to potentially increase typing resolution.

Keywords Typing · Discriminatory power · Intra-genotype variability · Highly polymorphic regions · Multilocus sequence typing · Toxoplasmosis

Introduction

Toxoplasma gondii is a zoonotic protozoon that infects a large variety of warm-blooded species and can cause clinical disease in animals and humans. Felids are the definitive hosts of this parasite with sexual reproduction stage occurring only in their intestines [1–5]. In a European study, *T. gondii* was ranked second out of 24 important foodborne parasites [6]. At a global level, *T. gondii* has a complex population structure [7]. While clonal lineages dominate many regions [8], the *T. gondii* population is diverse in other parts of the world, like South America [7, 9].

A frequently used genotyping method for *T. gondii* assesses up to 15 microsatellite (MS) markers located in 11 different chromosomes. This method includes eight lineage typing and seven fingerprinting markers, the latter being more polymorphic and thus able to detect variability

Extended author information available on the last page of the article

within archetypal (type I, II or III) and non-archetypal lineages [10]. MS typing represents the current reference standard for genotyping and fingerprinting. Harmonized guidelines were recently established to reach consistency between different laboratories [11]. Since data analysis cannot be completely automated, interpretation of MS typing results is affected by user experience and software for data analyses [11].

Another commonly used method to type *T. gondii* is PCR-restriction fragment length polymorphism (PCR-RFLP). This method involves up to 11 markers, distributed over eight chromosomes and the apicoplast genome [12]. It can differentiate genotypes, but cannot detect intra-genotype variability.

Multilocus sequence typing (MLST) of *T. gondii* [13, 14] targets specific regions in the parasite genome and was in past studies based on Sanger sequencing. If the amount of *T. gondii* DNA is not limited, MLST is an efficient technique due to its high typing resolution [15], because it displays the whole variability of a sequenced region. Due to the broad application of next generation sequencing (NGS) and the advantages compared to Sanger sequencing, NGS should replace Sanger sequencing for MLST of *T. gondii*. While only a single DNA fragment can be sequenced at a time with Sanger sequencing, millions of fragments are sequenced simultaneously per run with NGS. This allows to multiplex several highly polymorphic regions (HPRs) and different samples in a single sequencing run. Compared to Sanger sequencing, NGS also has higher sensitivity in detecting rare variants due to deep sequencing.

Furthermore, whole genome sequencing (WGS) provides the most detailed information about genetic variability. However, WGS requires highly concentrated DNA and may not be suitable for laboratories with more limited resources [16]. In addition, WGS of *T. gondii* is a bioinformatically challenging task due to the size of its genome, approximately 65 Mb [9].

T. gondii type II is the predominant clonal genotype in Europe, but MS typing and WGS analysis revealed genetic variability within this lineage [8, 9, 17, 18]. A high resolution MLST method, which is easy to interpret, is needed to improve our understanding of *T. gondii* transmission pathways, to analyze outbreaks and trace infection sources in a setting such as Europe. We aimed to establish a NGS-based typing method with a high typing resolution among closely related type II strains that allows for automated and standardized data analysis. This new typing method should help to better understand the molecular epidemiology and transmission pathways of *T. gondii* in Europe.

Material and methods

Collection of specimens, DNA extraction and quantification

In total, 170 *T. gondii* specimens, including 123 cell-culture isolates and 47 clinical samples, were analyzed according to the workflow depicted in Fig. 1 (also see Supplementary Figure 1). The sample set comprised specimens (Supplementary Table 1) from 19 different countries on the European continent (Fig. 2) and seven non-European countries or locations. Clinical samples originated from 12 different matrices and 15 animal species, including domestic, wild-living and zoo animals. Isolates were cell-cultured as described [17, 19, 20]. The isolates cultivated at Friedrich-Loeffler-Institut (FLI) included the type I reference strains RH_{FLI} and $GT1_{FLI}$, the type II references $ME49_{FLI}$ and NTE_{FLI} and the type III reference NED_{FLI}. DNA was extracted by standard methods from cellular pellets or clinical material (Supplementary Table 1). A real-time PCR targeting TgREP-529 [21] was used to characterize DNAs quantitatively [22] (Supplementary Note 1).

Genotyping by MS analysis and PCR-RFLP

All specimens were genotyped using 15 MS markers [10]. For the markers N60, M102 and AA, the fluorophore (FL) $Atto550_{Fl}$ was used instead of NED_{Fl} for primer labelling. The reported fragment sizes of these three markers were numerically adjusted based on published guidelines [11]. Furthermore, all isolates were genotyped using nine

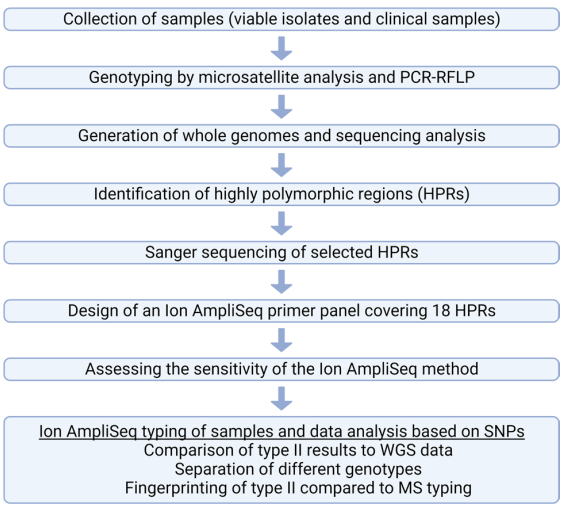

Fig. 1 Workflow of the establishment of the Ion AmpliSeq method. Created with BioRender.com

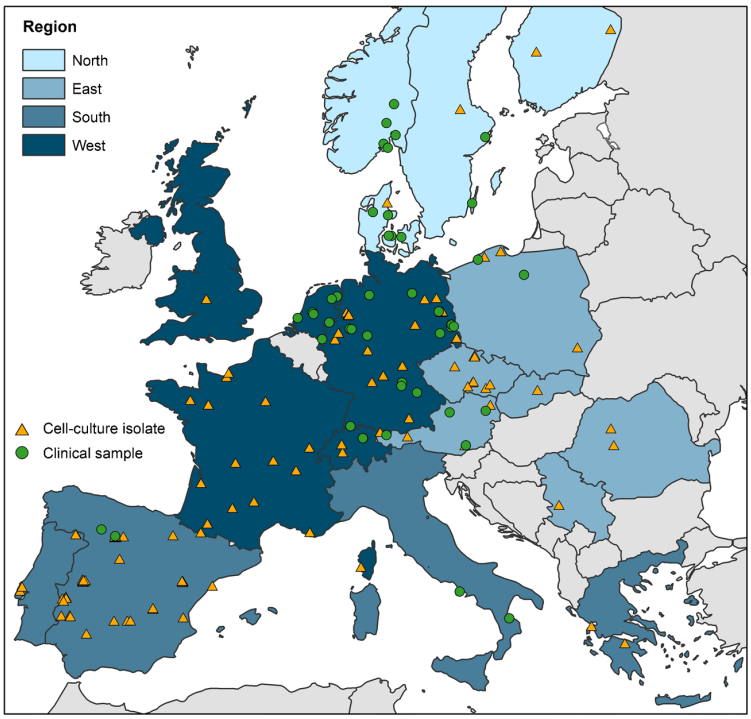

Fig. 2 Geographic origin of 110 European *T. gondii* isolates and 47 clinical samples, which were genotyped with the Ion AmpliSeq method. The geographic origin of the reference strains PRU and CZ-H3 not genotyped with the Ion AmpliSeq method is also included. Thirteen non-European isolates, also genotyped with the Ion AmpliSeq method are excluded. Details are described in Supplementary Table 1

PCR-RFLP markers [12, 23]. In both methods, the reference strains RH_{FLI}, $ME49_{FLI}$ and NED_{FLI} were used as positive controls and water as a negative control. The PCR-RFLP and MS genotypes of the reference strains PRU and CZ-H3 described in the literature (Supplementary Table 1) were included in the data analysis.

Generation of whole genomes and sequence analysis

Whole genome sequencing of 59 *T. gondii* isolates (Supplementary Table 1) was conducted with the Illumina NovaSeq 6000 system in 150 bp paired-end mode (Biodiversa s.r.l., Treviso, Italy). Raw read data of these genomes was processed bioinformatically with 21 publicly available and three reference genomes, ME49, PRU and CZ-H3 (accession numbers in Supplementary Table 1) as described in detail in Supplementary Note 2. Finally, genetic variants were detected and converted into genomic variant call format (gVCF) for further use.

Sanger sequencing

HPRs ($n = 55$) identified in the gVCF files of the WGS analysis were assessed by Sanger sequencing (details in Supplementary Note 3 and Supplementary Table 2). Sanger HPR sequences obtained in this study for three representative isolates and $ME49_{FLI}$ were aligned to a publicly available ME49 sequence (ToxoDB release 47), using the software Geneious Prime (version 2021.0.1), and SNPs detected by Sanger sequencing were compared to those identified by WGS analysis.

Library preparation and Ion AmpliSeq sequencing

Library preparation was performed using the Ion AmpliSeq™ Library Kit Plus and IonCode™ Barcode Adaptors (Thermo Fisher Scientific, Waltham, MA, USA), following the manufacturer's instructions.

For the initial multiplex PCR, an Ion AmpliSeq™ custom panel was designed (Ion AmpliSeq Designer, version 7.49, Thermo Fisher Scientific) by using a ME49 genome sequence (ToxoDB release 47), a BED file containing information about genetic variants of a subset ($n = 43$) of all available *T. gondii* genomes (Supplementary Note 2) and the locations of 24 Sanger sequencing confirmed target regions. Since six regions were excluded by the Ion AmpliSeq Designer, the panel consisted of 68 primers divided into two pools covering 18 regions (Supplementary Table 3). The final target regions were larger compared to the target regions initially identified by WGS analysis or covered these only partially (T16, T32, T51), as illustrated in Supplementary Figure 2a–b. PCR cycling conditions were 99 °C for 2

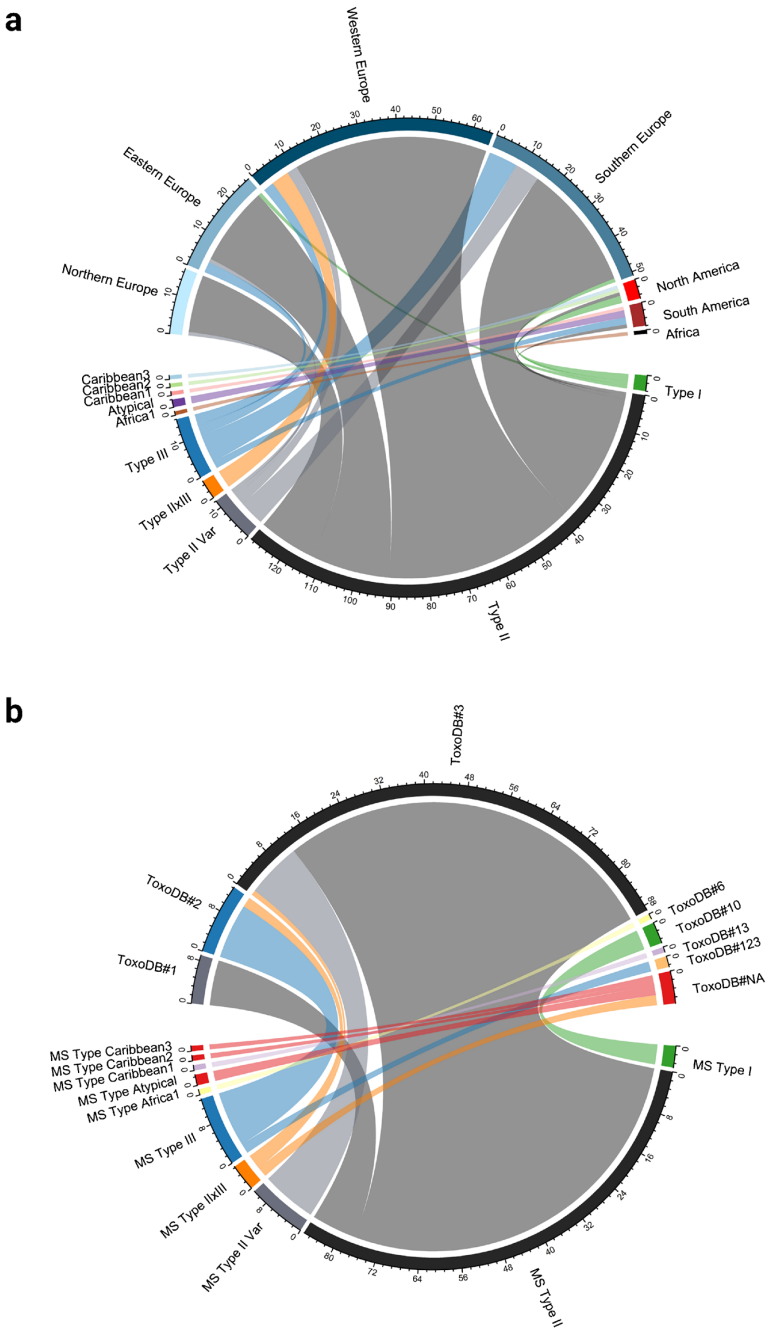

Fig. 3 Microsatellite (MS) typing of *T. gondii* specimens using 15 markers. **a** Ten different categories of MS genotypes were reported for DNA from 170 specimens genotyped in this study and additionally for MS genotypes of the reference strains PRU and CZ-H3 described in the literature in relation to their regional origin. Seven regions were defined, consisting of Northern Europe (Denmark, Finland, Sweden, Norway), Eastern Europe (Austria, Czech Republic, Poland, Romania, Serbia, Slovakia), Southern Europe (Greece, Italy, Portugal, Spain), Western Europe (France, Germany, Netherlands, Switzerland, UK), Africa, North America, and South America. **b** Ten different categories of MS genotypes were reported for 123 isolates genotyped in this study and MS genotypes of the reference strains PRU and CZ-H3 described in the literature in relation to their PCR-RFLP genotyping results

min, followed by 24, 26 or 28 cycles at 99 °C for 15 s and 60 °C for 8 min.

Adapter ligation was followed by size selection as described [24]. Library quality was checked using the High Sensitivity DNA Kit on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) or by using the 4200 TapeStation (Agilent Technologies). Libraries were quantified with the QIAseq™ Library Quant Assay (Qiagen Sciences, Germantown, MD, USA), pooled including the Ion S5 Calibration Standard and the pools sequenced on an Ion 530 chip with an Ion S5 XL System (Thermo Fisher Scientific) in 400 bp-mode according to the manufacturer's instructions.

Data analysis of the Ion AmpliSeq sequencing results

For data analyses, a reference sequence set (accessible at https://zenodo.org/; DOI: 10.5281/zenodo.8377016), in the following referred to as AmpliSeq-ME49-Reference, was created using the sequence data of each target region in the genome of ME49 (ToxoDB release 53) with additional 10 bp added to the 5′- and 3′-ends of the regions. The final target region corresponded in 17 of 18 targets to the amplicons generated by the primer panel as described in Supplementary Figure 2a–b and Supplementary Table 4. In the case of T26, the final target region was shortened for data analysis, because runs of consecutive thymine nucleotides (poly[T]) had led to ambiguous sequencing results**.** Sequence reads of each library were analyzed by reference mapping with the Torrent Mapping Alignment Program (TMAP-ion, version 3.4.0) to generate bam files for further use. Mapping quality (MQ) of the reads against the AmpliSeq-ME49-Reference was analyzed by Qualimap bamqc (v2.3) [25]. Furthermore, several BCFtools (v1.15.1 [using htslib 1.16]) [26] were employed, including the "mpileup" command to call variants in each Ion AmpliSeq record with mapped reads to generate library-specific variant call format (VCF) files. All Ion AmpliSeq VCF files were merged into a multiVCF file using the "bcftools merge" command and all variants were filtered by VCFtools (v0.1.16-20) using the hard filter criteria of MQ > 30 and read depth (DP) > 10. Moreover, the combination of Samtools faidx and the "bcftools consensus" command [26] was used to convert the VCF data into the FASTA format. The program Snp-sites (2.5.1) [27] was applied to extract the variable sites from the FASTA sequences. If parts of the AmpliSeq-ME49-Reference were not covered by the reads of specific genotypes, the corresponding nucleotides were indicated as "N" in the FASTA file. The aligned FASTA file containing respective library-specific SNPs was then converted into the NEXUS format and incorporated into SplitsTree4 software (version 4.18.1) [28] to generate unrooted phylogenetic networks using a neighbour-net method and 1000 bootstrap replicates. Sample IDs were replaced by numbers (Supplementary Table 1).

To verify the results of the automated analysis described above, the sequence reads of each library were also mapped to a ME49 genome (ToxoDB release 53) using the Geneious Prime mapper (version 2021.0.1) and default settings. Coverage of the target regions was analyzed, and positions of potential SNPs were visually inspected.

Assessing the sensitivity of the Ion AmpliSeq method

The analytical sensitivity of the Ion AmpliSeq method was assessed with a set of serially diluted DNA of $ME49_{FLI}$ previously used in a ring trial to harmonize MS typing [11]. The three dilutions were characterized by real-time PCR with Ct values of 23.79, 27.43 and 30.26, corresponding to DNA concentrations of 1 ng/µl, 0.1 ng/µl and 0.01 ng/µl. Each dilution was amplified with 24, 26 and 28 cycles in the Ion AmpliSeq multiplex PCR. The experiment was repeated three times.

Genotyping of specimens with the Ion AmpliSeq method

Library preparation of 170 specimens was performed as described above. Based on the results of the sensitivity assessment, 24 cycles were defined as the standard protocol, but five isolates with Ct values > 24.5 and all clinical samples with Ct values > 22.0 were amplified with 28 cycles (Supplementary Table 1).

Results

MS and PCR-RFLP typing results

By MS typing, most specimens ($n = 129$) were genotyped as type II (Fig. 3, Supplementary Table 1). In addition, twelve specimens were categorized as type II variants as they showed a deviation on one MS lineage typing marker. Eight were W35, two TgM-A variants and one specimen each was a XI.1 or a B18 variant. Four specimens belonged to type I, 16 to type III and five specimens were categorized as type II × III recombinants. Furthermore, the six non-archetypal strains were classified as Africa 1, Caribbean 1, Caribbean 2, Caribbean 3 and Atypical.

Nine of the 86 type II isolates were PCR-RFLP genotyped as ToxoDB#1, while the remaining 77 type II and all type II variant isolates belonged to ToxoDB#3 (Fig. 3, Supplementary Table 1). MS type I corresponded to ToxoDB#10 and 11/13 type III isolates belonged to ToxoDB#2. Two type III isolates from Argentina were classified as ToxoDB#123

Table 1 Number of SNPs in 43 *T. gondii* type II whole genomes compared to a ME49 genome (ToxoDB release 47) in non-overlapping windows of 333 bp of the genome. Four prioritization categories of target regions for the new Ion AmpliSeq-based typing method were defined based on the number of SNPs

Chromosome	Number of SNPs relative to ME49 in 43 *T. gondii* type II whole genomes per 333 bp windows					Total	SNPs/10 kb
	1–4	5-9 (4th priority targets)	10-14 (3rd priority targets)	15-19 (2nd priority targets)	20-35 (1st priority targets)		
Ia	1158	11	3	0	0	1172	6.30
Ib	1643	20	4	1	0	1668	8.53
II	1717	38	9	3	0	1767	7.53
III	1962	50	19	5	0	2036	8.04
IV	2145	35	8	1	1	2190	8.15
V	2677	68	29	11	9	2543	6.95
VI	2508	28	5	0	2	3858	8.49
VIIa	3784	60	6	4	4	3821	7.54
VIIb	3783	35	3	0	0	5543	7.95
VIII	5491	41	9	2	0	4803	7.59
IX	4704	77	18	3	1	2794	8.39
X	5622	132	15	6	1	5776	7.72
XI	4729	41	5	0	1	4776	7.21
XII	4804	37	3	1	0	4845	6.83
Total	46,727	673	136	37	19	47,592	8.00

by PCR-RFLP. One of the type II × III recombinant isolates belonged to ToxoDB#3 and two to ToxoDB#2. The two remaining recombinants and the isolates MS typed as atypical, Caribbean 2 and 3 could not be assigned to any known PCR-RFLP ToxoDB number. MS type Africa 1 corresponded to ToxoDB#6 and Caribbean 1 to ToxoDB#13.

Identification of HPRs in the nuclear genome of *T. gondii*

WGS data of 43 *T. gondii* type II genomes (Supplementary Table 1, Supplementary Figure 3) were used for the identification of HPRs (Supplementary Table 5). The mean number of reads per library was 62.1 M (range, 29.9–620.6 M) and the median depth of coverage after mapping to the ME49 genome, in the following referred to as ME49 reference, was 1077× in the case of ME49 (SRR6793863) and 15×–338× for the remaining 42 isolates. An average of 98.1% ± 1.4% standard deviation of each genome was mapped with over 10× coverage.

When mapping to ME49 reference, the SNPs found in the analyzed 43 *T. gondii* genomes sum up to a total of 65,006. The SNPs were used to identify target regions for the Ion AmpliSeq method (Table 1). SNPs were counted in non-overlapping windows of 333 bp, and four prioritization categories of target regions were defined. Nineteen target regions (Fig. 4) were categorized as first priority (20–35 SNPs), 37 as second priority (15–19 SNPs), 136 as third priority (10–14 SNPs) and 673 as fourth priority (5–9 SNPs). The first priority targets were located on seven chromosomes, mainly in subtelomeric regions. The second priority targets were located on ten chromosomes, while third and fourth priority targets were distributed all throughout the chromosomes (Fig. 4, Supplementary Table 5).

Confirmation of WGS findings using Sanger sequencing

Sanger sequencing confirmed the WGS data of 24/55 tested SNP dense regions (Supplementary Table 6). Of the sequenced targets, 15.4% (2/13) of first priority, 50.0% (8/16) of second priority, 52.1% (12/23) of third priority and 66.7% (2/3) of the fourth priority targets were confirmed. SNP analysis was not possible for 13/55 regions, due to overlapping peaks in the Sanger sequences resulting in low Phred quality scores. Furthermore, the sequences of 4/55 regions were too short to cover the whole region after mapping to ME49, and in the case of 10/55 regions, no SNPs were detected by Sanger sequencing or observed SNPs were not in accordance with WGS data. The Ion AmpliSeq primer panel was designed using all 24 confirmed regions, containing 336 different SNPs. Six of the confirmed 24 regions were excluded from primer design as detailed in Methods.

Establishment of the Ion AmpliSeq method

Analytical sensitivity of the Ion AmpliSeq method

The analytical sensitivity of the Ion AmpliSeq method was assessed with serially diluted DNA of $ME49_{FLI}$. All

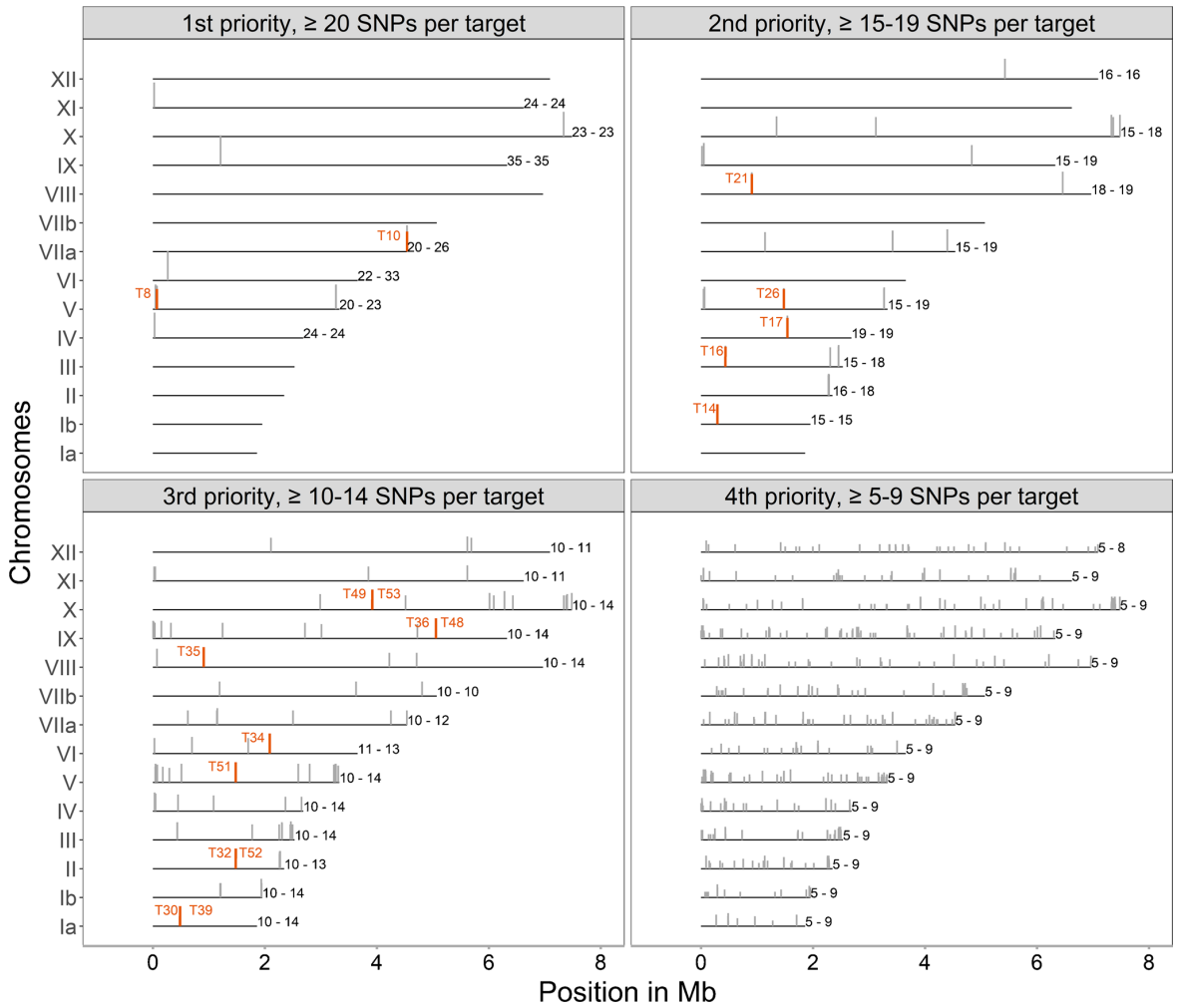

Fig. 4 SNP maps of all 14 *T. gondii* chromosomes based on the numbers of SNPs detected in non-overlapping windows of 333 bp in 43 type II genomes relative to the genome of ME49 (ToxoDB release 47). All identified highly polymorphic regions were categorized as first, second, third or fourth priority targets and their positions on the chromosomes are indicated with grey bars. Minimum and maximum numbers of SNPs per target are indicated on the right side of each chromosome. The 18 target regions used for Ion AmpliSeq typing are shown in orange

libraries were generated with an Ion AmpliSeq primer panel that included the 18 confirmed target regions located on 11 chromosomes (Supplementary Table 4). The mean number of reads per library was 261,220 and an average of 97.8% (range 90.3–99.8%) of the AmpliSeq-ME49-Reference was covered ≥ 30× by each library after mapping (Supplementary Table 7).

The coverage per target region was analyzed based on alignments with the entire ME49 genome. Target region T8 was identified as repetitive, since the reads were mapped to a length of about 10 kb of the reference genome instead of the expected 600 bp. Therefore, this region was excluded from further analyses. The reads of the first and second dilution covered all 17 remaining regions in each of the replicates, regardless of the number of cycles in the multiplex PCR (Table 2, Fig. 5). Reduced coverage completeness was observed in the third dilution of the samples. In addition, we found that 9–12 regions were completely covered if 24

Table 2 Number of regions completely covered by the ME49 replicates used to assess the analytical sensitivity of the Ion AmpliSeq method after mapping to a ME49 reference genome (ToxoDB release 53). Coverage is shown in relation to the different dilutions and number of cycles used in the Ion AmpliSeq multiplex PCR

Dilution	PCR cycles	Completely covered regions, relative to a ME49 reference genome		
		ME49 Ion AmpliSeq replicate 1	ME49 Ion AmpliSeq replicate 2	ME49 Ion AmpliSeq replicate 3
10(-1)	24	17	17	17
10(-1)	26	17	17	17
10(-1)	28	17	17	17
10(-2)	24	17	17	17
10(-2)	26	17	17	17
10(-2)	28	17	17	17
10(-3)	24	12	10	9
10(-3)	26	14	13	9
10(-3)	28	16	15	14

cycles were used, while using 28 cycles increased the complete coverage up to 14–16 regions.

General sequencing results of specimens

In total, 170 libraries comprising the set of the 17 final target regions were generated with the Ion AmpliSeq primer panel. Six libraries (TgShSp12-15, TgShSp18 and TgShSp19) were excluded from analysis after mapping to the AmpliSeq-ME49-Reference, since their sequences did not completely cover any of the target regions with a DP > 10. The Ion AmpliSeq results of 164 remaining libraries were analyzed (Supplementary Table 8).

The mean number of reads per library was 183,937 (range, 9279–1,323,246). More than 99.0% of the reads per library could be mapped to the AmpliSeq-ME49-Reference. Overall, the median depth of coverage was 4324×. Furthermore, an average of 97.5% of the AmpliSeq-ME49-Reference was covered ≥ 30× by the reads of type II samples, 94.7% by type II × III and type III, 93.6% by Caribbean 1-3, 81.2% by type I, 79.7% by Africa 1 and 77.9% by the reads of the atypical specimens (Supplementary Figure 4).

The coverage of each target region was analyzed based on alignments with the entire ME49 genome. Most regions were covered by the reads of the libraries regardless of the genotype (Supplementary Figures 5a–b). Sequencing of target region T14 failed partially or completely in case of type I and atypical isolates. Sequencing of target region T30 failed in case of Africa 1. In addition, reads for the target regions T21 and T35 were missing in case of type I, Africa 1, Caribbean 1, Caribbean 2 and atypical specimens.

SNPs of *T. gondii* type II isolates detected by the Ion AmpliSeq method compared to WGS data

The results of type II isolates were compared to their WGS data, if available, for the validation of SNPs detected by the Ion AmpliSeq method relative to ME49 (Table 3, Supplementary Table 9). For simplification, only the number of SNPs is described, as the majority of SNPs identified by both methods (WGS, AmpliSeq) were located at the same positions. The minimum number of SNPs per isolate was the same for both methods and ranged between zero and six per region. The maximum number of SNPs per isolate detected by WGS was higher in the case of three regions compared to Ion AmpliSeq.

Comparing the total number of different SNPs per region, considering also the SNP positions, revealed further differences between both analyses. In six regions (T30, T39, T14, T17, T21, T49), no differences were detected between Ion AmpliSeq and WGS data. In seven regions, WGS analysis revealed a larger number of SNPs, mainly because some SNPs detected in the Ion AmpliSeq data were excluded due to the filter criterion MQ > 30. One or two additional SNPs were observed with the Ion AmpliSeq method in the remaining four regions (details in Supplementary Table 9).

Separation of different *T. gondii* genotypes

For testing the discriminatory power of the Ion AmpliSeq method among different genotypes, the number of SNPs detected per region relative to the AmpliSeq-ME49-Reference in 164 libraries (Supplementary Table 8) and their positions (Supplementary Table 10) were compared. In 7/17 target regions, the results were similar (Fig. 6, Supplementary Figure 6). Moreover, the SNPs of type III and Caribbean 1, 2 and 3 were consistent across the target regions T36 and T48 and type III and Caribbean 3 were identical across the target regions T21 and T35. The non-type II genotypes revealed a noticeably larger number of SNPs than type II in the target regions T52, T16, T49 and T53. In addition, type I and Africa 1 showed a large number of SNPs in the target regions T36 and T48.

In summary, based on these differences in SNP density and positions, several genotypes could be clearly distinguished by the Ion AmpliSeq method, which was also confirmed by neighbour-net analysis (Fig. 7). Caribbean 1, 2 and 3 resembled type III, while Africa 1 was more similar to type I. Furthermore, one of the five type II × III recombinant specimens could not be differentiated from type II and two other recombinants were similar to type III. The results of the remaining two recombinant specimens identified them as a mix of type II and type III.

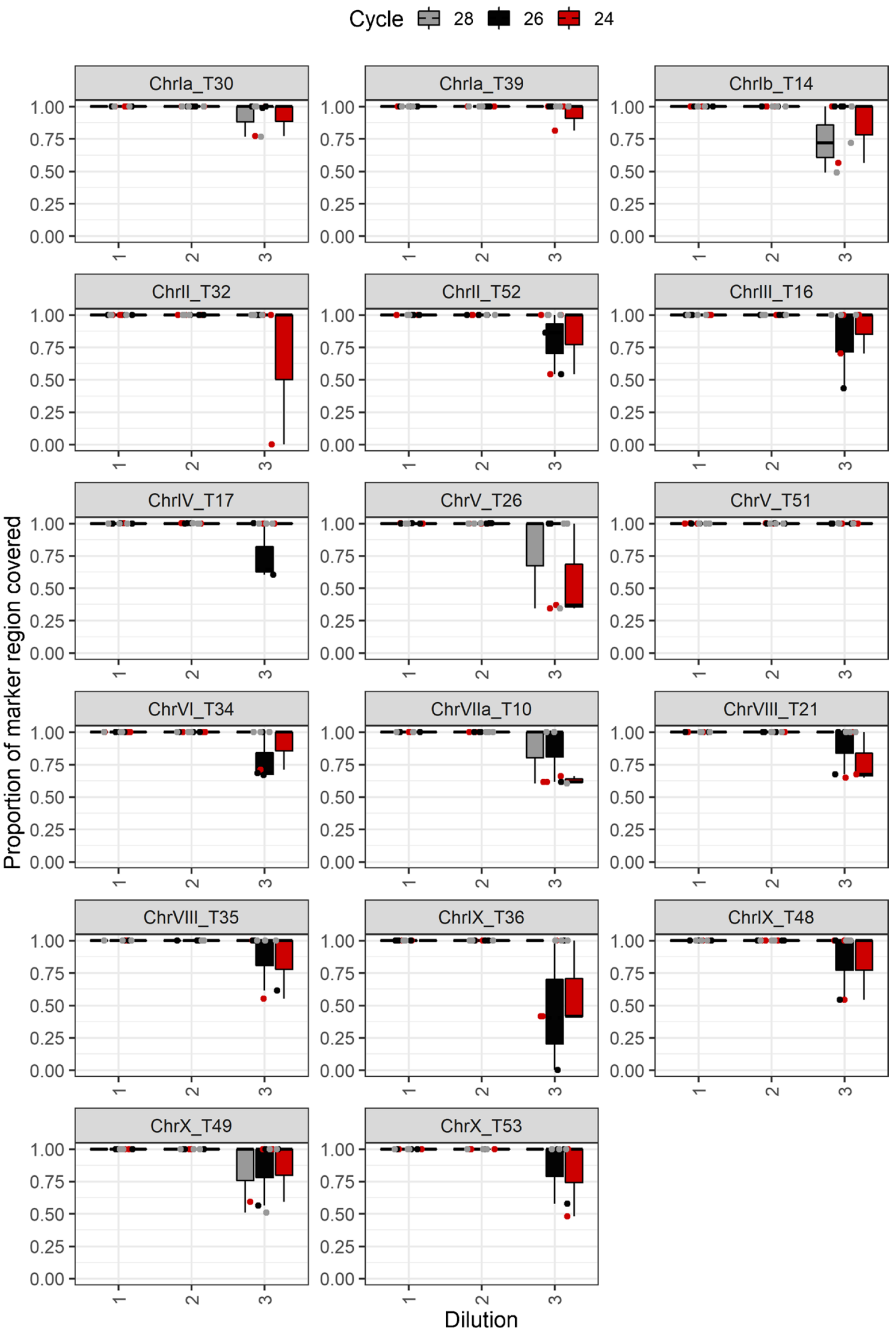

Fig. 5 Coverage of 17 target regions by ME49 replicates used to assess the analytical sensitivity of the Ion AmpliSeq method after mapping to mapped the genome of ME49 (ToxoDB release 53). The proportion of coverage of each region is shown in relation to the different dilutions (1, 2, and 3 correspond to *T. gondii* DNA concentrations of 1 ng/µl, 0.1 ng/µl and 0.01 ng/ µl) and the number of PCR cycles

Table 3 Comparison of the number of SNPs detected by Ion AmpliSeq typing and whole genome sequence (WGS) analysis relative to ME49 (ToxoDB release 53). For this comparison, 78 *T. gondii* type II isolates were used. An equal minimum or maximum number of SNPs implies that the SNPs are located at the same positions as well as the number of different SNPs per region considers the SNP positions

Chromosome	Target region	Minimum number of SNPs per isolate		Maximum number of SNPs per isolate		Number of different SNPs per region	
		Ion AmpliSeq	WGS	Ion AmpliSeq	WGS	Ion AmpliSeq	WGS
Ia	T30	0	0	5	5	20	20
Ia	T39	0	0	7	7	18	18
Ib	T14	0	0	7	7	28	28
II	T32	0	0	5	5	12	14
II	T52	1	1	6	6	17	18
III	T16	0	0	8	9	19	23
IV	T17	6	6	12	12	19	19
V	T26	0	0	5	6	13	17
V	T51	0	0	6	6	13	16
VI	T34	0	0	8	8	19	17
VIIa	T10	0	0	9	25	11	27
VIII	T21	0	0	4	4	23	23
VIII	T35	0	0	5	5	26	25
IX	T36	0	0	3	3	16	15
IX	T48	0	0	7	7	22	21
X	T49	0	0	7	7	16	16
X	T53	0	0	7	7	18	20

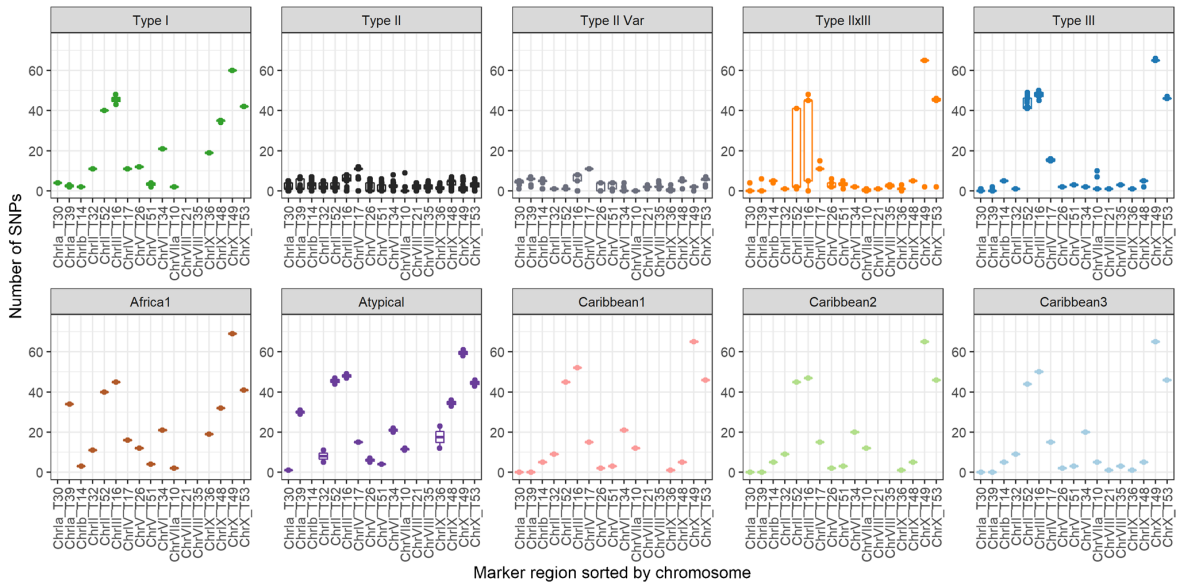

Fig. 6 Comparison of the numbers of SNPs detected by Ion AmpliSeq typing in *T. gondii* specimens relative to the AmpliSeq-ME49-Reference per region and per genotype. The numbers of specimens per genotype are not equally distributed. The figure includes results of 121 type II specimens, twelve type II variants, 14 type III (excluding C25 and C26, classified as ToxoDB #123 by PCR-RFLP typing) and four type I specimens. Only one specimen each was analyzed in case of Africa 1 and Caribbean 1, 2 and 3 and in addition, two atypical specimens and five type II × III recombinants were examined. If a boxplot is missing, the affected region was not covered by the reads of the respective genotype and no SNPs could be reported. This was the case for Africa 1 in target region T30 as well as for the atypical specimens in target region T14. In addition, target regions T21 and T35 were not covered by the reads of type I, Africa 1, Caribbean 1, Caribbean 2 and atypical specimens

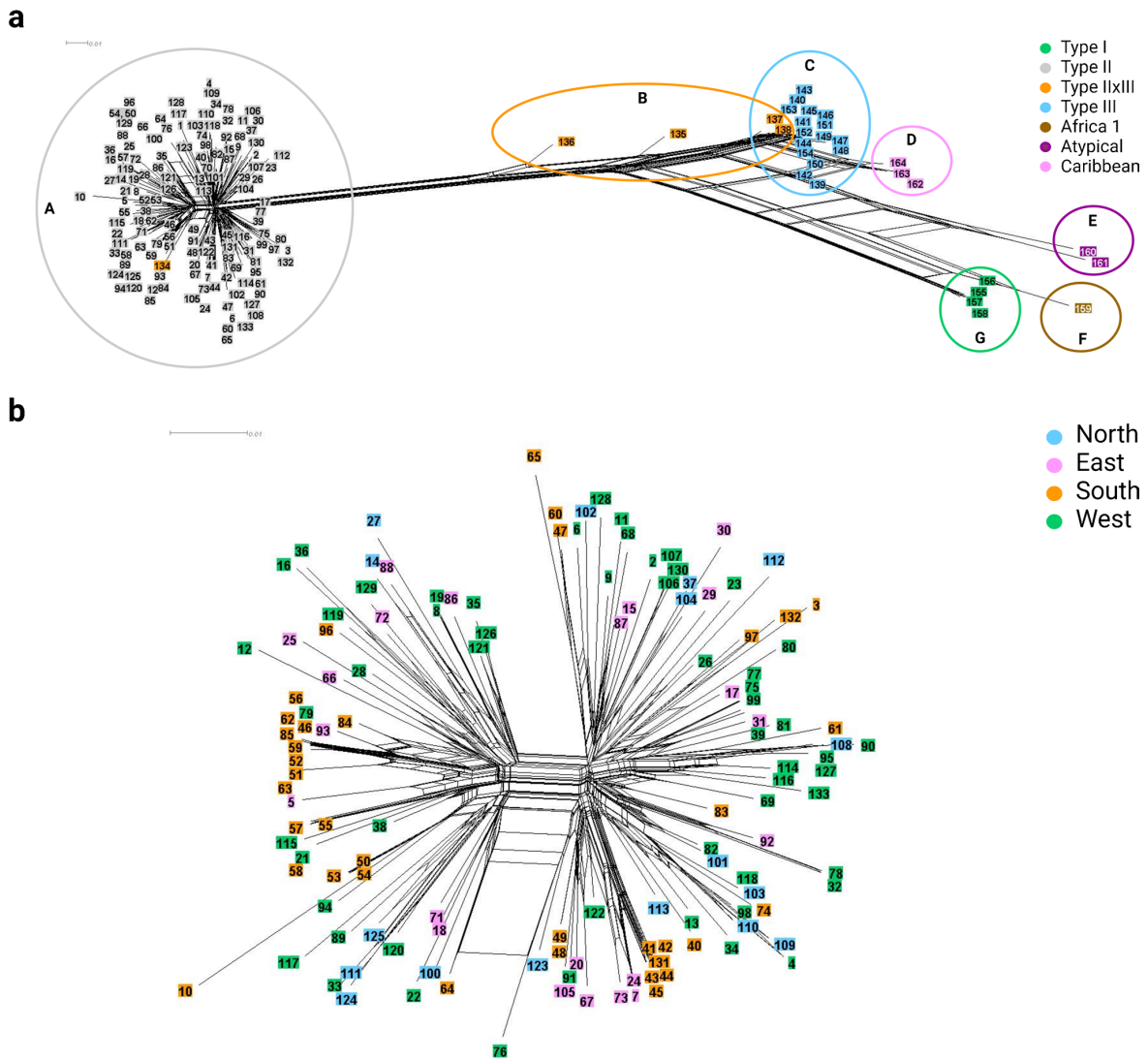

Fig. 7 Neighbour-net analysis of *T. gondii* specimens based on SNPs detected by Ion AmpliSeq typing relative to the AmpliSeq-ME49-Reference in 17 target regions (software SplitsTree4). **a** Analysis of 164 specimens belonging to different genotypes revealed seven groups. All type II specimens are located in group A, type III in group C and type I in group G. Four type II × III recombinant strains (coloured in orange) are in group B and one in group A. Group D is represented by the genotypes Caribbean 1, 2 and 3, group E by two atypical strains and one specimen typed as Africa 1 is located in group F. **b** Analysis of 131 European type II specimens distinguishing specimens from North, East, South and West Europe. No clear regional patterns can be observed. However, different passages from the same isolates were identified as identical (No. 7 and 24; No. 8 and 19; No. 32 and 78) as were specimens from abortion outbreaks (No. 42–45 and 131; No. 48 and 49). Furthermore, eight specimens (No. 46, 51, 52, 56, 59, 62, 79, 85), which showed the same variation in the MS marker W35, were identical or very similar

Ion AmpliSeq fingerprinting of *T. gondii* type II specimens compared to MS typing

All 131 sequenced and analyzed European type II specimens, including MS type II variants, were used to test the ability of the Ion AmpliSeq method to detect intra-genotype variability. The analysis was based on their SNPs relative to the AmpliSeq-ME49-Reference and the results were visualized by neighbour-net analysis (Fig. 7). Both, Ion AmpliSeq and MS typing, differentiated the same number of profiles (n = 115), of which 107 were unique. Eight profiles were detected in two or more libraries. Of three isolates, DNAs of

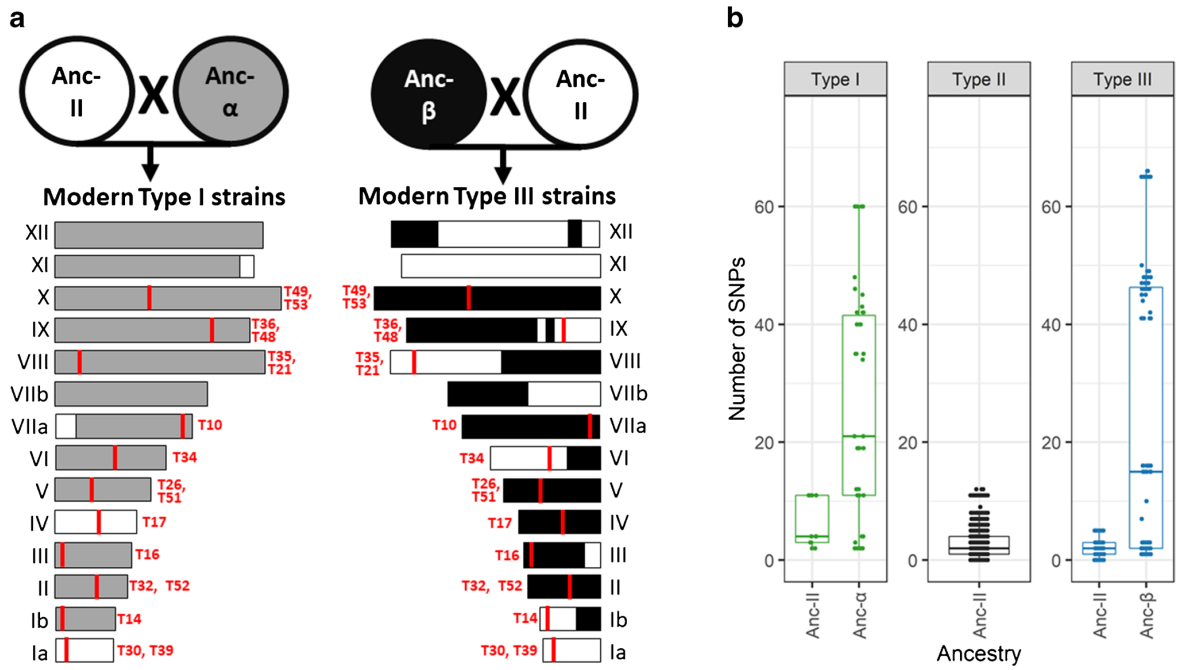

Fig. 8 Proposed genealogy of the *T. gondii* lineages type I and III and chromosome segregation during the proposed crosses, modified from Boyle et al. (2006) [37], combined with the number of SNPs detected by Ion AmpliSeq typing. **a** Chromosome segregation during the two proposed crosses (ancestral type II (Anc-II) × ancestral α (Anc-α) and ancestral type II × ancestral β (Anc-β)) (details in Supplementary Figure 4a-n). On the left (for type I) and right (for type III), all 14 chromosomes are represented schematically with their proposed ancestry coloured in grey (α), black (β), or white (type II). The positions and the names of the 17 Ion AmpliSeq target regions on the chromosomes are denoted in red. **b** Number of SNPs detected by Ion AmpliSeq typing within types I, II and III specimens relative to the AmpliSeq-ME49-Reference (details in Supplementary Figure 5). In case of type I and type III, the regions and the associated SNPs were differentiated into ancestral type II (Anc-II) and ancestral α (Anc-α) and ancestral β (Anc-β). Large numbers of SNPs per region are only observed in Ion AmpliSeq targets located in parts of the genome, for which Ancestral α or β origin was proposed

two different passages were analyzed, which revealed identical results by both methods (SplitsTree No. 7 and 24, 8 and 19, 32 and 78). Furthermore, No. 42–45 and 131 could not be differentiated, which was also true for No. 48 and 49. In both cases, the specimens originated from an abortion outbreak in a sheep flock. Moreover, No. 50, 53 and 54 were identical in both typing methods; they were all from adult sheep of the same farm. The specimens No. 55, 57 and 58 were also identical in the Ion AmpliSeq results; No. 57 and 58 originated from the same farm. Interestingly, No. 55 was different in MS typing as compared to specimens No. 57 and 58. In addition, out of eight specimens, classified as MS type II variants as they showed a deviation in the MS marker W35, five specimens (No. 46, 51, 56, 59 and 79; the first four originating from Spain and the remaining from France) were not differentiated by the Ion AmpliSeq method. In contrast, only the first three specimens (No. 46, 51, 56) had exactly the same profile by MS typing. On the other hand, three of the eight type II variants (No. 52, 62, 85), which had the same MS profile, were differentiated by the Ion AmpliSeq method.

Discussion

Genotyping of *T. gondii* is important to differentiate circulating strains, trace infection sources in outbreaks and characterize strains causing particular clinical forms of disease [17, 29]. Given the association of genotypes found in Central and South America with higher virulence and greater clinical relevance [18, 30], genotyping is also important to detect the introduction of genotypes into new areas such as Europe.

In this study, we aimed to develop an NGS-based typing method with high typing resolution among closely related type II strains, which may allow for automated and standardized data analysis.

Most European specimens were classified as type II by MS typing, which reflects the parasite (clonal) population structure in Europe [8]. The non-archetypal specimens originated from Central and South America and from Africa, where the population structure is much more diverse [7, 31].

WGS data of 43 *T. gondii* type II isolates provided the base for the identification of HPRs when mapped to the

genome of ME49. Approximately 6–8.5 SNPs per 10 kb were detected per chromosome. This corresponds to previous findings for type II, where about 10 SNPs per 10 kb were observed on all chromosomes [9, 17].

Fifty-five out of the 865 identified SNP dense regions were explored further using Sanger sequencing, as this study focused on the identification of genomic regions that were located on different *T. gondii* chromosomes that simultaneously harboured a large number of SNPs. This demonstrates the future potential to add additional targets to the Ion AmpliSeq primer panel. Some of the 55 tested regions were not confirmed by Sanger sequencing. Their majority, especially the first priority targets, was located in subtelomeric chromosomal regions. Subtelomeres are often affected by recombination events and thus repetitive sequence rich [32], which can cause sequencing problems.

In our study, the most frequent causes of inconclusive Sanger sequencing were overlapping peaks resulting in insufficient sequence quality. This may be due to the presence of repetitive sequences or multiple priming sites in the DNA template [33]. Discrepancies between SNPs detected by Sanger sequencing compared to WGS and the absence of expected SNPs in the Sanger sequences may also be explained by repetitive regions, as incorrect mapping of reads to the reference may lead to the identification of spurious SNPs.

The analytical sensitivity of the Ion AmpliSeq method was assessed with serial dilutions of ME49 DNA prepared for a previous ring trial [11]. Using the same set of dilutions ensured comparable results. Overall, the analytical sensitivity is comparable to MS typing, as failure of individual markers was only observed in the 3rd dilution with both methods [11]. Even in the 3rd dilution, at least half of the regions were completely covered by the Ion AmpliSeq sequences and failing regions were partially covered in most cases. Furthermore, increasing the number of cycles in the multiplex PCR improved coverage. This adjustment may help in the analysis of low concentrated samples. Nevertheless, it has to be noted that the use of too many cycles may increase nonspecific amplification and amplification errors. Therefore, we defined 24 cycles as the standard protocol, but used 28 cycles for specimens with higher Ct values.

For the validation of the Ion AmpliSeq results, SNPs detected in type II isolates were compared to those determined with WGS analysis. A few differences were observed when comparing the total number of different SNPs per region, mainly because SNPs detected in the Ion AmpliSeq data were excluded due to a MQ $\leq$ 30. It has to be considered that previous studies described lower quality scores for Ion Torrent bases compared to Illumina bases [34–36]. Since the base quality score affects the calculation of the MQ, this can also cause a higher MQ in the Illumina than in the Ion Torrent data. However, using a lower MQ as a filter criterion to adapt the Ion AmpliSeq results to WGS data bears the risk of detecting false positive SNPs.

Seven genotypes, two atypical and two recombinant specimens could be clearly distinguished by Ion AmpliSeq typing, due to differences in SNP density and positions relative to ME49. Sequencing of a few target regions failed partially or completely in case of type I, Africa 1, Caribbean 1, Caribbean 2 and the atypical specimens. In a neighbour-net analysis, type III showed less genetic distance to type II than type I to type II. Furthermore, Caribbean 1, 2 and 3 were grouped close to type III, while Africa 1 was more similar to type I as found in earlier studies on genetic distances [9, 17].

The genealogy of the clonal lineages type I, II and III indicates that types I and III originated from a cross between an ancestral type II strain and one of two ancestral strains, called α or β [37]. Regions, where the number of SNPs detected by the Ion AmpliSeq method was similar between the three clonal lineages, are likely to be of ancestral type II origin (Fig. 8, Supplementary Figure 7a–n, Supplementary Figure 8). If SNPs observed in type I or type III specimens clearly differed from type II, it is assumed that the corresponding regions originated at least in most cases from the ancestral strains, α or β. These previous findings could also explain why the sequencing of three regions failed in type I, as the Ion AmpliSeq primer panel was designed using a type II genome and local variations in the genomes of different genotypes might interfere with primer binding.

The specimens MS typed as Caribbean 1, 2 and 3 were previously characterized as a result of recombination between different ancestral strains [17]. Chromosome VIII of Caribbean 1 and 2 contains more type I origin segments compared to Caribbean 3, where chromosome VIII is dominated by sequences of type III origin. This fits to the fact that sequencing of the Ion AmpliSeq target regions on chromosome VIII failed in Caribbean 1 and 2, while the results of Caribbean 3 were identical with type III. Only two of the five type II × III recombinants were clearly classified as such by the Ion AmpliSeq method. This may be explained by different targets used for MS typing and depend on the site of recombination in the genome.

In total, 115 different profiles were identified by the Ion AmpliSeq method among 131 European type II specimens. MS typing, which served as a reference, distinguished the same number, but not exactly the same specimens. In our study, all specimens classified as identical by both methods were expected to be identical, as they were passages from the same isolates or originated from an abortion outbreak or the same sheep farm. This indicates the ability of the Ion AmpliSeq method to trace back infection sources in outbreaks. Ion AmpliSeq and MS typing revealed discrepancies in the identification of profiles for eight MS type II variants. We argue that these eight type II variants are genetically very similar and that discrepancies are due to the use of different target regions in the two typing methods. The WGS data when available, for these isolates, supported this hypothesis.

Furthermore, the performed neighbour-net analysis of the European type II specimens did not show a clear proximity of specimens originating from the same European region. However, a detailed study of the correlation between genetic differences and the geographic origin of specimens requires specific cluster analyses, which was out of the scope of this study. To validate the results of a cluster analysis based on the data of the new Ion AmpliSeq method, a cluster analysis based on the whole genome sequences of the corresponding isolates is needed.

In conclusion, we established an Ion AmpliSeq method that can distinguish archetypal and non-archetypal genotypes of *T. gondii* and detect intra-genotype variability among European *T. gondii* type II specimens. In addition to DNA extracted from cell-cultured *T. gondii* isolates, parasites present in clinical samples of different matrices and from different animal species were successfully typed, indicating the suitability of the new method for analyzing a large variety of samples. This is a major benefit for investigations using a One Health approach. The Ion AmpliSeq method appears promising for tracing back infection sources in outbreaks and for the detection of recombinant or non-archetypal strains. Automated data analysis makes data interpretation objective. Furthermore, as only a selection of 55 out of 865 identified SNP dense regions within the *T. gondii* genome were further explored in this work, there is a huge potential to add further target regions to the method.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10096-023-04721-7.

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Author contribution MJ, PM, DH, RCB, MFE, PV, SCAC, LMO, PJ and GS contributed to the study conception and design. MJ, RCB, MFE, BK, RB, MGV, KS, NB, SSO, JS, WP, PK, WB, AM, LG, MLD, AB, FS, CS, MP, NS, AL, RKD, RT, HW, EDB, PV, SMC, LMO, PJ and GS provided key biological materials or data. Data collection and analysis were performed by MJ, PM, PV, SMC and GS with the support of DH and SCAL. The first draft of the manuscript was written by MJ, GS and PM and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data availability The Ion AmpliSeq sequences generated and analyzed as FASTQ files in this study and four additional files (i.e. "AmpliSeq-ME49-Reference.fasta", "Tgondii_43Genomes_SNPs_INDELs.bed", "Tgondii_AmpliSeq_Results_SNPs_VCF.gz" and "Tgondii_IonAmpliSeq_Results_SNPs.fasta") are available at https://zenodo.org/ with the DOI: 10.5281/zenodo.8377016. The data set of Ion AmpliSeq sequences comprises 164 FASTQ files named according to the DNA identifiers of the analyzing laboratory in Supplementary Table 1 as D170242-D170246, D180563, D180565, D180567, D180569, D200108-D200114, D200116-D200122, D200124-D200129, D200209-D200212, D200284-D200289, D200389-D200394, D201429-D201439, D201444, D201445, D201448-D201464, D210002, D212711, D212719, D212822, D212823, D212937, D212938-D212940, D212961, D213025, D213050, D213302, D213370, D213419, D213421, D213426, D213428, D213432, D213580, D213618, D213622, D220012, D220063, D220071, D220083, D220102, D220116, D220176, D220214-D220219, D220221, D220224-D220228, D220234, D220249, D220252, D220254, D220269, D220324, D220526, D220529, D220530, D220537, D220557, D220606, D220758, D220798, D220820, D220856, D220881, D221297, D221302, D221313, D221316, D221362, D221364-D221380, D221394, D221398-D221402, GT1, RH, ME49, NTE, NED. The 59 genomes sequenced in this study are available upon reasonable request at https://zenodo.org/ with the DOIs: 10.5281/zenodo.8214035 and 10.5281/zenodo.8378855 under the accession numbers given in Supplementary Table 1. The Ion AmpliSeq primer panel used for this study can be shared through the corresponding author to all those which have an account at the Ion AmpliSeq Designer home page (https://www.ampliseq.com).

Declarations

Ethics approval Not applicable

Consent to participate Not applicable

Conflict of interest The authors declare no competing interests.

References

1. Schlüter D, Däubener W, Schares G et al (2014) Animals are key to human toxoplasmosis. Int J Med Microbiol 304:917–929. https://doi.org/10.1016/j.ijmm.2014.09.002
2. Robert-Gangneux F, Dardé M-L (2012) Epidemiology of and diagnostic strategies for toxoplasmosis. Clin Microbiol Rev 25:264–296. https://doi.org/10.1128/CMR.05013-11
3. Dubey JP (2022) Toxoplasmosis of animals and humans, Third edn. CRC Press, Boca Raton
4. Stelzer S, Basso W, Benavides Silván J et al (2019) *Toxoplasma gondii* infection and toxoplasmosis in farm animals: risk factors and economic impact. Food Waterborne Parasitol 15:e00037. https://doi.org/10.1016/j.fawpar.2019.e00037
5. Dámek F, Swart A, Waap H et al (2023) Systematic review and modelling of age-dependent prevalence of *Toxoplasma gondii* in

livestock, wildlife and felids in Europe. Pathogens 12:97. https://doi.org/10.3390/pathogens12010097
6. Bouwknegt M, Devleesschauwer B, Graham H et al (2018) Prioritisation of food-borne parasites in Europe, 2016. Euro Surveill 23:17-00161. https://doi.org/10.2807/1560-7917.ES.2018.23.9.17-00161
7. Galal L, Hamidović A, Dardé ML et al (2019) Diversity of *Toxoplasma gondii* strains at the global level and its determinants. Food Waterborne Parasitol 15:e00052. https://doi.org/10.1016/j.fawpar.2019.e00052
8. Fernández-Escobar M, Schares G, Maksimov P et al (2022) *Toxoplasma gondii* Genotyping: A Closer Look Into Europe. Front Cell Infect Microbiol 12:842595. https://doi.org/10.3389/fcimb.2022.842595
9. Lorenzi H, Khan A, Behnke MS et al (2016) Local admixture of amplified and diversified secreted pathogenesis determinants shapes mosaic *Toxoplasma gondii* genomes. Nat Commun 7:10147. https://doi.org/10.1038/ncomms10147
10. Ajzenberg D, Collinet F, Mercier A et al (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
11. Joeres M, Cardron G, Passebosc-Faure K et al (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
12. 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 36:841–848. https://doi.org/10.1016/j.ijpara.2006.03.003
13. Khan A, Fux B, Su C et al (2007) Recent transcontinental sweep of *Toxoplasma gondii* driven by a single monomorphic chromosome. Proc Natl Acad Sci U S A 104:14872–14877. https://doi.org/10.1073/pnas.0702356104
14. Bertranpetit E, Jombart T, Paradis E et al (2017) Phylogeography of *Toxoplasma gondii* points to a South American origin. Infect Genet Evol 48:150–155. https://doi.org/10.1016/j.meegid.2016.12.020
15. Su C, Shwab EK, Zhou P et al (2010) Moving towards an integrated approach to molecular detection and identification of *Toxoplasma gondii*. Parasitology 137:1–11. https://doi.org/10.1017/S0031182009991065
16. Vilares A, Borges V, Sampaio D et al (2020) Towards a rapid sequencing-based molecular surveillance and mosaicism investigation of *Toxoplasma gondii*. Parasitol Res 119:587–599. https://doi.org/10.1007/s00436-019-06523-3
17. Galal L, Ariey F, Gouilh MA et al (2022) A unique *Toxoplasma gondii* haplotype accompanied the global expansion of cats. Nat Commun 13:5778. https://doi.org/10.1038/s41467-022-33556-7
18. Shwab EK, Saraf P, Zhu X-Q et al (2018) Human impact on the diversity and virulence of the ubiquitous zoonotic parasite *Toxoplasma gondii*. Proc Natl Acad Sci U S A 115:E6956–E6963. https://doi.org/10.1073/pnas.1722202115
19. Fernández-Escobar M, Calero-Bernal R, Regidor-Cerrillo J et al (2020) Isolation, genotyping, and mouse virulence characterization of *Toxoplasma gondii* from free ranging iberian pigs. Front Vet Sci 7:604782. https://doi.org/10.3389/fvets.2020.604782
20. Schares G, Bangoura B, Randau F et al (2017) High seroprevalence of *Toxoplasma gondii* and probability of detecting tissue cysts in backyard laying hens compared with hens from large free-range farms. Int J Parasitol 47:765–777. https://doi.org/10.1016/j.ijpara.2017.07.003
21. Talabani H, Asseraf M, Yera H et al (2009) Contributions of immunoblotting, real-time PCR, and the Goldmann-Witmer coefficient to diagnosis of atypical toxoplasmic retinochoroiditis. J Clin Microbiol 47:2131–2135. https://doi.org/10.1128/JCM.00128-09
22. Schares G, Joeres M, Rachel F et al (2021) Molecular analysis suggests that Namibian cheetahs (*Acinonyx jubatus*) are definitive hosts of a so far undescribed *Besnoitia* species. Parasit Vectors 14:201. https://doi.org/10.1186/s13071-021-04697-3
23. Herrmann DC, Pantchev N, Vrhovec MG et al (2010) Atypical *Toxoplasma gondii* genotypes identified in oocysts shed by cats in Germany. Int J Parasitol 40:285–292. https://doi.org/10.1016/j.ijpara.2009.08.001
24. Wylezich C, Papa A, Beer M et al (2018) A versatile sample processing workflow for metagenomic pathogen detection. Sci Rep 8:13108. https://doi.org/10.1038/s41598-018-31496-1
25. Okonechnikov K, Conesa A, García-Alcalde F (2016) Qualimap 2: advanced multi-sample quality control for high-throughput sequencing data. Bioinformatics 32:292–294. https://doi.org/10.1093/bioinformatics/btv566
26. Danecek P, Bonfield JK, Liddle J et al (2021) Twelve years of SAMtools and BCFtools. Gigascience 10:giab008. https://doi.org/10.1093/gigascience/giab008
27. Page AJ, Taylor B, Delaney AJ et al (2016) SNP-sites: rapid efficient extraction of SNPs from multi-FASTA alignments. Microb Genom 2. https://doi.org/10.1099/mgen.0.000056
28. Huson DH, Bryant D (2006) Application of phylogenetic networks in evolutionary studies. Mol Biol Evol 23:254–267. https://doi.org/10.1093/molbev/msj030
29. Khan A, Shaik JS, Behnke M et al (2014) NextGen sequencing reveals short double crossovers contribute disproportionately to genetic diversity in *Toxoplasma gondii*. BMC Genom 15:1168. https://doi.org/10.1186/1471-2164-15-1168
30. Khan A, Jordan C, Muccioli C et al (2006) Genetic divergence of *Toxoplasma gondii* strains associated with ocular toxoplasmosis, Brazil. Emerg Infect Dis 12:942–949. https://doi.org/10.3201/eid1206.060025
31. Galal L, Ajzenberg D, Hamidović A et al (2018) *Toxoplasma* and Africa: one parasite, two opposite population structures. Trends Parasitol 34:140–154. https://doi.org/10.1016/j.pt.2017.10.010
32. Contreras SM, Zambrano Siri RT, Rivera EM et al (2022) Architecture, chromatin and gene organization of *Toxoplasma gondii* subtelomeres. Epigenomes 6:29. https://doi.org/10.3390/epigenomes6030029
33. Crossley BM, Bai J, Glaser A et al (2020) Guidelines for Sanger sequencing and molecular assay monitoring. J Vet Diagn Invest 32:767–775. https://doi.org/10.1177/1040638720905833
34. Quail MA, Smith M, Coupland P et al (2012) A tale of three next generation sequencing platforms: comparison of Ion Torrent, Pacific Biosciences and Illumina MiSeq sequencers. BMC Genom 13:341. https://doi.org/10.1186/1471-2164-13-341
35. Escalona M, Rocha S, Posada D (2016) A comparison of tools for the simulation of genomic next-generation sequencing data. Nat Rev Genet 17:459–469. https://doi.org/10.1038/nrg.2016.57
36. Uelze L, Borowiak M, Bönn M et al (2020) German-wide interlaboratory study compares consistency, accuracy and reproducibility of whole-genome short read sequencing. Front Microbiol 11:573972. https://doi.org/10.3389/fmicb.2020.573972
37. Boyle JP, Rajasekar B, Saeij JPJ et al (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. https://doi.org/10.1073/pnas.0510319103

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3.4 Drivers of infection with *Toxoplasma gondii* genotype type II in Eurasian red squirrels (*Sciurus vulgaris*)

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3.4.1 Declaration of author contributions

Contribution of Maike Joeres: Study conception, DNA extraction, qPCR analysis of DNA samples, microsatellite typing of DNA samples, reviewing/editing the manuscript, co-authoring the manuscript.

Contributions of other authors: Conceptualization: SRW, MGEM, MJLK, PM, MO, MM, GS; Methodology: SRW, MGEM, MJLK, GC, GL, GS; Formal analysis and investigation: SRW, PM, GS; Writing - original draft preparation: SRW, GS; Writing - review and editing: SRW, MGEM, MJLK, PM, MO, MM, GS.

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Parasites & Vectors

RESEARCH

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Drivers of infection with *Toxoplasma gondii* genotype type II in Eurasian red squirrels (*Sciurus vulgaris*)

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Abstract

Background In September 2014, there was sudden upsurge in the number of Eurasian red squirrels (*Sciurus vulgaris*) found dead in the Netherlands. High infection levels with the parasite *Toxoplasma gondii* were demonstrated, but it was unclear what had caused this increase in cases of fatal toxoplasmosis. In the present study, we aimed to gain more knowledge on the pathology and prevalence of *T. gondii* infections in Eurasian red squirrels in the Netherlands, on the *T. gondii* genotypes present, and on the determinants of the spatiotemporal variability in these *T. gondii* infections. The presence of the closely related parasite *Hammondia hammondi* was also determined.

Methods Eurasian red squirrels that were found dead in the wild or that had died in wildlife rescue centres in the Netherlands over a period of seven years (2014–2020) were examined. Quantitative real-time polymerase chain reaction was conducted to analyse tissue samples for the presence of *T. gondii* and *H. hammondi* DNA. *Toxoplasma gondii*-positive samples were subjected to microsatellite typing and cluster analysis. A mixed logistic regression was used to identify climatic and other environmental predictors of *T. gondii* infection in the squirrels.

Results A total of 178 squirrels were examined (49/178 *T. gondii* positive, 5/178 *H. hammondi* positive). Inflammation of multiple organs was the cause of death in 29 squirrels, of which 24 were also *T. gondii* polymerase chain reaction positive. *Toxoplasma gondii* infection was positively associated with pneumonia and hepatitis. Microsatellite typing revealed only *T. gondii* type II alleles. *Toxoplasma gondii* infection rates showed a positive correlation with the number of days of heavy rainfall in the previous 12 months. Conversely, they showed a negative association with the number of hot days within the 2-week period preceding the sampling date, as well as with the percentage of deciduous forest cover at the sampling site.

Conclusions *Toxoplasma gondii* infection in the squirrels appeared to pose a significant risk of acute mortality. The *T. gondii* genotype detected in this study is commonly found across Europe. The reasons for the unusually high infection rates and severe symptoms of these squirrels from the Netherlands remain unclear. The prevalence of *T. gondii* in the deceased squirrels was linked to specific environmental factors. However, whether the increase in the number of dead squirrels indicated a higher environmental contamination with *T. gondii* oocysts has yet to be established.

Keywords Toxoplasmosis, Zoonoses, Parasite, Oocyst, Sentinel, Monitoring, Squirrel, Microsatellite typing, Population structure

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Background

The protozoan parasite *Toxoplasma gondii* is the causative agent of toxoplasmosis, a widespread zoonotic disease that affects warm-blooded animals globally [1]. Felines, including domestic cats (*Felis catus*), European wildcats (*Felis silvestris*), and Eurasian lynxes (*Lynx lynx*), are its definitive hosts in Europe [2–5]. Following initial infection with *T. gondii*, felines excrete millions of environmentally resistant oocysts in their faeces [6, 7], which put other animals and humans at risk of exposure [8, 9]. Various species have been identified as intermediate hosts, with tissue cysts developing especially in their muscle tissues and brain [10]. *Toxoplasma gondii* infection in livestock can lead to substantial economic losses due to reproductive failure, e.g. in goats and sheep [11–13]. Animals intended for human consumption that harbour tissue cysts of *T. gondii* pose a risk to public health [14]. Humans can become infected through eating undercooked meat containing tissue cysts, by ingesting oocysts present in water, soil or on raw unwashed fruits and vegetables, and by congenital transmission [7, 15, 16]. Although most humans infected with *T. gondii* do not have specific symptoms, infection can cause severe disease, or even death in foetuses and immunocompromised individuals [17].

Toxoplasma gondii is also of concern for wildlife, as in certain species infection may be lethal [7, 18–25]. In September 2014, there was a sudden increase in the number of dead Eurasian red squirrels (*Sciurus vulgaris*) reported to the Dutch Wildlife Health Centre (DWHC) and the Dutch Mammal Society [26]. In 20 of the 37 squirrels examined, the cause of death (COD) was disseminated *T. gondii* infection [22]. There is limited information on *T. gondii* in squirrels [18, 23, 27], and it is unclear whether the cases of toxoplasmosis and the increase in the reported number of dead squirrels were related to climatic or other environmental factors, or whether specific genotypes of *T. gondii* with a higher virulence in squirrels may have been associated with the increase in the number of deaths [28]. Gaining knowledge about *T. gondii* infections in Eurasian red squirrels is not only important for the conservation of this species, which already suffers from various threats [29], but also from a One Health perspective, as the rate of toxoplasmosis in squirrels may represent a proxy for the exposure of humans living in the same areas as these animals to *T. gondii*.

To increase our understanding of *T. gondii* in red squirrels, we retrospectively examined data collected by the DWHC from 2014 to 2020. One objective of this study was to gain insight into the determinants of the spatiotemporal characteristics of the *T. gondii* infection rates in these squirrels. Another aim was to assess the pathology of *T. gondii* infection. To assess associations with pathogenicity, we compared the microsatellite (MS) genotypes of *T. gondii* in squirrels from the Netherlands with other MS genotypes observed in Europe. The presence of the parasite *Hammondia hammondi* was also determined, as it is a closely related parasite and has similar biology to *T. gondii*, which may lead to misdiagnosis of the infectious agent.

Methods

Study area and populations

Eurasian red squirrels that were found dead in the wild or that had died in wildlife rescue centres were collected by the DWHC between 2014 and 2020 to examine the COD (Additional file 4). A carcass was accepted for examination depending on its freshness (< 24 h since death), the time that the squirrel had spent in captivity in the rescue centre (< 24 h), and the capacity of the DWHC to examine the carcass. The number of squirrels examined by the DWHC was temporarily increased in 2019 and 2020 because squirrels were then accepted that had been dead for < 48 h or had been in captivity in rescue centres for < 48 h before death, and because the DWHC gave priority to squirrels over other animals at that time. The squirrels were sexed and weighed; age could not reliably be assessed. Geographical coordinates were determined based on the site of collection (i.e. coordinates were not based on the location of the rescue centre).

Pathological and histopathological examination

Post-mortem examination included gross examination and cytological and histopathological analyses. Immunohistochemical, bacteriological and viral investigations were performed when feasible. Cytological examination was performed on the liver, spleen, lungs, and intestinal contents, which were stained with Hemacolor quick stain (Merck, Darmstadt, Germany). For histopathological examination, tissue samples of internal organs (brain, spleen, lung, liver, kidney, and heart) were fixed in 4% phosphate-buffered formalin, embedded in paraffin, cut into 4-µm sections and stained with hematoxylin and eosin. Duplicate slides were examined in the immunohistochemical assay, which used polyclonal antibodies against *T. gondii* (avidin–biotin complex immunoperoxidase stain, goat anti rabbit/biotin; Dako E0432; Klinipath, the Netherlands).

Real-time polymerase chain reaction analyses

Lung, liver and heart samples were examined for *T. gondii* DNA by quantitative real-time polymerase chain reaction (qPCR) targeting a 529-base pair repetitive element, TgREP-529 [30, 31]. A quantitative real-time qPCR for *H. hammondi* was also performed [32]. DNA was extracted using the NucleoMag Tissue kit (Macherey–Nagel,

Düren, Germany) in accordance with the manufacturer's instructions with a previously described adjustment [33] whereby the volume of lysis buffer and proteinase K was adapted to the weight of the individual tissue samples, which ranged from 25 to 500 mg. Sequences of primers and probes (Eurofins, Ebersberg, Germany), and the final concentrations used are provided in Additional file 1: Table S1. To monitor qPCR inhibition, a heterologous plasmid DNA resembling the gene that encodes enhanced green fluorescent protein (EGFP) [34] was added to the reaction mix, which included the primers EGFP1-F and EGFP2-R and the probe EGFP1 [35]. The volume of the final quantitative real-time qPCR reaction was 25 µl, and a commercial master mix was used (5× PerfeCTa qPCR ToughMix; VWR, Darmstadt, Germany). Amplification was done on a CFX96 instrument (Bio-Rad, Munich, Germany).

MS typing

For each *T. gondii*-positive animal [quantification cycle (Cq) values < 34], the sample with the lowest Cq value was assessed by MS typing [36]. Extracted DNA was amplified in a multiplex PCR [37], using 15 unlinked MS markers. These markers included eight typing markers (TUB2, W35, TgM-A, B18, B17, M33, IV.1, XI.1) and seven fingerprinting markers (M48, M102, N83, N82, AA, N61, N60). These fingerprinting markers display a high level of polymorphism within the clonal lineages type I, type II and type III [37]. Primers were used at a concentration 0.2 pmol/µl. The only divergence from the original method was that, in the case of M102, AA and N60, the fluorophore Atto 550 was used instead of NED to label amplicons during multiplex qPCR. MS typing followed recently described guidelines [36].

Population genetic analyses of MS typing data

MS data conversion and processing were done using the R package adegenet (version 2.1.9) [38]. The genetic distance matrix for relative distances between MS genotypes was calculated using Bruvo's method [39, 40]. In the computation of Bruvo's distance, raw allele calls were divided by their respective repeat lengths, followed by rounding up. This methodology introduces a potential error when repeat lengths are of even magnitude, owing to adherence to standard IEC 60559 [41, 42]. The MS data were therefore normalized by employing R package poppr (version 2.9.3) [39]. This step was executed before the calculation of Bruvo's distance to improve the accuracy of the results. Prior to the determination of clustering among the MS data, their clustering tendency was assessed by applying the Hopkins statistic [43, 44]. A Hopkins statistic value above 0.5 was used to conclude if the dataset had a significant clustering tendency [44]. Affinity propagation clustering (APC) was used to identify subpopulations from the *T. gondii* DNA samples for which MS data for all 15 markers were available. APC is an unsupervised classification model that clusters MS multi-locus genotypes (MLGs) according to their similarity, which is inferred by Bruvo's distance [45]. To this end, Bruvo's distance matrix was squared and converted by inverting the values [45]. The APC algorithm determines a single MLG from the set of input MLGs for each potential cluster that is most representative of that cluster. The optimal number of clusters was defined as the largest range of input parameters for which a constant number of clusters was calculated [45].

Selection of spatiotemporal covariables

An overview of the explanatory variables is provided in Additional file 1: Table S2. To estimate the presence of domestic cats, four variables were chosen as indirect proxies (due to the limited population of wild cats solely present in the south of the province of Limburg, these species were not included [46]): annual estimate of the domestic cat population (determined by a public survey [47]; outcomes of that survey were available by personal communication with B. Beekhof, Nederlandse Voedingsindustrie Gezelschapsdieren) in the Netherlands per region [i.e. northern region (provinces of Drenthe, Friesland, and Groningen), eastern region (provinces of Gelderland and Overijssel), southern region (provinces of Limburg and North Brabant), western region (provinces of Flevoland, North Holland, Utrecht and South Holland) [48]); distance to the nearest urban area; human population density; and farm density (i.e. agriculture, horticulture, and livestock). The proportion of cats per region was assumed to remain stable over time. Distance to urban areas [functional land use map (BBG) category 2 [49]] was calculated as the Euclidean distance from the geolocation of the collected squirrel to the nearest urban area (metres). Both human population [50] and farm density [51] were calculated within a 3-km-diameter buffer zone (i.e. based on our estimation of cat and squirrel home ranges) centred around the geographical location where individual squirrels had been collected (Additional file 1: Table S2).

To explain the potential spatial variability of *T. gondii* infections, we considered landscape composition [8, 52], determined from data in the national land use database of the Netherlands (5×5-m^2 resolution; Wageningen Environmental Research). Land cover was aggregated into seven categories: agricultural, urban, deciduous forest, coniferous forest, infrastructure, surface water, and nature reserve. The proportion per land cover type was calculated within a 3-km-diameter buffer zone. As infection with *T. gondii* may occur as a result of exposure to

infective oocysts present in contaminated water [53], the nearest Euclidean distance (metres) from the geographical location of the collected squirrel to water bodies (BBG category 7 [49]) was also included (Additional file 2: Table S2).

Environmental infection pressure is determined by factors that affect the sporulation and survival of oocysts in the environment. To estimate the climatic parameters favourable for oocyst survival and sporulation, we retrieved interpolated data at 1-km^2 spatial resolution from 34 weather stations in the Netherlands (Royal Netherlands Meteorological Institute) for 2013–2020 (Additional file 2: Table S2). These gridded layers were used to derive values [54] for the maximum length of a dry spell; the number of summer days when the maximum temperature was > 25 ℃ (SU25); the number of days with heavy rainfall (i.e. ≥ 25 mm) (R25mm); and the number of days when it froze (minimum temperature ≤ −6 ℃) (FD6) preceding the date of sampling of individual squirrels. The first three parameters were chosen based on the following hypotheses: oocyst survival is adversely affected by high temperatures and drought [55–57]; the survival of oocysts increases with levels of environmental moisture [58, 59]. Both sporulation and survival of oocysts are affected by low temperatures. Temperatures around 4 ℃, and most likely also temperatures below 4 ℃, have a negative effect on the survival of non-sporulated *T. gondii* oocysts; in one study [60], almost all non-sporulated oocysts died over a period of 2–3 months at temperatures around 4 ℃. Sporulated oocysts can survive at very low temperatures (down to −21 ℃) [2, 57, 61]. However, they are very susceptible to repeated freezing and thawing events [57], which are likely to occur under natural, non-experimental conditions. Moreover, sporulation of oocysts does not occur below ≤ −6 ℃, independent of moisture levels [62]. The climatic variables were calculated for the 5 × 5-km^2 area around each individual squirrel geolocation point for the 2-week, 1-, 3-, 6-, and 12-month periods preceding the date of specimen collection (Additional file 1: Table S2).

Squirrels use food sources such as nuts, seeds, fruits, fungi, grains, vegetables, and roots, which requires a varying amount of time and activity on the ground. The ratios of the components of the diet of squirrels vary according to their availability [63]. We hypothesized that squirrels may have increased exposure to *T. gondii* in years with a higher Living Planet Index (LPI) of fungi compared to the general trend. This effect may be amplified if mast production is low in the same years. Mast year was determined for beech (*Fagus sylvatica*) and oak (*Quercus robur*) [64]. Data from the National Databank of Flora and Fauna were used to aggregate trends (LPI) for saprotrophic and ectomycorrhizal fungal species ($n = 88$), as described by van Strien et al. [65]. In short, the first step involved calculating the year-to-year degree of change per fungal species by dividing the index value for a given year by the index value of the preceding year [65]. Extreme increase was capped at 10, extreme decline was restricted to 0.1, and all instances where the value was < 1 were substituted by 1 [66]. Aggregated annual indices per region were calculated by using the yearly geometric mean of the degree of change. These geometric means were transformed to annual indices (LPI), with the first year (1994) set to 100 [65]. Trend lines were derived by using smoothed conditional means (Additional file 2: Figure S1).

Statistical analysis

An exact binomial test was used to determine confidence intervals (CIs) for infection rates. The association between squirrel *T. gondii* infection status and sex were assessed using a chi-squared test. Univariable logistic regression was used to assess patterns over the years, between regions, and between seasons. A generalized linear mixed model (GLMM) analysis was performed to investigate associations between positivity for *T. gondii* and potential risk factors (Additional file 1: Table S2). A random year effect was used to account for any unobserved factors that may have differed between years and may have affected infection risk [67]. Pre-processing of data included standardization by using the *z*-score (e.g. the mean was subtracted from each observation and the result divided by the SD), exclusion of variables with zero variance, and the removal of observations for which values were missing [68]. We first built univariable mixed logistic regression models to select potential candidate variables for the final GLMM. Predictor variables with $P \leq 0.25$ were retained as input variables for the final GLMM. Correlations between potential predictor variables were assessed [69]. Where variables were highly correlated ($r_s > 0.6$) or there was multicollinearity between predictor variables (variance inflation factor < 10) [70], the predictor variable with the lowest *P*-value in the univariable mixed logistic regression was retained (Additional file 2: Figure S2). Forward and backward model selection based on the corrected Akaike information criterion was conducted [71]. To measure the strength of associations, the odds ratio and the 95% CIs were determined. *P*-values below 0.05 were considered to indicate statistical significance.

Software

Quantitative real-time qPCR results were analysed using CFX Manager software version 1.6 (Bio-Rad). MS data

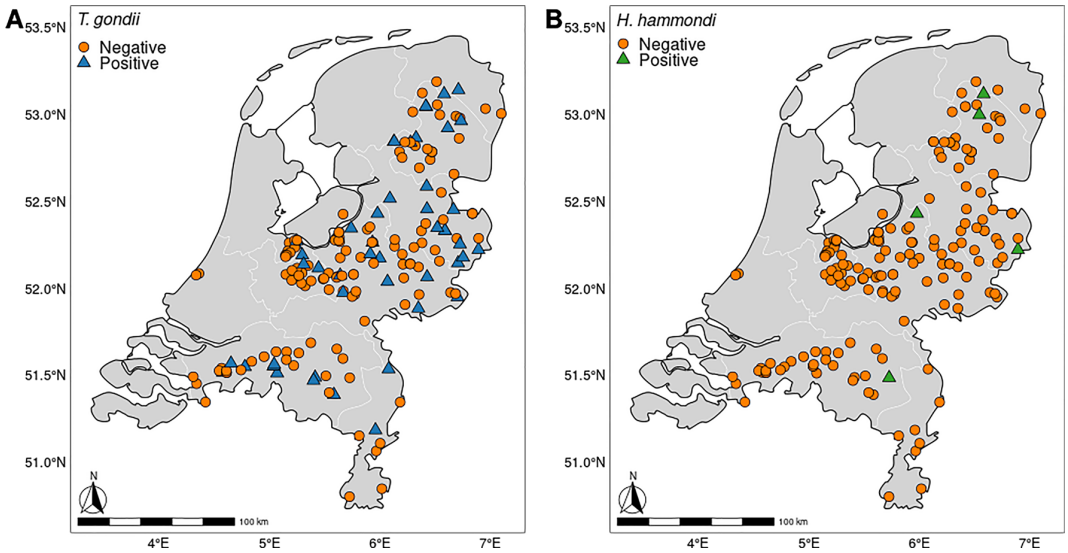


Fig. 1 Geographical distribution of the squirrels that were quantitative real-time polymerase chain reaction (qPCR) positive or negative for *Toxoplasma gondii* (**A**) or *Hammondia hammondi* (**B**)

analyses, covariate data extraction, and statistical analyses were performed in R version 4.3.1.

Results

In total, 178 Eurasian red squirrels were tested for the presence of *T. gondii* and *H. hammondi* by quantitative real-time qPCR (Fig. 1). Of these squirrels, 27.5% (49/178, 95% CI 21.1–34.7%) tested positive for *T. gondii* and 2.8% (5/178, 95% CI 0.9–6.4%) for *H. hammondi* (Fig. 1B). Three of the squirrels were positive for both *H. hammondi* and *T. gondii*. Of the 174 specimens for which sex could be determined, 78 were female and 96 were male. The proportion of squirrels infected with *T. gondii* did not differ between the sexes (chi-squared test, $\chi^2 = 0.022$, $df = 1$, $P = 0.88$), and was 28.2% for females (95% CI 18.6–39.5%), and 26.0% for males (95% CI 17.6–36.0%).

Toxoplasma gondii infection rates significantly decreased over the years, with positivity ranging from 52.9% in 2014 to 9.1% in 2020. Seasonal differences were detected. In autumn, significantly more *T. gondii*-positive squirrels were observed in comparison to winter and summer. Autumn also represented the period in which the largest proportion [40.4% (72/178), 95% CI 33.2–48.0%] of squirrels had been collected. There was no evidence of regional differences in *T. gondii*-positive samples (Table 1; Additional file 1: Table S3). Since only five squirrels were positive for *H. hammondi*, and each of them had been collected in a different year and month (Additional file 1: Table S4), no statistical analyses were performed on their data.

Pathological and histopathological examination

In 24 of the 49 squirrels (49.0%) that tested positive for *T. gondii* by quantitative real-time qPCR, inflammation of multiple organs (i.e. inflammation of two or more organs) was determined to be the main COD (Fig. 2A). A combination of the following conditions was detected: hepatitis (20/24), pneumonia (20/24), splenitis (9/24), myocarditis (8/24), encephalitis (4/24), peritonitis (1/24), lymphadenitis (1/24), dermatitis (1/24), liver necrosis (1/24), enteritis (1/24), and thymus necrosis (1/24). Pneumonia-only was detected in 12.2% (6/49) of the positive animals, hepatitis-only in 2.0% (1/49), and sepsis in 2.0% (1/49). For six animals the COD was unknown. *Toxoplasma gondii* infection was positively associated with pneumonia (chi-squared test, $\chi^2 = 46.6$, $df = 1$, $P < 0.001$), and with hepatitis (chi-squared test, $\chi^2 = 28.7$, $df = 1$, $P < 0.001$). In all 24 squirrels with inflammation of two or more organs, the inflammation was acute, i.e. there was no visible signs of fibrosis. Trauma was the COD in 22.4% (11/49) of the 49 squirrels, and no inflammation was seen that could have been the COD.

In *T. gondii*-negative animals, trauma was shown to be the leading COD (51.2%, 66/129; Fig. 2B). In the remaining animals, the COD included pneumonia (9.3%, 12/129), inflammation of multiple organs (3.9%, 5/129), and sepsis (3.9%, 5/129). In five squirrels with inflammation of multiple organs combinations of the following

Table 1 Overview of quantitative real-time polymerase chain reaction (qPCR) *Toxoplasma gondii*-positive squirrels ($n=178$) per sampling year, season, and region, with odds ratios (*OR*) from univariable logistic regression analysis

	% Positive (*n* positive/*n*; 95% CI)	OR (95% CI)	*P*-value
Sampling year			
2014	52.9 (18/34; 35.3–70.2)	1	–
2015	33.3 (9/27; 16.5–54.0)	0.44 (0.15–1.24)	0.128
2016	30.0 (6/20; 11.9–54.3)	0.38 (0.11–1.19)	0.106
2017	20.0 (3/15; 4.3–48.1)	0.22 (0.04–0.85)	0.040*
2018	15.4 (2/13; 1.9–45.4)	0.16 (0.02–0.72)	0.030*
2019	22.2 (8/36; 10.1–39.1)	0.25 (0.09–0.70)	0.009*
2020	9.1 (3/33; 1.9–24.3)	0.09 (0.02–0.31)	< 0.001*
Season			
Autumn (September–November)	44.4 (32/72; 32.7–56.6)	1	–
Winter (December–February)	8.0 (2/25; 0.98–26.0)	0.11 (0.02–0.40)	0.004*
Spring (March–May)	27.1 (13/48; 15.3–41.8)	0.46 (0.21–1.01)	0.056
Summer (June–August)	6.1 (2/33; 0.74–20.2)	0.08 (0.01–0.29)	0.001*
Region			
East	29.9 (23/77; 20.0–41.3)	1	–
South	28.2 (11/39; 15.0–44.9)	0.92 (0.38–2.13)	0.852
West	16.7 (5/30; 5.6–34.7)	0.47 (0.14–1.30)	0.169
North	31.2 (10/32; 16.1–50.0)	1.07 (0.42–2.57)	0.886

CI Confidence interval

* $P < 0.05$

conditions were observed: hepatitis (3/5), lymphadenitis (1/5), myocarditis (1/5), pleuritis (1/5), pneumonia (5/5), and splenitis (1/5). In 34 animals the COD could not be determined (26.4%, 34/129). Further details on the COD are provided in Additional file 1: Table S5.

In the *T. gondii*-positive animals, the trachea often contained foam, and the lungs were hyperemic and oedematous. In general, the liver was enlarged and pale, and the spleen was also enlarged. Pulmonary interstitial lymphoplasmacytic and neutrophilic infiltrates with oedema and numerous intra-alveolar macrophages were also revealed. The *T. gondii*-negative animals showed different types of infiltrates, which were not consistently noted.

Data from immunohistochemical analyses (IHC) were evaluated for a total of 56 squirrels. In 43 of these, IHC indicated a *T. gondii* infection. In 15 of these 43 squirrels (34.9%), *T. gondii* infection was confirmed by quantitative real-time qPCR (Additional file 1: Table S5). One squirrel shown to be positive for *T. gondii* by quantitative real-time qPCR was not shown to be infected according to the IHC results. One squirrel considered to be positive for *T. gondii* according to the IHC results was shown to be negative for the parasite by quantitative real-time qPCR but positive for *H. hammondi* (Additional file 1: Tables S6, S7). Four of the five quantitative real-time qPCR-positive *H. hammondi* samples were not subjected to IHC testing. The remaining sample was shown to be positive for *T. gondii* by IHC.

Toxoplasma gondii and *H. hammondi* detection per organ

Thirty-five of the 49 *T. gondii*-positive squirrels tested positive for all organs. In these 35 squirrels, the Cq values for the heart, lung and liver correlated to each other (Additional file 2: Figure S6). In 33 of these squirrels, the Cq-values ranged between 15 and 25, whereas in two of the squirrels the Cq value was around 30 or above.

Cq values were higher for squirrels that died from trauma compared to the other CODs (Wilcoxon rank sum test, $Z=-3.51$, $P<0.001$; Fig. 3). Cq values were lower for the liver [median, 18.3 (interquartile range (IQR), 3.3)] than for the lung [median, 19.4 (IQR, 2.5); Wilcoxon rank sum test, $Z=-3.42$, $P<0.001$]. Cq values for heart samples [median, 21.6 (IQR 2.2)] were higher than those for the liver (Wilcoxon rank sum test, $Z=-4.53$, $P<0.001$) and lung samples (Wilcoxon rank sum test, $Z=-3.66$, $P<0.001$; Additional file 2: Figure S3).

Of the remaining 14 squirrels, seven tested positive for *T. gondii* in the lungs only (14.3%, 7/49); one squirrel in the liver only (2.0%, 1/49); and one squirrel in the heart only (2.0%, 1/49). Four squirrels tested positive in the lungs and liver (8.2%, 4/49), but not in the heart (for

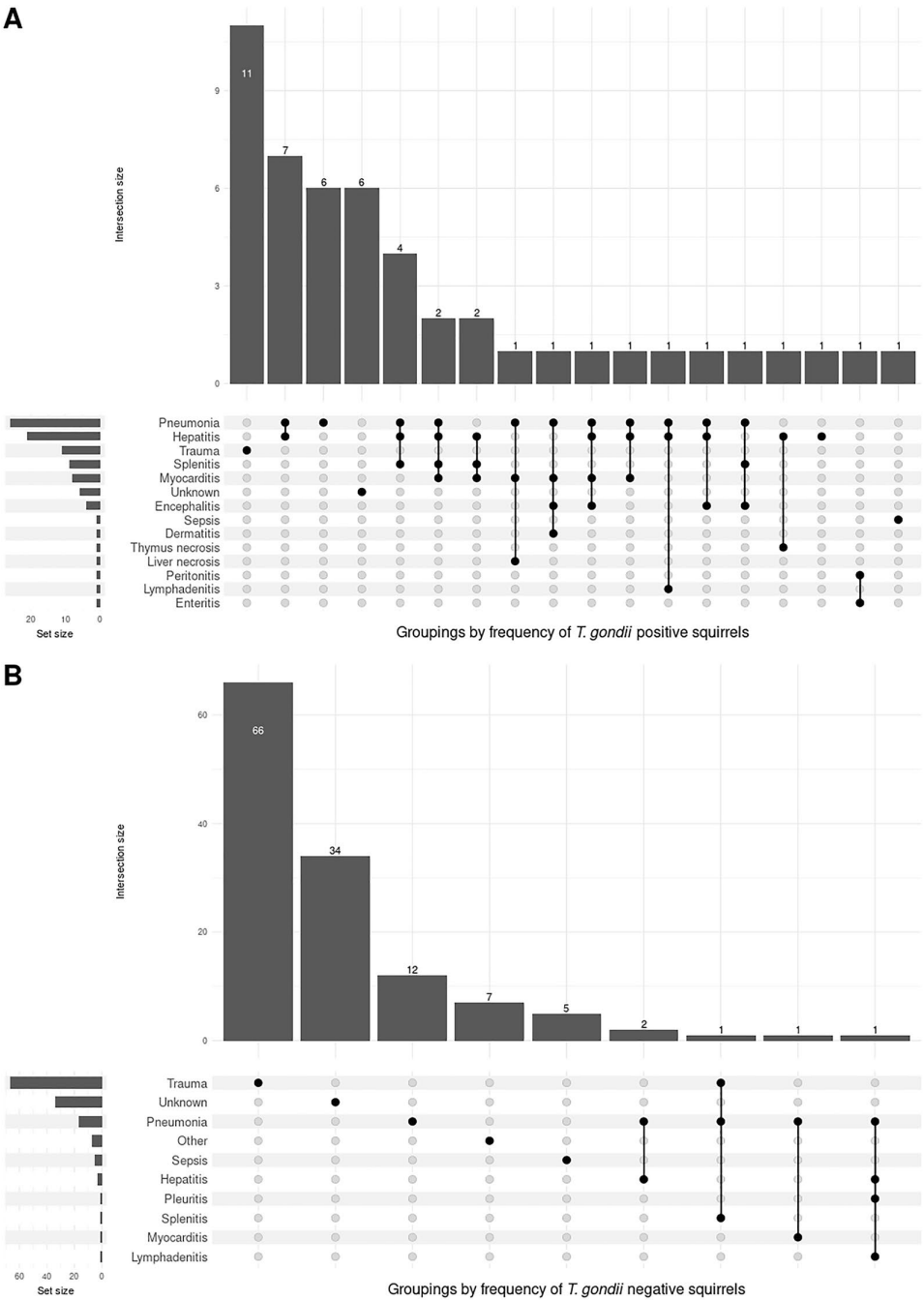


Fig. 2 Cause of death determined from pathological examination of quantitative real-time polymerase chain reaction (qPCR) *Toxoplasma gondii*-positive squirrels (**A**) and real-time qPCR *T. gondii*-negative squirrels (**B**). Connections between dots indicate co-occurring conditions

one squirrel no heart sample was available). One squirrel tested positive in both heart and liver samples; no lung sample was available for this individual (Additional file 2: Figures S4, S5).

In four of the five squirrels that tested positive for *H. hammondi*, only the heart samples were positive (Additional file 2: Figures S4, S5). The remaining squirrel tested positive in the lungs (20.0%, 1/5). The Cq value for *H.*

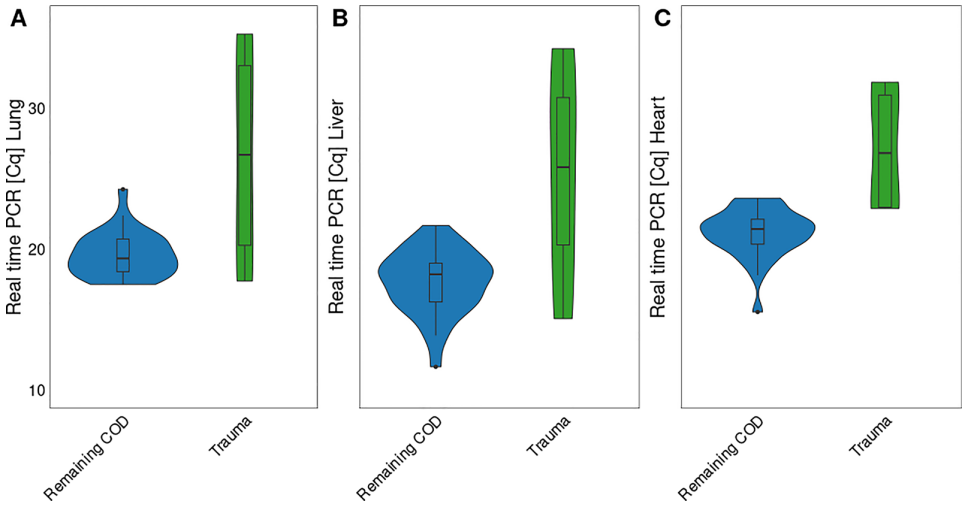


Fig. 3 Quantification cycle (*Cq*) values of *Toxoplasma gondii* by quantitative real-time polymerase chain reaction (qPCR) for cause of death (*COD*) [comparison of *Trauma* and other (*Remaining*) CODs; these included inflammation of multiple organs, pneumonia, and undetermined causes] for individual organs [lung ($n=35$) (**A**); liver ($n=35$) (**B**); heart ($n=35$) (**C**)]

Table 2 Characteristics of the 15 microsatellite (MS) markers used in this study, displayed for completely ($n=39$) or partially ($n=5$) typable *Toxoplasma gondii* samples

	Marker	Alleles	
		Completely typable	Partially typable
Typing markers	B18	158	158
	M33	169	169
	TUB2	289	289
	XI.1	356	356
	TgM-A	207	207
	W35	242	242
	IV.1	272–274	274
	B17	336	336
Fingerprinting markers	N61	85–117	95–101
	M48	211–235	219–227
	N83	308–316	310–312
	N82	109–123	111
	N60	140—144	140—142
	M102	172—182	174–182
	AA	259–289	261–271

For an overview of the population genetic analyses of *Toxoplasma gondii* MS data, see Additional file 3

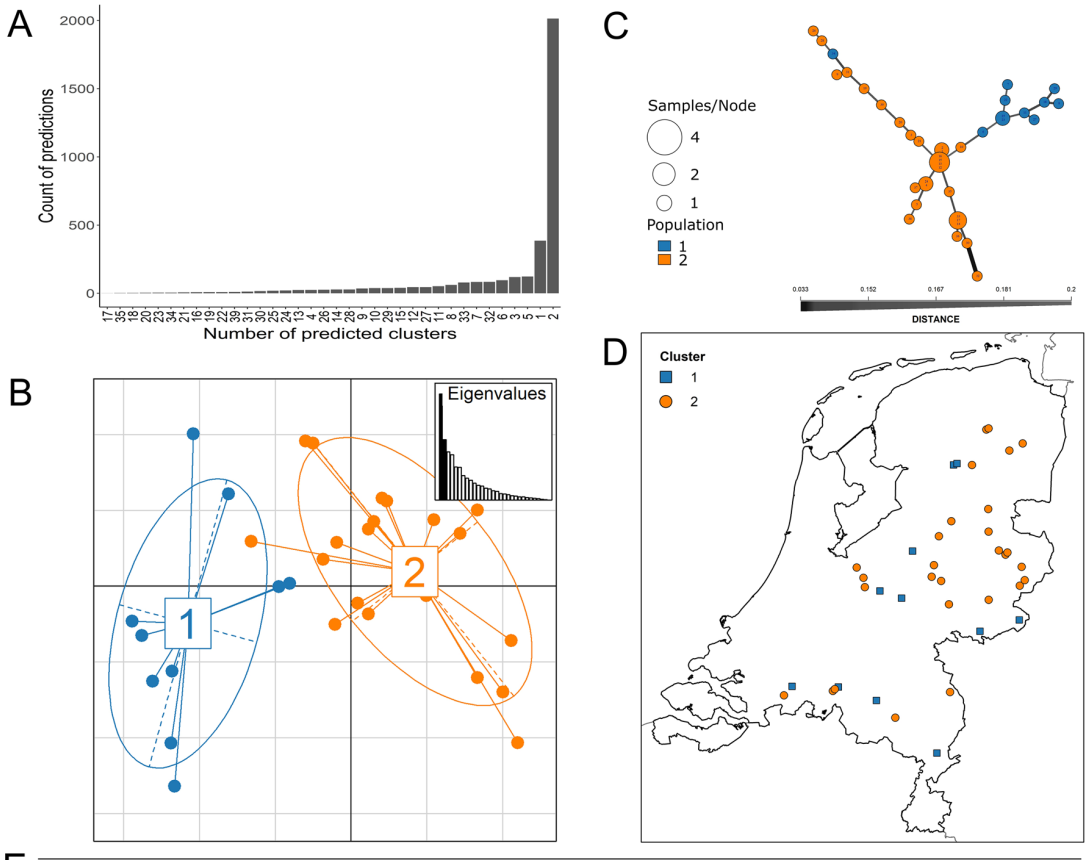
hammondi infections ranged from 30.2 to 31.5 for the heart samples, and was 34.4 for the lung sample.

Population genetic analyses of *T. gondii* MS typing data

MS typing was used to analyse samples of 44 of the 49 *T. gondii*-positive squirrels. Five samples with a Cq value above 35 were impossible to type. Thirty-nine samples could be typed completely, and were shown to be *T. gondii* type II (Table 2; Additional file 3). Five samples were partially typable and showed only *T. gondii* type II alleles (Table 2; Additional file 3). By MS fingerprinting a total of 32 different MS types were observed among the 39 *T. gondii* for which the DNA was completely typed (Additional file 3). The value of the Hopkins statistic determined for the squirrel MS data, 0.65, strongly supported rejection of the null hypothesis positing the absence of a clustering tendency within the dataset.

APC revealed two clusters of *T. gondii* type II in the sampled squirrels (Additional file 3; Fig. 4A). Principal coordinate analysis, based on Bruvo's distances separated by the principal coordinates with the highest eigenvalues, i.e. principal coordinate 1 and principal coordinate 2, explaining 20.3% and 11.6% of the variation, respectively (Fig. 4B), and the minimum spanning network (MSN) of the haplotypes (Fig. 4C) revealed that the clusters were well separated from each other. The MSN was inferred by assuming the principle of parsimony, and was consistent with the clustering indicated by the APC. Representatives of both clusters were observed throughout the Netherlands (Fig. 4D). There was no statistical difference in geographical location of representatives of these two *T. gondii* clusters over time (Fig. 4E).

When the APC analysis included clonal *T. gondii* type II MS typing results for European type II genotypes that



E

Period (September until August of the following year)	Microsatellite type (MT)																													Total number typed	Number MTs
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32		
2014-2015	2	1	4	2		1	1	1	1	1	1	1	1																				17	12
2015-2016		1			2									1	1	1	1	1															8	7
2016-2017																			1	1													2	2
2017-2018																					1	1											2	2
2018-2019																							1	1	1	1							4	4
2019-2020																											1	1	1	1	1	1	6	6
Total	2	2	4	2	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	39	NA

Fig. 4 **A**–**E** Results of affinity propagation clustering (APC) using microsatellite (MS) genotyping data ($n=39$), based on Bruvo's distance. **A** Variation in the number of parasite clusters indicated by APC. **B** Results of the principal coordinate analysis (colours correspond to the two clusters identified by APC). **C** Minimum spanning network of haplotypes (colour denotes parasite clusters according to APC; circle size corresponds to the total number of individuals with the same MS type; branch thickness is proportional to inferred genetic distance between haplotypes). **D** Geographic distribution of *Toxoplasma gondii*-positive squirrels ($n=39$) harbouring the *T. gondii* MS types ($n=32$; colours correspond to those of the clusters in **B**). **E** Representatives of the parasite clusters over time. Representatives of each of the clusters were observed for all of the periods, except for 2018–2019, for which there were only four typed samples

had been reported in a recent review ($n=444$; Additional file 3) [28] in addition to those of the present study ($n=39$), three clusters were determined (designated EurCluster1, EurCluster2, EurCluster3; Table 3; Additional file 3). The clustering tendency of the merged MS data was confirmed as statistically significant by the Hopkins statistic (0.9). A comparison of the proportions of these clusters among various northwestern European countries (Table 3) showed no significant differences between the following: France, Belgium, Netherlands, Germany, Denmark, and Norway (chi-square test, $\chi^2=9.44$, $df=12$, $P=0.665$).

Table 3 Proportions of three European clusters (EurCluster1, EurCluster2, EurCluster3) of clonal *Toxoplasma gondii* type II determined by affinity propagation clustering of merged MS data[a]

Country	EurCluster1	EurCluster2	EurCluster3
Portugal	9% (1/11)	0% (0/11)	*91*% (10/11)
Spain	0% (0/1)	*100*% (1/1)	0% (0/1)
England	0% (0/1)	*100*% (1/1)	0% (0/1)
France	26% (78/296)	*37*% (108/296)	*37*% (110/296)
Belgium	21% (6/28)	*43*% (12/28)	*36*% (10/28)
The Netherlands	22% (9/39)	*39*% (15/39)	*39*% (15/39)
Germany	15% (3/20)	*40*% (8/20)	*45*% (9/20)
Norway	0% (0/5)	*60*% (3/5)	*40*% (2/5)
Denmark	0% (0/12)	*50*% (6/12)	*50*% (6/12)
Austria	*63*% (38/60)	28% (17/60)	8% (5/60)
Czech Republic	*100*% (4/4)	0% (0/4)	0% (0/4)
Romania	0% (0/6)	17% (1/6)	*83*% (5/6)

[a] Proportions > 30% are shown italic

Predictor variables of *T. gondii* in the dead squirrels

The variables identified by univariable mixed logistic regression as putative predictor variables of infection with *T. gondii* in GLMM were the following: human population density, distance to nearest urban area, percentage of urban area, percentage of deciduous forest, distance to nearest water body, maximum length of dry spell (in the previous 2 weeks), SU25 (in the previous 2 weeks), R25mm (in the previous 12 months), and FD6 (in the previous 6 months) (Table 4; Additional file 1: Tables S2, S8). As percentage of infrastructure, SU25 (in the previous month), and FD6 (in the previous 12 months) were highly correlated to other predictor variables, they were excluded from further analyses (Table 4; Additional file 2: Figure S2). After model selection, the following predictors were determined to be significantly associated with *T. gondii* infection: percentage of deciduous forest at the sampling site (negative association), R25mm (in the previous 12 months; positive association), and SU25 (in the previous 2 weeks; negative association) (Table 4; Additional file 1: Table S9).

Discussion

The primary objectives of this study were to enhance our understanding of the drivers of *T. gondii* infection in Eurasian red squirrels found dead in the Netherlands following the *T. gondii* outbreak of 2014, to assess the pathology

Table 4 Overview of univariable mixed logistic regression ($P < 0.25$) and final mixed logistic regression using data on squirrels ($n = 175$) from the Netherlands to identify predictors for *Toxoplasma gondii*-positive findings by quantitative real-time polymerase chain reaction (qPCR)

	Univariable regression		Final mixed logistic regression[d]	
Predictor variables[a]	*P*-value	OR (95% CI)	*P*-value	OR (95% CI)
Human population density	0.153	1.28 (0.91–1.79)	–	–
Nearest distance to urban areas	0.055	0.58 (0.34–1.01)	0.098	0.60 (0.33–1.10)
Land use-% Urban	0.151	1.29 (0.91–1.81)	–	–
Land use-% Deciduous forest	0.101	0.72 (0.49–1.07)	*0.020*	*0.60* (*0.39–0.92*)
Land use-% Infrastructure[b]	0.193	1.26 (0.89–1.77)	–	–
Distance to nearest water body	0.031*	0.60 (0.38–0.96)	–	–
Maximum length of dry spell–2 weeks[c]	0.170	1.27 (0.90–1.78)	0.113	1.36 (0.93–2.00)
R25mm–12 months	0.031*	1.48 (1.04–2.11)	*0.037*	*1.58* (*1.03–2.43*)
SU25–2 weeks	0.007*	0.31 (0.13–0.72)	*0.008*	*0.27* (*0.10–0.71*)
SU25–1 month[b]	0.020*	0.51 (0.29–0.90)	–	–
FD6–6 months	0.047*	0.56 (0.32–0.99)	0.144	0.62 (0.33–1.17)
FD6–12 months[b]	0.236	0.74 (0.45–1.22)	–	–
Mast year–Oak	0.073	0.65 (0.41–1.04)	0.302	0.75 (0.44–1.29)

R25mm–12 months Days with heavy rainfall (≥ 25 mm) in the 12 months preceding sampling; *SU25–2 weeks* Summer days with maximum temperature > 25 ℃ in the 2 weeks preceding sampling; *SU25–1 month* summer days with maximum temperature > 25 ℃ in the month preceding sampling; *FD6–6 months* Days when it froze (minimum temperature ≤ −6 ℃) in the 6 months preceding sampling; *FD6–12 months* days when it froze (minimum temperature ≤ −6 ℃) in the 12 months preceding sampling

* $P < 0.05$

[a] For further details on predictor variables, see Additional file 1: Table S2

[b] $r_s > 0.6$; for further details, see Additional file 2: Figure S2

[c] Dry days in the 2-week period preceding the sampling date

[d] Model random effects, σ^2 (random effect variance)—3.29; marginal R^2/conditional R^2—0.478/0.504

of the infections, and to compare MS genotypes of *T. gondii* from the Netherlands with those observed in other European countries. We detected an overall *T. gondii* infection rate of 27.5%. Infection rates decreased over the years, but it remains to be determined whether this was due to declining environmental pressure or other, non-investigated, factors. There were no marked differences between regions, which possibly reflects the widespread geographic distribution of *T. gondii* in domestic cats in the Netherlands.

The five animals that tested positive for *H. hammondi* represent, to the best of our knowledge, the first records of *H. hammondi* infection in squirrels [72]. In its other intermediate host species, *H. hammondi* is typically not virulent [73]. Two of these five squirrels, including the one in which the parasite was only detected in the lung, were thought to have died of trauma, while hepatitis and pneumonia were thought to have been the COD in two of the remaining animals and sepsis in the other one. Because *H. hammondi* DNA was only observed in the heart samples of four of these animals, and in the lung in the remaining one, it is very unlikely that *H. hammondi* infection contributed to their deaths.

Inflammation of multiple organs was the leading COD in *T. gondii*-positive animals (49.0%), followed by trauma (22.4%), and pneumonia (12.2%). In contrast, in *T. gondii*-negative animals, the leading COD was trauma (51.2%), followed by pneumonia (9.3%), and inflammation of multiple organs (3.9%). Pathological examination of the *T. gondii*-positive animals predominantly revealed pneumonia and hepatitis, which are signs of disseminated/acute toxoplasmosis [74]. Although lesions in the liver and lungs of red squirrels infected with *T. gondii* have been reported [24, 75, 76], in the present work conclusions must be drawn with care due to the design of the study. Only red squirrels that were found dead or that had died after being rescued and taken to wildlife rescue centres were examined. Thus, although *T. gondii* infection appears to have played a role in acute mortality in the squirrels, we cannot draw any firm conclusions on its lethality as we do not know how many squirrels survived infection with this parasite. Our results do, however, suggest that red squirrels are highly susceptible to infection with *T. gondii* type II. Therefore, it would be interesting to further investigate whether squirrel mortality due to *T. gondii* infection is indicative of a high environmental load of the parasite, as this could provide valuable insights into *T. gondii*-contaminated areas and consequently information of relevance to public health.

IHC appeared to be less specific than quantitative real-time qPCR for the detection of *T. gondii*. Only 15 of the 43 *T. gondii*-positive infections shown by IHC were confirmed by quantitative real-time qPCR. Additionally, one squirrel that was *T. gondii*-positive according to IHC but negative according to the results of quantitative real-time qPCR tested positive by qPCR for *H. hammondi*, which suggests that the parasite was misidentified by IHC [77]. As the morphology and antigenic composition of *T. gondii* and *H. hammondi* are similar [78], molecular tools are required for their differentiation [79]. The sensitivity of both IHC and quantitative real-time qPCR are lower when the *T. gondii* burden is low [80]. Both methods perform better in cases of disseminated/acute toxoplasmosis than in cases of chronic infection with unevenly distributed tissue cysts. Of the 49 squirrels testing positive for *T. gondii* by qPCR, 35 tested positive for three organs, five tested positive for two organs, and nine for one organ only. In *T. gondii*-negative individuals or organs, cysts may have been present at concentrations below the limit of detection [81]. Sampling multiple organs of an individual animal reduces the chance of false negatives. Due to the retrospective character of this study, it was not possible to use tissue cyst-specific antibodies to re-examine tissue sections for *T. gondii*.

The surprisingly high concentration of *T. gondii* DNA in most of the squirrels, which indicated a high infection load, undoubtedly contributed to the high success rate of the MS typing. The success rate was, to the best of our knowledge, exceptionally high for wildlife specimens [28]. The MS typing indicated the presence of *T. gondii* type II in the squirrels, which is the prevailing *T. gondii* lineage in both wild and domestic animals in Europe [82, 83]. Only *T. gondii* type II alleles were detected in studies undertaken in the Netherlands that examined samples from sheep [84] and squirrels [22], though the results were based on sequencing of the *GRA6* gene only. The two *T. gondii* type II clusters observed in the present study for squirrels from the Netherlands are in agreement with those identified for Europe (EurClusters 1-3) [28] and thus are not thought to represent specific genotypic entities.

Cases of *Toxoplasma gondii* were identified year-round, but both the number of reported dead squirrels and *T. gondii* infection rates peaked in autumn. A study undertaken in a neighbouring country [85] revealed that the proportion of domestic cats shedding *T. gondii* oocysts varied over the year. The reasons for this pattern were not entirely clear, but the seasonality of oocysts shedding could be modelled using climatic data [85]. Interestingly, the pattern of *T. gondii* oocysts shedding by felines in the earlier study [85] appeared to align with the increased rates of *T. gondii* infections in squirrels in autumn observed in this study. Moderate temperatures and high levels of humidity can promote the survival and sporulation of oocysts in the environment [86, 87]. In the present study, *T. gondii* infection was positively

associated with heavy rainfall over the 12 months preceding sampling and negatively associated with hot summer days during the 2-week period preceding sampling. Our results also indicated that *T. gondii* infection was negatively associated with the percentage of deciduous forest in a 3-km-diameter buffer zone arround the site of sampling; however, this particular association is difficult to interpret. Deciduous forest cover was correlated with lower human density and possibly fewer cats; however, the effect of this association may be partly accounted for by inclusion of distance to urban areas in the final multivariable model. It can also be hypothesized that squirrels have different foraging behaviour in deciduous forest than in other habitats, which may influence their risk of exposure to *T. gondii*. Other parameters, including most categories of land use, the presence of domestic cats, and the availability of food, were not found to be significantly associated with *T. gondii* infection. This may have be due to the number of samples examined in this study, which was relatively low for modelling purposes. Also, cat density data were only available at the regional level, which may not have always reflected cat density at the locations where the squirrels were found. Data on human density and distance to urban area were available at a higher resolution and might therefore have been better proxies for cat density.

As information on toxoplasmosis in red squirrels is limited, it is impossible to know whether the situation determined in the present study is comparable to those of neighbouring countries with similar habitats and climatic conditions. In comparison to available data for the rest of Europe, the infection rates of *T. gondii* determined for Eurasian red squirrels in the present study, and especially for 2014 (52.9%), were relatively high. For instance, infection rates were notably lower in squirrels in Jersey [2.1% (7/337)] [75] and Finland [16% (3/19)] [18]. The discrepancies between these results may indicate that the high proportion of infected squirrels observed in 2014 was an exception. In sum, we are unable to provide a comprehensive explanation for the sudden upsurge in the number of *T. gondii*-related deaths in 2014 and its subsequent decline based on the predictor variables determined in the present study.

Conclusions

Toxoplasma gondii type II was observed in 27.5% of the Eurasian red squirrels examined. Most of the infected squirrels had pneumonia and hepatitis. MS typing revealed *T. gondii* type II genotypes similar to those previously reported for Europe. Factors associated with oocyst survival rather than squirrel feeding behaviour were found to be putative risk factors of infection, which suggests that the death of squirrels due to infection with *T. gondii* is likely indicative of a high environmental load of infectious oocysts. The findings of this study contribute to our understanding of the epidemiology of *T. gondii*, and indicate the importance of this parasite as a cause of death in Eurasian red squirrels in the Netherlands.

Abbreviations

APC	Affinity propagation clustering
BBG	Functional land use map
CI	Confidence interval
COD	Cause of death
DWHC	Dutch Wildlife Health Centre
EGFP	Enhanced green fluorescent protein
FD	Freezing days, minimum temperature ≤ − 6 ℃
GLMM	Generalized linear mixed model
IHC	Immunohistochemical analyses
IQR	Interquartile range
LPI	Living Planet Index
MLG	Multi-locus genotype
MS	Microsatellite
MSN	Minimum spanning network
qPCR	Quantitative real-time PCR
R25mm	Heavy rainfall days, mean precipitation ≥ 25 mm
SU25	Summer days, maximum temperature > 25 ℃

Supplementary Information

The online version contains supplementary material, which is available at https://doi.org/10.1186/s13071-023-06068-6.

Additional file 1: Table S1. Sequences of primers, probes, and final concentrations used in the quantitative real-time qPCR. **Table S2.** Description, spatial resolution and source of explanatory variables included in the analysis to assess risk factors for *Toxoplasma gondii* quantitative real-time qPCR positivity in squirrels. **Table S3.** Geographical origin of *Toxoplasma gondii* quantitative real-time qPCR-positive Eurasian red squirrels; proportion per region and per province. **Table S4**. *Hammondia hammondi* quantitative real-time qPCR-positive samples per year, month, and province. **Table S5**. Grouped and detailed causes of death implicated by pathological examination compared to quantitative real-time qPCR results for *Toxoplasma gondii*. **Table S6.** Results of IHC and quantitative real-time qPCR for *Toxoplasma gondii* and *Hammondia hammondi*. **Table S7.** Samples positive for *Toxoplasma gondii* by IHC versus those positive by IHC but negative by quantitative real-time qPCR, per year. **Table S8.** Results of the univariable mixed logistic regression used to assess risk factors for *Toxoplasma gondii* quantitative real-time qPCR positivity in squirrels ($n = 175$ squirrels). **Table S9.** Assessment of multicollinearity for variables included in the final model, as determined by variance inflation factor.

Additional file 2: Figure S1. Living Planet Index (LPI) per region (north sandy region, central sandy region, south sandy region, and other regions of the Netherlands). **Figure S2**. Correlation matrix between predictor variables selected by univariable analysis to assess risk factors for *Toxoplasma gondii* quantitative real-time qPCR positivity in squirrels. **Figure S3.** Results of a *Toxoplasma gondii*-specific quantitative real-time qPCR for lung, liver, and heart samples. The Wilcoxon rank sum test was restricted to data on squirrels in which *T. gondii* qPCR was positive for all of the three organs ($n = 35$). Cq values of heart samples were significantly higher than those of the liver and lung, and those of the lung were significantly higher than those of the liver (Wilcoxon rank sum test, $P < 0.001$). **Figure S4.** Fig. S4A: Number of *Toxoplasma gondii*- and *Hammondia hammondi*-positive samples per month. Fig. S4B: Proportion of *Toxoplasma gondii*- and *Hammondia hammondi*-positive samples per month. Samples were grouped into the following categories: *T. gondii* positive (*Tgo*), *H. hammondi* positive (*Hha*), *T. gondii* and *H. hammondi* positive (*Tgo&Hha*), negative in qPCR (*Neg*). **Figure S5.** Fig. S5A: Number of *Toxoplasma gondii*- and *Hammondia hammondi*-positive samples per year. Fig. S5B: Proportion of *Toxoplasma gondii*- and *Hammondia hammondi*-positive samples per year. Samples

were grouped into the following categories: Tgo, Hha, Tgo&Hha, Neg.
Figure S6. Statistically significant correlation ($P < 0.001$) of Cq values obtained for different organs of squirrels by *Toxoplasma gondii* quantitative real-time qPCR, i.e. liver vs. lung [residual SE (RSE), 2.432, *df* 33, multiple R^2 0.5753, adjusted R^2 0.5624, F 44.69, $P < 0.001$; Fig. S6A], heart vs. lung (RSE, 1.477, *df* 33, multiple R^2 0.7469, adjusted R^2 0.7392, F 97.36, $P < 0.001$; Fig. S6B), and heart vs. liver (RSE, 2.109, *df* 33, multiple R^2 0.4837, adjusted R^2 0.468, F 30.91, $P < 0.001$; Fig. S6C). Adjusted R^2 values were obtained by linear regression (regression line and 95% CIs are displayed).

Additional file 3: Dataset S1. Population genetic analyses of *Toxoplasma gondii* MS typing data.

Additional file 4: Dataset S2. Squirrels in the Netherlands.

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Availability of data and materials
The data used in this study are available from the Additional files.

Declarations

Ethics approval and consent to participate
The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. No ethical approval was required for this study as the Eurasian red squirrels included in it were dead on arrival at the DWHC and none of them were euthanized for the purposes of this study.

Consent for publication
All of the authors have given their consent for the publication of this article.

Competing interests
The authors declare that they have no competing interests. This is an original research article that is not under review for publication in another journal.

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References

1. Tenter AM, Heckeroth AR, Weiss LM. *Toxoplasma gondii*: from animals to humans. Int J Parasitol. 2000;30:1217–58.
2. Dubey J. *Toxoplasma gondii* oocyst survival under defined temperatures. J Parasitol. 1998;84:862–5.
3. Frenkel J, Dubey J, Miller NL. *Toxoplasma gondii*: fecal forms separated from eggs of the nematode *Toxocara cati*. Science. 1969;164:432–3.
4. Hutchison W, Dunachie J, Siim JC, Work K. Life cycle of *Toxoplasma gondii*. BMJ. 1969;4:806.
5. Scherrer P, et al. Exploring the epidemiological role of the Eurasian lynx (*Lynx lynx*) in the life cycle of *Toxoplasma gondii*. Int J Parasitol Parasites Wildl. 2023;21:1–10.
6. Fritz H, et al. Methods to produce and safely work with large numbers of *Toxoplasma gondii* oocysts and bradyzoite cysts. J Microbiol Methods. 2012;88:47–52.
7. Dubey JP. Toxoplasmosis of animals and humans. Boca Raton: CRC Press; 2021.
8. Stensgaard A, et al. Sero-prevalence and risk factors of *Toxoplasma gondii* infection in wild cervids in Denmark. Int J Parasitol Parasites Wildl. 2022;17:288–94.
9. Boyer K, et al. Unrecognized ingestion of *Toxoplasma gondii* oocysts leads to congenital toxoplasmosis and causes epidemics in North America. Clin Infect Dis. 2011;53:1081–9.
10. Dubey JP. The history of *Toxoplasma gondii*—the first 100 years. J Eukaryot Microbiol. 2008;55:467–75.
11. Stelzer S, et al. *Toxoplasma gondii* infection and toxoplasmosis in farm animals: risk factors and economic impact. Food Waterborne Parasitol. 2019;15:e00037.
12. Dubey J, et al. Economic and public health importance of *Toxoplasma gondii* infections in sheep: 2009–2020. Vet Parasitol. 2020;286:109195.
13. Dubey JP, Murata F, Cerqueira-Cézar C, Kwok O. Public health and economic importance of *Toxoplasma gondii* infections in goats: the last decade. Res Vet Sci. 2020;132:292–307.
14. Opsteegh M, Maas M, Schares G, van der Giessen J. 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 Rep EFSA Support Publ. 2016;13:996E.
15. Flegr J, Prandota J, Sovičková M, Israili ZH. Toxoplasmosis–a global threat. Correlation of latent toxoplasmosis with specific disease burden in a set of 88 countries. PLoS ONE. 2014;9:e90203.
16. Kapperud G, et al. Risk factors for *Toxoplasma gondii* infection in pregnancy: results of a prospective case-control study in Norway. Am J Epidemiol. 1996;144:405–12.
17. Dubey J, et al. Congenital toxoplasmosis in humans: an update of worldwide rate of congenital infections. Parasitology. 2021;148:1406–16.
18. Jokelainen P, Nylund M. Acute fatal toxoplasmosis in three Eurasian red squirrels (*Sciurus vulgaris*) caused by genotype II of *Toxoplasma gondii*. J Wildl Dis. 2012;48:454–7.
19. Howe L, Hunter S, Burrows E, Roe W. Four cases of fatal toxoplasmosis in three species of endemic New Zealand birds. Avian Dis. 2014;58:171–5.
20. Work TM, et al. Fatal toxoplasmosis in free-ranging endangered 'alala from Hawaii. J Wildl Dis. 2000;36:205–12.
21. Szabo K, et al. Fatal toxoplasmosis in a bald eagle (*Haliaeetus leucocephalus*). J Parasitol. 2004;90:907–8.
22. Kik M, et al. *Toxoplasma gondii* in wild red squirrels, the Netherlands, 2014. Emerg Infect Dis. 2015;21:2248.
23. Duff J, Higgins R, Sainsbury A, Macgregor S. Zoonotic infections in red squirrels. Vet Rec. 2001;148:123–4.
24. Simpson VR, et al. Causes of mortality and pathological lesions observed post-mortem in red squirrels (*Sciurus vulgaris*) in Great Britain. BMC Vet Res. 2013;9:1–14.
25. Dubey J, et al. Epidemiological significance of *Toxoplasma gondii* infections in wild rodents: 2009–2020. J Parasitol. 2021;107:182–204.

26. DWHC. Red squirrels dying from toxoplasmosis. 2015. https://dwhc.nl/en/red-squirrels-dying-from-toxoplasmosis/. Accessed 16 Mar 2023.
27. Rodhain J. La grande réceptivité des sciuridés aux toxoplasmes. Compte rendu des seances de la Societe de biologie. 1950;144:717–9
28. Fernández-Escobar M, et al. *Toxoplasma gondii* genotyping: a closer look into Europe. Front Cell Infect Microbiol. 2022;12:305.
29. Chantrey J, et al. European red squirrel population dynamics driven by squirrelpox at a gray squirrel invasion interface. Ecol Evol. 2014;4:3788–99.
30. Talabani H, et al. Contributions of immunoblotting, real-time PCR, and the Goldmann-Witmer coefficient to diagnosis of atypical toxoplasmic retinochoroiditis. J Clin Microbiol. 2009;47:2131–5.
31. Legnani S, et al. Emergence of cutaneous neosporosis in a dog receiving immunosuppressive therapy: molecular identification and management. Vet Dermatol. 2016;27:49-e14.
32. Schares G, et al. A real-time quantitative polymerase chain reaction for the specific detection of *Hammondia hammondi* and its differentiation from *Toxoplasma gondii*. Parasit Vectors. 2021;14:1–11.
33. Grochow T, et al. Establishment and validation of a guinea pig model for human congenital toxoplasmosis. Parasit Vectors. 2021;14:1–18.
34. Hoffmann B, Depner K, Schirrmeier H, Beer M. A universal heterologous internal control system for duplex real-time RT-PCR assays used in a detection system for pestiviruses. J Virol Methods. 2006;136:200–9.
35. Schares G, et al. Sensitive, quantitative detection of *Besnoitia darlingi* and related parasites in intermediate hosts and to assess felids as definitive hosts for known and as-yet undescribed related parasite species. Int J Parasitol Parasites Wildl. 2020;11:114–9.
36. Joeres M, et al. A ring trial to harmonize *Toxoplasma gondii* microsatellite typing: comparative analysis of results and recommendations for optimization. Eur J Clin Microbiol Infect Dis. 2023;42:1–16.
37. Ajzenberg D, et al. Genotyping of *Toxoplasma gondii* isolates with 15 microsatellite markers in a single multiplex PCR assay. J Clin Microbiol. 2010;48:4641–5.
38. Jombart T, Ahmed I. Adegenet 1.3–1: new tools for the analysis of genome-wide SNP data. Bioinformatics. 2011;27:3070–1.
39. Kamvar ZN, Tabima JF, Grünwald NJ. 2014. Poppr: an R package for genetic analysis of populations with clonal, partially clonal, and/or sexual reproduction. PeerJ 2:e281.
40. Bruvo R, Michiels N, D'Souza T, Schulenburg H. A simple method for the calculation of microsatellite genotype distances irrespective of ploidy level. Mol Ecol. 2004;13:2101–6.
41. ISO/IEC TS 18661–1:2014. Information technology—programming languages, their environments, and system software interfaces. Floating-point extensions for C. Part 1. Binary floating-point arithmetic. https://www.iso.org/standard/63146.html. Accessed 5 July 2023.
42. Goldberg D. What every computer scientist should know about floating-point arithmetic. ACM Comput Surv (CSUR). 1991;23:5–48.
43. Hopkins B, Skellam JG. A new method for determining the type of distribution of plant individuals. Ann Bot. 1954;18:213–27.
44. Kassambara A, Mundt F. 2020. Factoextra: Extract and Visualize the Results of Multivariate Data Analyses. R Package Version 1.0.7. https://CRAN.R-project.org/package=factoextra. Accessed 5 July 2023.
45. Fischer S, et al. Defining objective clusters for rabies virus sequences using affinity propagation clustering. PLoS Negl Trop Dis. 2018;12:e0006182.
46. National Databank of Flora and Fauna. *Felis silvestris* Schreber, 1777. 2023. https://www.verspreidingsatlas.nl/8496095#. Accessed 20 June 2023.
47. Beekhof B. Aantallen katten. 2023, I&O research. https://www.ioresearch.nl/. Accessed 27 June 2023.
48. Borst N, et al. Feiten & Cijfers Gezelschapsdierensector. HAS Kennistransfer, Hogeschool HAS Den Bosch. 2011. https://edepot.wur.nl/186568. Accessed 28 June 2023.
49. CBS, Bestand Bodemgebruik 2017 (BBG2017). 2017. https://nationaalgeoregister.nl/geonetwork/srv/dut/catalog.search#/metadata/d9bf1ce1-5197-409e-b19e-340e50afafc4?tab=general. Accessed 20 June 2023.
50. CBS. Kaart van 500 meter bij 500 meter met statistieken. 2020. https://www.cbs.nl/nl-nl/dossier/nederland-regionaal/geografische-data/kaart-van-500-meter-bij-500-meter-met-statistieken. Accessed 20 June 2023.
51. KVK. Overzicht standaard bedrijfsindeling (SBI-codes) voor activiteiten. 2023. https://www.kvk.nl/over-het-handelsregister/overzicht-standaard-bedrijfsindeling-sbi-codes-voor-activiteiten/. Accessed 14 Feb 2023.
52. Afonso E, et al. Environmental determinants of spatial and temporal variations in the transmission of *Toxoplasma gondii* in its definitive hosts. Int J Parasitol Parasites Wildl. 2013;2:278–85.
53. Miller M, et al. Coastal freshwater runoff is a risk factor for *Toxoplasma gondii* infection of southern sea otters (Enhydra lutris nereis). Int J Parasitol. 2002;32:997–1006.
54. Climdex. Indices. 2013. https://www.climdex.org/learn/indices/. Accessed 24 Feb 2023.
55. Frenkel J, Ruiz A, Chinchilla M. Soil survival of *Toxoplasma* oocysts in Kansas and Costa Rica. Am J Trop Med Hyg. 1975;24:439–43.
56. Patz JA, Graczyk TK, Geller N, Vittor AY. Effects of environmental change on emerging parasitic diseases. Int J Parasitol. 2000;30:1395–405.
57. Bergler K, Erber M, Boch J. Untersuchungen zur Überlebensfähigkeit von Sporozysten bzw. Oozysten von *Sarcocystis*, *Toxoplasma*, *Hammondia* und *Eimeria* unter Labor- und Freilandbedingungen. Berl Münch Tierärztl Wochenschr. 1980;93:288–93.
58. Afonso E, Thulliez P, Gilot-Fromont E. Local meteorological conditions, dynamics of seroconversion to *Toxoplasma gondii* in cats (*Felis catus*) and oocyst burden in a rural environment. Epidemiol Infect. 2010;138:1105–13.
59. Beral M, et al. Environmental factors associated with the seroprevalence of *Toxoplasma gondii* in wild boars (*Sus scrofa*). France EcoHealth. 2012;9:303–9.
60. Lindsay D, Blagburn B, Dubey J. Survival of nonsporulated *Toxoplasma gondii* oocysts under refrigerator conditions. Vet Parasitol. 2002;103:309–13.
61. Frenkel J, Dubey J. Effects of freezing on the viability of *Toxoplasma* oocysts. J Parasitol. 1973;59:587–8.
62. Dumètre A, Dardé M-L. How to detect *Toxoplasma gondii* oocysts in environmental samples? FEMS Microbiol Rev. 2003;27:651–61.
63. Krauze-Gryz, Gryz J. *A* review of the diet of the red squirrel (*Sciurus vulgaris*) in different types of habitats. Red squirrels: ecology, conservation and management in Europe. European Squirrel Initiative, Stoneleigh Park, Warwickshire CV8 2LG, England, 2015: p. 39–50.
64. Vereniging Wildbeheer Veluwe. Jaarrapportage grofwild 2020–2021. Vereniging Wildbeheer Veluwe. 2021. p. 1–76. https://www.vereniging wildbeheerveluwe.nl/Portals/24/Downloads/Jaarverslagen/NBRF2021%20V1.0.pdf?ver=2021-09-02-173610-487&timestamp=1630597270472. Accessed 20 June 2023.
65. Van Strien AJ, et al. Modest recovery of biodiversity in a western European country: the Living Planet Index for the Netherlands. Biol Cons. 2016;200:44–50.
66. Soldaat LL, et al. A Monte Carlo method to account for sampling error in multi-species indicators. Ecol Ind. 2017;81:340–7.
67. Gotteland C, et al. Toxoplasmosis in natural populations of ungulates in France: prevalence and spatiotemporal variations. Vector-Borne Zoonotic Dis. 2014;14:403–13.
68. Kuhn M, Silge J. Tidy modeling with R. Sebastopol: O'Reilly Media Inc; 2022.
69. Kassambara A. 2023. Package ggcorrplot. R Package Vers 0.1.4.1. https://cran.r-project.org/web/packages/ggcorrplot/index.html. Accessed 22 Mar 2023.
70. Lüdecke D, Ben-Shachar MS, Patil I, Waggoner P, Makowski D. performance: An R package for assessment, comparison, and testing of statistical models. J Open Sour Softw. 2021;6(3139).
71. Ripley B, et al. Package MASS. CRAN R. 2013;538:113–20.
72. Dubey J, Sreekumar C. Redescription of *Hammondia hammondi* and its differentiation from *Toxoplasma gondii*. Int J Parasitol. 2003;33:1437–53.
73. Wong ZS, et al. Head-to-head comparisons of *Toxoplasma gondii* and its near relative *Hammondia hammondi* reveal dramatic differences in the host response and effectors with species-specific functions. PLoS Pathog. 2020;16:e1008528.
74. Brown C, Baker D, Barker I. Alimentary system. Maxie, MG, Ed, Jubb, Kennedy & Palmer's Pathology of Domestic Animals. 5th Edition. Volume 2. Saunders Elsevier, Edinburgh; 2007. p. 1–296.
75. Blackett TA, et al. Mortalities, amyloidosis and other diseases in free-living red squirrels (*Sciurus vulgaris*) on Jersey, Channel Islands. Veterinary Record. 2018;183:503–503.

76. Fayyad A, et al. Fatal systemic *Toxoplasma gondii* infection in a red squirrel (*Sciurus vulgaris*), a Swinhoe's striped squirrel (*Tamiops swinhoei*) and a New World porcupine (*Erethizontidae* sp.). J Comp Pathol. 2016;154:263–7.
77. Gondim LF, Mineo JR, Schares G. Importance of serological cross-reactivity among *Toxoplasma gondii*, *Hammondia* spp., *Neospora* spp. *Sarcocystis* spp. and *Besnoitia besnoiti*. Parasitology. 2017;144:851–68.
78. Riahi H, Bouteille B, Darde M-L. Antigenic similarity between *Hammondia hammondi* and *Toxoplasma gondii* tachyzoites. J Parasitol. 1998;84:651–3.
79. Schares G, et al. Occurrence of *Toxoplasma gondii* and *Hammondia hammondi* oocysts in the faeces of cats from Germany and other European countries. Vet Parasitol. 2008;152:34–45.
80. Casartelli-Alves L, et al. Sensitivity and specificity of serological tests, histopathology and immunohistochemistry for detection of *Toxoplasma gondii* infection in domestic chickens. Vet Parasitol. 2014;204:346–51.
81. Kinnunen RP, Schmidt C, Hernández-Ortiz A, Garroway CJ. No evidence of *Toxoplasma gondii* infection in urban and rural squirrels. Biorxiv. 2021. https://doi.org/10.1101/2020.09.28.313866.
82. Khan A, et al. Recent transcontinental sweep of *Toxoplasma gondii* driven by a single monomorphic chromosome. Proc Natl Acad Sci. 2007;104:14872–7.
83. Lorenzi H, et al. Local admixture of amplified and diversified secreted pathogenesis determinants shapes mosaic *Toxoplasma gondii* genomes. Nat Commun. 2016;7:10147.
84. Opsteegh M, et al. Direct detection and genotyping of *Toxoplasma gondii* in meat samples using magnetic capture and PCR. Int J Food Microbiol. 2010;139:193–201.
85. Schares G, et al. Seasonality in the proportions of domestic cats shedding *Toxoplasma gondii* or *Hammondia hammondi* oocysts is associated with climatic factors. Int J Parasitol. 2016;46:263–73.
86. Smith DD, Frenkel J. Prevalence of antibodies to *Toxoplasma gondii* in wild mammals of Missouri and east central Kansas: biologic and ecologic considerations of transmission. J Wildl Dis. 1995;31:15–21.
87. Dubey JP, Beattie C. Toxoplasmosis of animals and man. Boca Raton: CRC Press Inc; 1988.

Publisher's Note

4 Discussion

T. gondii has a complex population structure (Galal et al. 2019, Lorenzi et al. 2016, Shwab et al. 2014). High resolution genotyping is extremely instrumental to analyze and understand the population structure of this parasite. The relevant methods can generally be used to determine the worldwide distribution and frequency of different genotypes (Shwab et al. 2014, Su et al. 2012). Genotyping makes it also possible to notice the entry of particular genotypes into areas, in which they were previously rare or absent. Moreover, genotyping and especially fingerprinting serve to improve our understanding of the transmission routes of *T. gondii* and to identify infection sources in outbreaks.

The present work focused on genotyping of *T. gondii* in Europe. First, the available information on *T. gondii* genotypes circulating in Europe and the methods used for typing were summarized in a review (Fernández-Escobar et al. 2022). It was observed that there is a lack of comparability between the genotyping methods used, such as the MS method, which is the current reference standard for fingerprinting (Fernández-Escobar et al. 2022). Therefore, as a second objective of this work, a ring trial was performed to compare and finally harmonize the application and results of MS typing in different laboratories (Joeres et al. 2023a). Furthermore, the review included in this work (Fernández-Escobar et al. 2022) confirmed the predominance of *T. gondii* type II in Europe, but a summary of MS genotyping results also showed genetic variability among European type II. To further explore these differences, a third objective was to develop an NGS-based MLST method for genotyping of *T. gondii* and particularly for fingerprinting of type II (Joeres et al. 2023b), since a high typing resolution has been described for MLST (Su et al. 2010). The MS typing method, after harmonization based on the ring trial results (Joeres et al. 2023a), was used as a reference to validate the new method, i.e. NGS-based MLST. Finally, as a fourth objective, the harmonized MS genotyping method was applied in a local Dutch study (Wijburg et al. 2024), allowing to put the genotyping results of this study into the European context. The application of the new Ion AmpliSeq method would not have made it possible to place the results in a larger European context due to the limited availability of AmpliSeq typing data from Europe and other parts of the world.

PCR-based methods for genotyping of *T. gondii* have been used for about 30 years (Howe and Sibley 1995, Dardé et al. 1992, Sibley and Boothroyd 1992). Initially, however, only single or a few loci were used for typing, limiting the ability to explore genetic diversity. Then, about 15-20 years ago, multilocus PCR-RFLP typing (Su et al. 2006) and multilocus MS typing (Ajzenberg et al. 2010) of *T. gondii* were developed. This PCR-RFLP typing method can reliably distinguish different clonal lineage genotypes (i.e. type I, type II, type III) and in principle

also detect recombination between clonal lineages, but it is not able to detect intra-lineage genotype variability. In contrast, the MS method is suitable for fingerprinting and therefore serves as the current reference standard for this purpose (Ajzenberg et al. 2010). In addition to these two methods, MLST was used as a PCR-based typing method (Su et al. 2010).

The review included in this work (Fernández-Escobar et al. 2022) summarized the information of European studies that used one or more of these three PCR-based methods for genotyping *T. gondii* isolates. It was noted that, particularly with PCR-RFLP typing, often only a partial set of the available markers was used for typing. When the MS method was applied, all 15 available markers were more often used, but still there was no information on the comparability of results from different laboratories at that time (Fernández-Escobar et al. 2022). It was therefore concluded that, on the one hand, the genetic diversity in Europe may not have been fully recorded so far. On the other hand, a lack of consistency in the application of methods for genotyping of *T. gondii*, e.g. in the number of markers used or in the interpretation of the results in the European studies, was observed (Fernández-Escobar et al. 2022). In addition, some European countries were overrepresented in the studies, while there was no or limited information on other parts of Europe (Fernández-Escobar et al. 2022).

These findings suggested the urgent need for a ring trial to harmonize the MS typing of *T. gondii*, which was organized and carried out as part of this thesis (Joeres et al. 2023a). The aim of the ring trial was to identify differences between the analyzing laboratories and to develop standardized guidelines for the application of MS typing in order to harmonize the genotyping of *T. gondii* as much as possible. During the study, some differences in the performance of the MS method and in the evaluation of the data were found (Joeres et al. 2023a). For example, the participating laboratories used different fluorophores to label the primers, different capillary sequencers, and different software to analyze the data. The use of different fluorophores caused the most significant differences in the results (Joeres et al. 2023a). Therefore, specific experiments were performed with primers labeled with different fluorophores to assess the resulting differences in fragment lengths numerically.

Guidelines for MS typing were established based on the results of the ring trial and published as supplementary data of the respective publication to avoid or reduce differences in MS typing results among laboratories in the future (Joeres et al. 2023a). Thus, with the help of these guidelines, data generated with fluorophores that deviate from the original protocol (Ajzenberg et al. 2010) can be numerically corrected to be consistent with literature data. Furthermore, the guidelines provide detailed information on how to perform MS typing in a standardized way

and point out potential difficulties (Joeres et al. 2023a). This may help to compare *T. gondii* genotyping results obtained in different laboratories if they apply the guidelines.

One issue that cannot be solved with the guidelines is the use of different analysis software, which in some cases makes a standardized analysis of the data difficult. Therefore, in addition to the guidelines, *T. gondii* reference DNAs will be provided by the participants of the ring trial to other laboratories on request. The MS profiles of these reference DNAs are well known and if they are used as positive controls for MS typing, the results can be standardized more easily.

Overall, the publication of the ring trial results together with the guidelines provides a good base for laboratories intending to use the MS method for genotyping of *T. gondii*. Due to the relatively low costs of the method, more European laboratories may perform MS typing in the future. This could help to fill the gaps identified in the review paper (Fernández-Escobar et al. 2022), i.e. countries, where no or only few samples were MS typed in the past. In addition, it may become possible to combine datasets from different European studies, if MS typing has been performed homogeneously or the results have been numerically corrected.

The MS method has been further optimized as the current reference standard for fingerprinting of *T. gondii* based on the interlaboratory comparison (Joeres et al. 2023a). This made it possible to use it as a reference method for the new NGS-based typing method (Joeres et al. 2023b), which was established within this work. Fingerprinting by MS typing already revealed that there is genetic variability within *T. gondii* type II (Fernández-Escobar et al. 2022, Galal et al. 2022, Shwab et al. 2018). To explore this further, a new NGS-based typing method with high discriminatory power among European *T. gondii* type II samples (Joeres et al. 2023b) was developed as part of this thesis. This new Ion AmpliSeq method can discriminate the same number of *T. gondii* type II samples as the MS method and the sensitivity is comparable to that of the MS method.

Currently, the low costs and easy application of the MS method is an advantage over the Ion AmpliSeq method. However, it is important to consider that performing NGS has become much less expensive in the last few years, and it could become even cheaper in the future due to its current broad application. Yet, the quality of data analysis depends on the software used and on the experience of the person performing the analysis, although generally speaking, the MS method seems to be easier to use. The Ion AmpliSeq method, on the other hand, can be standardized by using uniform criteria for analysis. This makes the data evaluation more objective than with MS typing.

Both, the MS method and the Ion AmpliSeq method, can discriminate different *T. gondii* genotypes. Within type II, the Ion AmpliSeq method was able to distinguish the same number of samples as the MS method, thus showing an equivalent typing resolution in fingerprinting for both methods. However, it has to be taken into consideration that samples with different MS typing profiles were preferably selected for Ion AmpliSeq typing in order to confirm the discriminatory power of the Ion AmpliSeq method. If, on the other hand, samples were specifically selected that showed the same profiles in the MS method, although they originated for example not from the same farm, the Ion AmpliSeq method might have a better discriminatory power than the MS method. Furthermore, the whole genome sequence analysis performed as a basis for this work identified a large number of highly polymorphic regions, of which only 18 were covered by the Ion AmpliSeq primer panel. Expanding the primer panel to cover more regions may also lead to a better discriminatory power of the Ion AmpliSeq method.

In summary, a new Ion AmpliSeq method was developed for the genotyping of *T. gondii* that is comparable to the MS method in terms of its discriminatory power and sensitivity (Joeres et al. 2023b). With future cost reductions of NGS, the advantage of objective data evaluation, and the possibility of expanding the Ion AmpliSeq primer panel, a broader application of the Ion AmpliSeq method is imaginable in the future. So far, however, MS typing is the reference method for fingerprinting, which was further optimized through the performed ring trial (Joeres et al. 2023a).

The MS method adapted to the published guidelines was used in a local study to genotype *T. gondii* DNA extracted from the tissue of red squirrels (*Sciurus vulgaris*) found dead in the Netherlands (Wijburg et al. 2024). The background of this study was the sudden increase in the number of squirrels found dead in 2014, which led to the retrospective examination of data and the tissue samples of squirrels that died in the Netherlands from 2014 to 2020. Objectives were to analyze the risk factors of the *T. gondii* infection in these squirrels, to evaluate the pathology and to characterize the *T. gondii* MS genotypes detected in the tissue of the squirrels. It was hypothesized that specific *T. gondii* genotypes circulating in the Netherlands might be responsible for the rise in squirrel toxoplasmosis; it was thus the aim to compare MS profiles observed in the Netherlands in squirrels with other MS genotypes observed in Europe.

Factors associated with oocyst survival were found to be potential risk factors of *T. gondii* infection (Wijburg et al. 2024). This suggests that squirrels dying as a result of *T. gondii* infection may be indicators of a high environmental load of infectious oocysts. Such a high load of oocysts harbors the risk of genetic recombination of different genotypes (Herrmann et al. 2010). Furthermore, different *T. gondii* genotypes have been described, which varied in their

virulence in laboratory mice (Sibley and Boothroyd 1992) or were associated with different degrees of clinical severity in humans (Blaizot et al. 2019, Demar et al. 2012, Gilbert et al. 2008). These facts were reasons to MS-type the *T. gondii*-positive DNA samples. The use of the harmonized MS method made it possible to include MS typing results from previous studies in a cluster analysis of the Dutch data. This example shows that in the future different local studies on MS typing of *T. gondii* in Europe can be combined to perform population and cluster analyses.

Within the 39 *T. gondii* DNA samples analyzed, MS typing identified 32 different *T. gondii* type II MS profiles and no recombination events between *T. gondii* type II and other clonal lineages were observed (Wijburg et al. 2024). Nevertheless, recombination among different *T. gondii* type II strains is possible and may have contributed to the large variety of MS typing profiles. A few samples showed identical profiles, although they were sampled in different years or originated from different regions. These samples would thus be well suitable for genotyping with the Ion AmpliSeq method, which might detect different Ion AmpliSeq profiles in samples with identical MS profiles. Due to the long survival time of oocysts in the environment (Dubey et al. 1970a), it is possible that *T. gondii* samples collected from intermediate hosts in different years but from the same region also have the same genotype, as they could originate from a single oocyst source.

As part of this work, nearly 250 European *T. gondii* DNAs from clinical samples of animals were fully typed using the MS method and the results were adjusted based on the developed guidelines (Joeres et al. 2023a). The *T. gondii* DNA samples extracted from red squirrel tissue described above are a subset of these samples, which was used for a local study (Wijburg et al. 2024). This study identified genetic clusters, but without a clear regional association. One possible explanation may be that with the 39 typed *T. gondii* DNA samples, consisting of 32 different MS profiles, there was not enough data available to detect potential regional differences. Moreover, the samples were collected over seven years, which may be an interval too long for such analyses given the relatively small number of typed samples. Yet, further regional studies with the collected data in the future, e.g. with oocyst samples from Germany, may reveal region associated clusters or help to understand their spatial distribution. In addition, all MS data collected for this work can be combined with the data available in the literature to get a better overview of the population structure in Europe. This may help to fill gaps that were described initially in the review paper about *T. gondii* genotyping in Europe (Fernández-Escobar et al. 2022).

Of the almost 250 *T. gondii* samples completely typed by the MS method, only 47 have also been typed using the Ion AmpliSeq method to date. In this case, further experiments can be performed in the future, to compare the Ion AmpliSeq method with the MS method more extensively or to test, whether the Ion AmpliSeq method is able to detect regional clusters within a larger data set.

To summarize, in the context of the present work, the MS method was further optimized and harmonized for genotyping of *T. gondii* (Joeres et al. 2023a). In addition, a new genotyping method for *T. gondii* was developed (Joeres et al. 2023b). This raises general questions, such as why genotyping of *T. gondii* is necessary and why its optimization is important.

T. gondii is a zoonotic parasite capable of infecting virtually all warm-blooded species (Dámek et al. 2023, Dubey 2022). One of the pathways of infection is through oocysts excreted by cats, which can contaminate soil, water, and fruits and vegetables (Dubey et al. 1970a). As a result, *T. gondii* extends its impact as a pathogen into the fields of human and veterinary medicine, and the environment, making it a good example to apply the One Health approach. Interdisciplinary collaboration in line with the One Health concept has become increasingly important in recent years.

At first glance, human infections with *T. gondii* are the main interest of people. The clinical picture of toxoplasmosis is diverse, often asymptomatic, or associated with nonspecific symptoms (Dubey 2022, Montoya and Liesenfeld 2004). However, a primary infection with *T. gondii* during pregnancy can lead to severe damage to the child, and toxoplasmosis can be lethal in immunocompromised individuals (Dubey 2022, Montoya and Liesenfeld 2004). Transmission of *T. gondii* between humans occurs extremely rarely, except for transplacental transmission, and only in specific cases, such as through a contaminated blood transfusion or organ transplantation (Montoya and Liesenfeld 2004). Therefore, animals are highly relevant as a source of a *T. gondii* infection for humans, both through the meat of infected intermediate hosts and through oocysts excreted by cats that can contaminate food or soil (Montoya and Liesenfeld 2004).

Veterinary medicine focuses on animal health. As in humans, the clinical picture of toxoplasmosis is diverse, ranging from asymptomatic courses to epidemic abortions (Dubey 2022). Certain species or immunocompromised animals can also die as the result of a *T. gondii* infection (Dubey 2022).

Diagnosing *T. gondii* infections does not necessarily require genotyping. Infection with this protozoon can be detected indirectly by testing for *T. gondii*-specific antibodies or directly by

demonstrating *T. gondii* DNA in host tissues (Weiss and Dubey 2009, Grover et al. 1990). Genotyping, however, becomes an important tool, when the source of infection, for example during an abortion event in a livestock population, needs to be identified. Finding the same *T. gondii* genotype in all samples may suggest that the outbreak has a single source, while detecting different genotypes could imply multiple sources of infection. Genotyping can also be used to identify potential sources of infection. If there is a *T. gondii* outbreak in a livestock population, for instance, small mammals from the surrounding area or fecal samples from cats living on the farm could be tested for *T. gondii*. In this case, a matching genotype may indicate a link between the outbreak and particular intermediate or definitive hosts.

In this work, the focus was on the genotyping of European *T. gondii* samples. In Europe, *T. gondii* has a clonal population structure, with the clonal lineage type II being predominant (Fernández-Escobar et al. 2022, Shwab et al. 2014). However, through fingerprinting it is possible to distinguish different genotypes within *T. gondii* type II. Previous studies have shown that genetic variation within type II lineage exists (Galal et al. 2022, Bertranpetit et al. 2017). Detecting intra-lineage genotype variability, as described above, provides the opportunity to investigate outbreaks in more detail, analyze transmission pathways and identify potential sources of infection.

Moreover, there is the possibility to perform cluster analyses and investigate associations between geographical and genetic distances to gain a more precise understanding of the population structure of *T. gondii* in Europe (Galal et al. 2022, Fischer et al. 2018, Lorenzi et al. 2016, Su et al. 2012). As part of this work, a cluster analysis based on MS data from European *T. gondii* samples was conducted, but the identified clusters did not reveal a clear regional association (Wijburg et al. 2024). Further cluster analyses should be included in future European studies on genotyping of *T. gondii* to investigate possible temporal and spatial associations in more detail. In this context, new datasets could be examined, and existing and new genotyping results could be integrated into large datasets, thus increasing the statistical power of cluster analysis. Furthermore, cluster analyses should not only be conducted based on MS data. It is possible to use also results from MLST or, if available, whole genome sequences.

Another reason to perform genotyping of *T. gondii* is the fact that different genotypes can cause clinical disease of varying severity (Gilbert et al. 2008, Carme et al. 2002, Grigg et al. 2001). The predominant *T. gondii* lineage type II in Europe is described to be of intermediate virulence for laboratory mice (Sibley and Boothroyd 1992), and it is believed that there has likely been an adaptation between the hosts and *T. gondii* type II in Europe during evolution (Shwab et al.

2018). In South America, however, the population structure of *T. gondii* is highly diverse, and a higher incidence of severe toxoplasmosis is reported in this context (Gilbert et al. 2008, Carme et al. 2002). For example, a Brazilian study described a larger number of cases and more severe courses of ocular toxoplasmosis as a result of congenital toxoplasmosis compared to Europe (Gilbert et al., 2008). Furthermore, in French Guiana, a high number of non-archetypal *T. gondii* strains were observed in association with severe cases of toxoplasmosis (Blaizot et al., 2019; Demar et al., 2012; Carme et al., 2002).

In our globalized world, non-archetypal *T. gondii* genotypes can be introduced into Europe through the import of animals (Galal et al. 2019, Bertranpetit et al. 2017). Moreover, humans can become infected with *T. gondii* while traveling and take the parasite to their country of origin when they return (Seers et al. 2021, Anand et al. 2012, Cook et al. 2000). Therefore, despite the clonal population structure in Europe, occasional infections with potentially more virulent *T. gondii* genotypes can occur. In such cases, it should be possible to monitor potential introduction or the presence of these genotypes through genotyping. Furthermore, the occurrence of natural recombination of *T. gondii* strains has been described in Europe (Herrmann et al. 2010). Recombination can lead to new characteristics of the parasite. It is therefore of interest to have techniques available that allow monitoring and analyzing such situations.

The MS method can reliably detect various genotypes of *T. gondii* and is also suitable for fingerprinting (Joeres et al. 2023a, Ajzenberg et al. 2010). In addition, it can reveal recombination, if the genomic loci, where the MS markers are located, are affected by the recombination event. Through the harmonization of the MS method, future population genetic studies based on large datasets originating from different smaller studies can be conducted (Joeres et al. 2023a). Furthermore, the established guidelines may allow more laboratories in Europe to apply the MS method. This may help to fill the geographic gaps in information about existing *T. gondii* genotypes in Europe, which were described in the review paper (Fernández-Escobar et al. 2022).

The new Ion AmpliSeq method is also suitable for distinguishing different *T. gondii* genotypes and for fingerprinting (Joeres et al. 2023b). Moreover, during its establishment, it was able to detect recombination. Therefore, like the MS method, it is suitable for investigating outbreak events more closely and identifying the introduction of rare genotypes to Europe and the occurrence of recombination. In the future, results from Ion AmpliSeq typing may also be used for cluster analyses and thus for studies on the population genetics of *T. gondii*.

5 Zusammenfassung

Erforschung der genetischen Vielfalt von *Toxoplasma gondii* in Europa durch molekulare Feincharakterisierung

T. gondii ist ein zoonotisches Protozoon das vermutlich alle warmblütigen Spezies auf allen Kontinenten infizieren kann (Dámek et al. 2023, Dubey 2022, Stelzer et al. 2019, Schlüter et al. 2014, Robert-Gangneux and Dardé 2012). Der Parasit hat eine komplexe Populationsstruktur (Galal et al. 2019), die Länder und Kontinente umfasst, welche von nur wenigen klonalen Linien dominiert werden (Shwab et al. 2018, Shwab et al. 2014), während die *T. gondii*-Populationen in Südamerika sehr vielfältig sind (Galal et al. 2019, Lorenzi et al. 2016). Die in Mittel- und Südamerika beschriebene genetische Vielfalt wird mit höherer Virulenz und schwereren Fällen von Toxoplasmose beim Menschen in Verbindung gebracht (Shwab et al. 2018, Khan et al. 2006), weshalb es wichtig ist, die Einschleppung solcher Genotypen nach Europa zu erkennen. Die vorliegende Arbeit konzentrierte sich auf die Genotypisierung von *T. gondii* in Europa, wo die klonale Linie Typ II vorherrschend ist (Galal et al. 2022, Shwab et al. 2018, Lorenzi et al. 2016).

Die Dominanz von *T. gondii* Typ II in Europa wurde in der Vergangenheit im Rahmen globaler Studien zur Populationsgenetik von *T. gondii* beschrieben (Galal et al. 2019, Shwab et al. 2014), aber es gab keine Studien, die sich ausschließlich auf Europa konzentrierten. Daher wurden die verfügbaren Informationen zur Genotypisierung von *T. gondii* in Europa in einer Übersicht zusammengefasst (Fernández-Escobar et al. 2022) und die Verteilung der zirkulierenden Stämme in Europa abgebildet, wobei festgestellt wurde, dass für einige Länder nur wenige oder gar keine Informationen verfügbar waren. Außerdem wurde festgestellt, dass die Anwendung der Genotypisierungsmethoden in den verschiedenen Laboren nicht einheitlich war.

Eine häufig genutzte Genotypisierungsmethode für *T. gondii* basiert auf Mikrosatelliten (MS)-Markern (Ajzenberg et al. 2010). Sie stellt den aktuellen Referenzstandard für die Genotypisierung und „Fingerprinting“ dar. Um eine einheitliche MS-Typisierung von *T. gondii* zu erreichen, wurde ein Ringversuch mit fünf europäischen Laboratorien durchgeführt. Die Ergebnisse wurden zusammen mit Leitlinien für eine harmonisierte Anwendung der MS-Typisierung veröffentlicht (Joeres et al. 2023a). Auf dieser Grundlage können die Ergebnisse der MS-Typisierung in Zukunft besser vergleichbar werden, was notwendig ist, um größere Datensätze zu *T. gondii*-Genotypen aus verschiedenen Studien zu kombinieren. Die Leitlinien für die MS-Typisierung könnten auch mehr Labore in Europa dazu bewegen, die MS-Methode

anzuwenden, was dazu beitragen könnte, die festgestellten Lücken bei den Genotypisierungs-Daten zu schließen.

Die MS-Typisierung und die Analyse des gesamten Genoms zeigten bereits die genetische Variabilität innerhalb von *T. gondii* Typ II (Fernández-Escobar et al. 2022, Galal et al. 2022, Shwab et al. 2018, Lorenzi et al. 2016). Um dies weiter zu erforschen, wurde eine auf Next-Generation Sequencing (NGS) basierende Typisierungsmethode mit einer hohen Typisierungsauflösung bei *T. gondii* Typ II-Stämmen entwickelt. Diese neue Ion AmpliSeq-Methode scheint geeignet zu sein, das Verständnis der Übertragungswege von *T. gondii* zu verbessern, Infektionsquellen bei Ausbrüchen zu identifizieren oder Rekombinationen und den Eintrag von Genotypen in neue Regionen zu erkennen.

Darüber hinaus wurde die harmonisierte MS-Methode in einer Studie zur Genotypisierung von *T. gondii*-DNA aus Gewebeproben von in den Niederlanden tot aufgefundenen Eichhörnchen (*Sciurus vulgaris*) angewandt. In dieser Studie konnten auf der Grundlage der MS-Typisierungsergebnisse genetische Cluster identifiziert werden, allerdings ohne offensichtlichen regionalen Zusammenhang. In künftigen Studien können Cluster-Analysen mit großen, kombinierten Datensätzen mit Hilfe der harmonisierten Methoden durch MS-Typisierung oder durch Ion AmpliSeq-Typisierung durchgeführt werden, um weitere Erkenntnisse über die Populationsstruktur von *T. gondii* in Europa zu gewinnen.

6 Summary

Exploring the genetic diversity of *Toxoplasma gondii* in Europe by molecular fine characterization

T. gondii is a zoonotic protozoon that can infect virtually all warm-blooded species on all continents (Dámek et al. 2023, Dubey 2022, Stelzer et al. 2019, Schlüter et al. 2014, Robert-Gangneux and Dardé 2012). The parasite has a complex population structure (Galal et al. 2019), including countries and continents dominated by only a few clonal lineages (Shwab et al. 2018, Shwab et al. 2014), while *T. gondii* populations in South America are highly diverse (Galal et al. 2019, Lorenzi et al. 2016). The genetic diversity described in Central and South America is associated with higher virulence and more severe cases of toxoplasmosis in humans (Shwab et al. 2018, Khan et al. 2006), making it important to detect the introduction of such genotypes into Europe. This work focused on genotyping of *T. gondii* in Europe where the clonal lineage type II is predominant (Galal et al. 2022, Shwab et al. 2018, Lorenzi et al. 2016).

The predominance of *T. gondii* type II in Europe was described in the past as part of global studies on population genetics of *T. gondii* (Galal et al. 2019, Shwab et al. 2014), but there were no studies that focused exclusively on Europe. Therefore, the available genotyping information on European *T. gondii* was summarized in a review (Fernández-Escobar et al. 2022) and the distribution of circulating strains in Europe was mapped, revealing that there was little or no information available for some countries. Moreover, it was recognized that the application of genotyping methods was not consistent between different laboratories.

A frequently used genotyping method for *T. gondii* is based on MS markers (Ajzenberg et al. 2010). It represents the current reference standard for genotyping and fingerprinting. To reach consistency in *T. gondii* MS typing, a ring trial among five European laboratories was organized and the results were published together with guidelines for a harmonized application of MS typing (Joeres et al. 2023a). On this basis, MS typing results may become more comparable in the future, which is necessary to combine larger data sets on *T. gondii* genotypes originating from different studies. Guidelines for MS typing may also encourage more laboratories in Europe to use the MS method, which may help to fill the identified gaps in genotyping data.

MS typing and WGS analysis revealed genetic variability within *T. gondii* type II (Fernández-Escobar et al. 2022, Galal et al. 2022, Shwab et al. 2018, Lorenzi et al. 2016). To explore this further, an NGS-based typing method with a high typing resolution among *T. gondii* type II strains was established. This new Ion AmpliSeq method appears to be suitable to improve the

understanding of transmission pathways of *T. gondii*, to trace infection sources in outbreaks or to detect recombination and the introduction of genotypes to new areas.

Furthermore, the harmonized MS method was applied in a study for genotyping of *T. gondii* DNA extracted from tissue samples of red squirrels (*Sciurus vulgaris*) found dead in the Netherlands. This study identified genetic clusters based on the MS typing results, but without obvious regional association. In future studies, cluster analyses can be performed with large, combined datasets using the harmonized MS typing method or Ion AmpliSeq typing to obtain further insights into the population structure of *T. gondii* in Europe.

7 References

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

Ajzenberg D, Collinet F, Mercier A, Vignoles P, Dardé M-L (2010): Genotyping of *Toxoplasma gondii* isolates with 15 microsatellite markers in a single multiplex PCR assay. J Clin Microbiol, 48, 4641–4645. DOI 10.1128/JCM.01152-10.

Ajzenberg D, Collinet F, Aubert D, Villena I, Dardé M-L, Devillard S (2015): The rural-urban effect on spatial genetic structure of type II *Toxoplasma gondii* strains involved in human congenital toxoplasmosis, France, 2002-2009. Infect Genet Evol, 36, 511–516. DOI 10.1016/j.meegid.2015.08.025.

Anand R, Jones C W, Ricks J H, Sofarelli T A, Hale D C (2012): Acute Primary Toxoplasmosis in Travelers Returning From Endemic Countries. J Travel Med, 19, 57–60. DOI 10.1111/j.1708-8305.2011.00564.x.

Beaman M H, Araujo F G, Remington J S (1994): Protective Reconstitution of the SCID Mouse against Reactivation of Toxoplasmic Encephalitis. J Infect Dis, 169, 375–383. DOI 10.1093/infdis/169.2.375.

Behnke M S, Dubey J P, Sibley L D (2016): Genetic Mapping of Pathogenesis Determinants in *Toxoplasma gondii*. Annu Rev Microbiol, 70, 63–81. DOI 10.1146/annurev-micro-091014-104353.

Berna L, Marquez P, Cabrera A, Greif G, Francia M E, Robello C (2021): Reevaluation of the *Toxoplasma gondii* and *Neospora caninum* genomes reveals misassembly, karyotype differences and chromosomal rearrangements. Genome Res, 31, 823–833. DOI 10.1101/2020.05.22.111195.

Berrébi A, Assouline C, Bessières M-H, Lathière M, Cassaing S, Minville V, Ayoubi J-M (2010): Long-term outcome of children with congenital toxoplasmosis. Am J Obstet Gynecol, 203, 552.e1-6. DOI 10.1016/j.ajog.2010.06.002.

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.

Beverley J K (1959): Congenital Transmission of Toxoplasmosis through Successive Generations of Mice. Nature, 183, 1348–1349. DOI 10.1038/1831348a0.

Black M W, Boothroyd J C (2000): Lytic cycle of *Toxoplasma gondii*. Microbiol Mol Biol Rev, 64, 607–623. DOI 10.1128/MMBR.64.3.607-623.2000.

Blaizot R, Nabet C, Blanchet D, Martin E, Mercier A, Dardé M-L, Elenga N, Demar M (2019): Pediatric Amazonian Toxoplasmosis Caused by Atypical Strains in French Guiana, 2002-2017. Pediatr Infect Dis J, 38, e39-e42. DOI 10.1097/INF.0000000000002130.

Bouwknegt M, Devleesschauwer B, Graham H, Robertson L J, van der Giessen J W (2018): Prioritisation of food-borne parasites in Europe, 2016. Euro surveillance : bulletin Europeen sur les maladies transmissibles = European communicable disease bulletin, 23. DOI 10.2807/1560-7917.ES.2018.23.9.17-00161.

Bowie W R, King A S, Werker D H, Isaac-Renton J L, Bell A, Eng S B, Marion S A (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 J P, Rajasekar B, Saeij J P J, Ajioka J W, Berriman M, Paulsen I, Roos D S, Sibley L D, White M W, Boothroyd J C (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.

Buxton D, Thomson K M, Maley S, Wright S, Bos H J (1993): Experimental challenge of sheep 18 months after vaccination with a live (S48) *Toxoplasma gondii* vaccine. Vet Rec, 133, 310–312. DOI 10.1136/vr.133.13.310.

Buxton D, Innes E A (1995): A commercial vaccine for ovine toxoplasmosis. Parasitology, 110, 11-6. DOI 10.1017/s003118200000144x.

Calero-Bernal R, Gennari S M, Cano S, Salas-Fajardo M Y, Ríos A, Álvarez-García G, Ortega-Mora L M (2023): Anti-*Toxoplasma gondii* Antibodies in European Residents: A Systematic Review and Meta-Analysis of Studies Published between 2000 and 2020. Pathogens, 12, 1430. DOI 10.3390/pathogens12121430.

Carme B, Bissuel F, Ajzenberg D, Bouyne R, Aznar C, Demar M, Bichat S, Louvel D, Bourbigot A M, Peneau C, Neron P, Dardé M L (2002): Severe acquired toxoplasmosis in

immunocompetent adult patients in French Guiana. J Clin Microbiol, 40, 4037–4044. DOI 10.1128/jcm.40.11.4037-4044.2002.

Castro B B P, Gennari S M, Lorenzi H, Su C (2020): A simple method to generate PCR-RFLP typing profiles from DNA sequences in *Toxoplasma gondii*. Infect Genet Evol, 85, 1–7. DOI 10.1016/j.meegid.2020.104590.

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

Cook A J, Gilbert R E, Buffolano W, Zufferey J, Petersen E, Jenum P A, Foulon W, Semprini A E, Dunn D T (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.

Dámek F, Swart A, Waap H, Jokelainen P, Le Roux D, Deksne G, Deng H, Schares G, Lundén A, Álvarez-García G, Betson M, Davidson R K, Györke A, Antolová D, Hurníková Z, Wisselink H J, Sroka J, van der Giessen J W B, Blaga R, Opsteegh M (2023): Systematic Review and Modelling of Age-Dependent Prevalence of *Toxoplasma gondii* in Livestock, Wildlife and Felids in Europe. Pathogens, 12, 97. DOI 10.3390/pathogens12010097.

Dardé M L, Bouteille B, Pestre-Alexandre M (1992): Isoenzyme analysis of 35 *Toxoplasma gondii* isolates and the biological and epidemiological implications. J Parasitol, 78, 786–794. DOI 10.2307/3283305.

de Moura L, Bahia-Oliveira L M G, Wada M Y, Jones J L, Tuboi S H, Carmo E H, Ramalho W M, Camargo N J, Trevisan R, Graça R M T, da Silva A J, Moura I, Dubey J P, Garrett D O (2006): Waterborne Toxoplasmosis, Brazil, from Field to Gene. Emerg Infect Dis, 12, 326–329. DOI 10.3201/eid1202.041115.

Delhaes L, Ajzenberg D, Sicot B, Bourgeot P, Dardé M-L, Dei-Cas E, Houfflin-Debarge V (2010): Severe congenital toxoplasmosis due to a *Toxoplasma gondii* strain with an atypical genotype: case report and review. Prenat Diagn, 30, 902–905. DOI 10.1002/pd.2563.

Demar M, Hommel D, Djossou F, Peneau C, Boukhari R, Louvel D, Bourbigot A-M, Nasser V, Ajzenberg D, Darde M-L, Carme B (2012): Acute toxoplasmoses in immunocompetent patients

hospitalized in an intensive care unit in French Guiana. Clin Microbiol Infect, 18, E221-31. DOI 10.1111/j.1469-0691.2011.03648.x.

Dubey J P, Miller N L, Frenkel J K (1970a): The *Toxoplasma gondii* oocyst from cat feces. J Exp Med, 132, 636–662. DOI 10.1084/jem.132.4.636.

Dubey J P, Miller N L, Frenkel J K (1970b): *Toxoplasma gondii* life cycle in cats. J Am Vet Med Assoc, 157, 1767–1770.

Dubey J P, Frenkel J K (1972): Cyst-Induced Toxoplasmosis in Cats. J Protozool, 19, 155–177. DOI 10.1111/j.1550-7408.1972.tb03431.x.

Dubey J P, Carpenter J L (1993): Histologically confirmed clinical toxoplasmosis in cats: 100 cases (1952-1990). J Am Vet Med Assoc, 203, 1556–1566.

Dubey J P, Lappin M R, Thulliez P (1995): Diagnosis of induced toxoplasmosis in neonatal cats. J Am Vet Med Assoc, 207, 179–185.

Dubey J P (1996): Infectivity and Pathogenicity of *Toxoplasma gondii O*ocysts for Cats. J Parasitol, 82, 957–961.

Dubey J P (1997): Bradyzoite-Induced Murine Toxoplasmosis: Stage Conversion, Pathogenesis, and Tissue Cyst Formation in Mice Fed Bradyzoites of Different Strains of *Toxoplasma gondii*. J Eukaryot Microbiol, 44, 592–602. DOI 10.1111/j.1550-7408.1997.tb05965.x.

Dubey J P, Speer C A, Shen S K, Kwok O C, Blixt J A (1997): Oocyst-Induced Murine Toxoplasmosis: Life Cycle, Pathogenicity, and Stage Conversion in Mice Fed *Toxoplasma gondii* Oocysts. J Parasitol, 83, 870–882.

Dubey J P, Lindsay D S, Speer C A (1998): Structures of *Toxoplasma gondii* tachyzoites, bradyzoites, and sporozoites and biology and development of tissue cysts. Clin Microbiol Rev, 11, 267–299. DOI 10.1128/CMR.11.2.267.

Dubey J P, Frenkel J K (1998): Toxoplasmosis of rats: a review, with considerations of their value as an animal model and their possible role in epidemiology. Vet Parasitol, 77, 1–32. DOI 10.1016/s0304-4017(97)00227-6.

Dubey J P (2001): Oocyst Shedding by Cats Fed Isolated Bradyzoites and Comparison of Infectivity of Bradyzoites of the VEG Strain *Toxoplasma gondii* to Cats and Mice. J Parasitol 87, 215–219. DOI 10.1645/0022-3395(2001)087[0215:OSBCFI]2.0.CO;2.

Dubey J P (2005): Unexpected oocyst shedding by cats fed *Toxoplasma gondii* tachyzoites: In vivo stage conversion and strain variation. Vet Parasitol, 133, 289–298. DOI 10.1016/j.vetpar.2005.06.007.

Dubey J P, Vianna M C B, Sousa S, Canada N, Meireles S, Da Correia Costa J M, Marcet P L, Lehmann T, Dardé M L, Thulliez P (2006): Characterization of *Toxoplasma gondii* isolates in free-range chickens from Portugal. J Parasitol, 92, 184–186. DOI 10.1645/GE-652R.1.

Dubey J P (2010): *Toxoplasma gondii* Infections in Chickens (*Gallus domesticus*): Prevalence, Clinical Disease, Diagnosis and Public Health Significance. Zoonoses Public Health, 57, 60–73. DOI 10.1111/j.1863-2378.2009.01274.x.

Dubey J P, Velmurugan G V, Rajendran C, Yabsley M J, Thomas N J, Beckmen K B, Sinnett D, Ruid D, Hart J, Fair P A, McFee W E, Shearn-Bochsler V, Kwok O C H, Ferreira L R, Choudhary S, Faria E B, Zhou H, Felix T A, Su C (2011): Genetic characterisation of *Toxoplasma gondii* in wildlife from North America revealed widespread and high prevalence of the fourth clonal type. Int J Parasitol, 41, 1139–1147. DOI 10.1016/j.ijpara.2011.06.005.

Dubey JP (2022): Toxoplasmosis of animals and humans. Third edition. Boca Raton: CRC Press. ISBN: 9781000431476.

Dunn D, Wallon M, Peyron F, Petersen E, Peckham C, Gilbert R (1999): Mother-to-child transmission of toxoplasmosis: risk estimates for clinical counselling. Lancet, 353, 1829–1833. DOI 10.1016/S0140-6736(98)08220-8.

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, 599–603. DOI 10.1007/BF00930955.

Fernández-Escobar M, Schares G, Maksimov P, Joeres M, Ortega-Mora L M, Calero-Bernal R (2022): *Toxoplasma gondii* Genotyping: A Closer Look Into Europe. Front Cell Infect Microbiol, 12, 842595. DOI 10.3389/fcimb.2022.842595.

Fischer S, Freuling C M, Müller T, Pfaff F, Bodenhofer U, Höper D, Fischer M, Marston D A, Fooks A R, Mettenleiter T C, Conraths F J, Homeier-Bachmann T (2018): Defining objective

clusters for rabies virus sequences using affinity propagation clustering. PLoS Negl Trop Dis, 12, e0006182. DOI 10.1371/journal.pntd.0006182.

Frenkel J K, Dubey J P, Miller N L (1970): *Toxoplasma gondii* in Cats: Fecal Stages Identified as Coccidian Oocysts. Science, 167, 893–896. DOI 10.1126/science.167.3919.893.

Frenkel J K (1973): *Toxoplasma* In and Around Us. BioScience, 23, 343–352. DOI 10.2307/1296513.

Galal L, Ajzenberg D, Hamidović A, Durieux M-F, Dardé M-L, Mercier A (2018): *Toxoplasma* and Africa: One Parasite, Two Opposite Population Structures. Trends Parasitol, 34, 140–154. DOI 10.1016/j.pt.2017.10.010.

Galal L, Hamidović A, Dardé M-L, Mercier M (2019): Diversity of *Toxoplasma gondii* strains at the global level and its determinants. Food Waterborne Parasitol, 15, e00052. DOI 10.1016/j.fawpar.2019.e00052.

Galal L, Ariey F, Gouilh M A, Dardé M-L, Hamidović A, Letourneur F, Prugnolle F, Mercier A (2022): A unique *Toxoplasma gondii* haplotype accompanied the global expansion of cats. Nat Commun, 13, 5778. DOI 10.1038/s41467-022-33556-7.

Gilbert R E, Freeman K, Lago E G, Bahia-Oliveira L M G, Tan H K, Wallon M, Buffolano W, Stanford M R, Petersen E (2008): Ocular sequelae of congenital toxoplasmosis in Brazil compared with Europe. PLoS Negl Trop Dis, 2, e277. DOI 10.1371/journal.pntd.0000277.

Grigg M E, Ganatra J, Boothroyd J C, Margolis T P (2001): Unusual Abundance of Atypical Strains Associated with Human Ocular Toxoplasmosis. J Infect Dis, 184, 633–639. DOI 10.1086/322800.

Grover C M, Thulliez P, Remington J S, Boothroyd J C (1990): Rapid Prenatal Diagnosis of Congenital *Toxoplasma* Infection by Using Polymerase Chain Reaction and Amniotic Fluid. J Clin Microbiol, 28, 2297–2301. DOI 10.1128/jcm.28.10.2297-2301.1990.

Hassan M A, Olijnik A-A, Frickel E-M, Saeij J P (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.

Herrmann D C, Pantchev N, Vrhovec M G, Barutzki D, Wilking H, Fröhlich A, Lüder C G K, Conraths F J, Schares G (2010): Atypical *Toxoplasma gondii* genotypes identified in oocysts shed by cats in Germany. Int J Parasitol, 40, 285–292. DOI 10.1016/j.ijpara.2009.08.001.

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

Hutchison W M (1965): Experimental Transmission of *Toxoplasma gondii*. Nature, 206, 961–962. DOI 10.1038/206961a0.

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

Joeres M, Cardron G, Passebosc-Faure K, Plault N, Fernández-Escobar M, Hamilton C M, O'Brien-Anderson L, Calero-Bernal R, Galal L, Luttermann C, Maksimov P, Conraths F J, Dardé M L, Ortega-Mora L M, Jokelainen P, Mercier A, Schares G (2023a): 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.

Joeres M, Maksimov P, Höper D, Calvelage S, Calero-Bernal R, Fernández-Escobar M, Koudela B, Blaga R, Vrhovec M G, Stollberg K, Bier N, Sotiraki S, Sroka J, Piotrowska W, Kodym P, Basso W, Conraths F J, Mercier A, Galal L, Dardé M L, Balea A, Spano F, Schulze C, Peters M, Scuda N, Lundén A, Davidson R K, Terland R, Waap H, Bruin E de, Vatta P, Caccio S, Ortega-Mora L M, Jokelainen P, Schares G (2023b): Genotyping of European *Toxoplasma gondii* strains by a new high-resolution next-generation sequencing-based method. Eur J Clin Microbiol Infect Dis, 43, 355-371. DOI 10.1007/s10096-023-04721-7.

Johnson J D, Butcher P D, Savva D, Holliman R E (1993): Application of the polymerase chain reaction to the diagnosis of human toxoplasmosis. J Infect, 26, 147–158. DOI 10.1016/0163-4453(93)92788-x.

Jokelainen P, Isomursu M, Näreaho A, Oksanen A (2011): Natural *Toxoplasma gondii* infections in European brown hares and mountain hares in Finland: proportional mortality rate, antibody prevalence, and genetic characterization. J Wildl Dis, 47, 154–163. DOI 10.7589/0090-3558-47.1.154.

Jones J L, Muccioli C, Belfort R, Holland G N, Roberts J M, Silveira C (2006): Recently Acquired *Toxoplasma gondii* Infection, Brazil. Emerg Infect Dis, 12, 582–587. DOI 10.3201/eid1204.051081.

Jones J L, Dubey J P (2010): Waterborne toxoplasmosis - Recent developments. Exp Parasitol, 124, 10–25. DOI 10.1016/j.exppara.2009.03.013.

Jones J L, Dubey J P (2012): Foodborne Toxoplasmosis. Clin Infect Dis, 55, 845–851. DOI 10.1093/cid/cis508.

Khan A, Jordan C, Muccioli C, Vallochi A L, Rizzo L V, Belfort R, Vitor R W A, Silveira C, Sibley L D (2006): Genetic divergence of *Toxoplasma gondii* strains associated with ocular toxoplasmosis, Brazil. Emerg Infect Dis, 12, 942–949. DOI 10.3201/eid1206.060025.

Khan A, Fux B, Su C, Dubey J P, Darde M L, Ajioka J W, Rosenthal B M, Sibley L D (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, Dubey J P, Su C, Ajioka J W, Rosenthal B M, Sibley L D (2011): Genetic analyses of atypical *Toxoplasma gondii* strains reveal a fourth clonal lineage in North America. Int J Parasitol, 41, 645–655. DOI 10.1016/j.ijpara.2011.01.005.

Kik M, IJzer J, Opsteegh M, Montizaan M, Dijkstra V, Rijks J, Gröne A (2015): *Toxoplasma gondii* in Wild Red Squirrels, the Netherlands, 2014. Emerg Infect Dis, 21, 2248–2249. DOI 10.3201/eid2112.141711.

Kuruca L, Uzelac A, Klun I, Lalošević V, Djurković-Djaković O (2019): *Toxoplasma gondii* genotypes circulating in domestic pigs in Serbia. Acta Vet Hung, 67, 204–211. DOI 10.1556/004.2019.022.

Lambert H, Hitziger N, Dellacasa I, Svensson M, Barragan A (2006): Induction of dendritic cell migration upon *Toxoplasma gondii* infection potentiates parasite dissemination. Cell Microbiol, 8, 1611–1623. DOI 10.1111/j.1462-5822.2006.00735.x.

Lavrard I, Chouaid C, Roux P, Poirot J L, Marteau M, Lemarchand B, Meyohas M C, Olivier J L (1995): Pulmonary toxoplasmosis in HIV-infected patients: usefulness of polymerase chain reaction and cell culture. Eur Respir J, 8, 697–700.

Liesenfeld O (2002): Oral Infection of C57BL/6 Mice with *Toxoplasma gondii*: A New Model of Inflammatory Bowel Disease? J Infect Dis, 185, 96–101. DOI 10.1086/338006.

Lilue J, Müller U B, Steinfeldt T, Howard J C (2013): Reciprocal virulence and resistance polymorphism in the relationship between *Toxoplasma gondii* and the house mouse. eLife. DOI 10.7554/eLife.01298.001.

Liu Q, Wang Z-D, Huang S-Y, Zhu X-Q (2015): Diagnosis of toxoplasmosis and typing of *Toxoplasma gondii*. Parasit Vectors, 8, 292. DOI 10.1186/s13071-015-0902-6.

Lorenzi H, Khan A, Behnke M S, Namasivayam S, Swapna L S, Hadjithomas M, Karamycheva S, Pinney D, Brunk B P, Ajioka J W, Ajzenberg D, Boothroyd J C, Boyle J P, Dardé M L, Diaz-Miranda M A, Dubey J P, Fritz H M, Gennari S M, Gregory B D, Kim K, Saeij J P J, Su C, White M W, Zhu X-Q, Howe D K, Rosenthal B M, Grigg M E, Parkinson J, Liu L, Kissinger J C, Roos D S, Sibley L D (2016): Local admixture of amplified and diversified secreted pathogenesis determinants shapes mosaic *Toxoplasma gondii* genomes. Nat Commun, 7, 10147. DOI 10.1038/ncomms10147.

McAuley J, Boyer K M, Patel D, Mets M, Swisher C, Roizen N, Wolters C, Stein L, Stein M, Schey W (1994): Early and Longitudinal Evaluations of Treated Infants and Children and Untreated Historical Patients with Congenital Toxoplasmosis: The Chicago Collaborative Treatment Trial. Clin Infect Dis, 18, 38–72. DOI 10.1093/clinids/18.1.38.

Mercier A, Ajzenberg D, Devillard S, Demar M P, Thoisy B de, Bonnabau H, Collinet F, Boukhari R, Blanchet D, Simon S, Carme B, Dardé M-L (2011): Human impact on genetic diversity of *Toxoplasma gondii*: Example of the anthropized environment from French Guiana. Infect Genet Evol, 11, 1378–1387. DOI 10.1016/j.meegid.2011.05.003.

Messaritakis I, Detsika M, Koliou M, Sifakis S, Antoniou M (2008): Prevalent genotypes of *Toxoplasma gondii* in pregnant women and patients from Crete and Cyprus. Am J Trop Med Hyg, 79, 205–209. DOI 10.4269/ajtmh.2008.79.205.

Miller N L, Frenkel J K, Dubey J P (1972): Oral Infections with *Toxoplasma* Cysts and Oocysts in Felines, Other Mammals, and in Birds. J Parasitol, 58, 928–937.

Montoya J G, Parmley S, Liesenfeld O, Jaffe G J, Remington J S (1999): Use of the polymerase chain reaction for diagnosis of ocular toxoplasmosis. Ophthalmology, 106, 1554–1563. DOI 10.1016/S0161-6420(99)90453-0.

Montoya J G (2002): Laboratory Diagnosis of *Toxoplasma gondii* Infection and Toxoplasmosis. J Infect Dis, 185, 73-82. DOI 10.1086/338827.

Montoya J G, Liesenfeld O (2004): Toxoplasmosis. Lancet, 363, 1965–1976. DOI 10.1016/S0140-6736(04)16412-X.

Munoz-Zanzi C A, Fry P, Lesina B, Hill D (2010): *Toxoplasma gondii O*ocyst-specific Antibodies and Source of Infection. Emerg Infect Dis, 16, 1591–1593. DOI 10.3201/eid1610.091674.

Nicolle C, Manceaux L H (1908): Sur une infection à corps de Leishman (ou organismes voisins) du gondi. Comptes Rendus Hebdomadaires des Séances de L'Academie des Sciences, 147, 763–766.

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.

Pena H F J, Soares R M, Amaku M, Dubey J P, Gennari S M (2006): *Toxoplasma gondii* infection in cats from São Paulo state, Brazil: Seroprevalence, oocyst shedding, isolation in mice, and biologic and molecular characterization. Res Vet Sci, 81, 58–67. DOI 10.1016/j.rvsc.2005.09.007.

Pfefferkorn L C, Pfefferkorn E R (1980): *Toxoplasma gondii*: Genetic Recombination between Drug Resistant Mutants. Exp Parasitol, 50, 305–316. DOI 10.1016/0014-4894(80)90034-x.

Pinkerton H, Weinman D (1940): Toxoplasma infection in man. Arch Pathol, 30, 374–392.

Powell C C, Lappin M R (2001): Clinical ocular toxoplasmosis in neonatal kittens. Vet Ophthalmol, 4, 87–92. DOI 10.1046/j.1463-5224.2001.00180.x.

Prestrud K W, Asbakk K, Mørk T, Fuglei E, Tryland M, Su C (2008): Direct high-resolution genotyping of *Toxoplasma gondii* in arctic foxes (*Vulpes lagopus*) in the remote arctic Svalbard archipelago reveals widespread clonal Type II lineage. Vet Parasitol, 158, 121–128. DOI 10.1016/j.vetpar.2008.08.020.

Robert-Gangneux F, Dardé M-L (2012): Epidemiology of and Diagnostic Strategies for Toxoplasmosis. Clin Microbiol Rev, 25, 264–296. DOI 10.1128/CMR.05013-11.

Sabin A B, Olitsky P K (1937): Toxoplasma and Obligate Intracellular Parasitism. Science, 85, 336–338. DOI 10.1126/science.85.2205.336.

Saraf P, Shwab E K, Dubey J P, Su C (2017): On the determination of *Toxoplasma gondii* virulence in mice. Exp Parasitol, 174, 25–30. DOI 10.1016/j.exppara.2017.01.009.

Sato K, Iwamoto I, Yoshiki K (1993): Experimental Toxoplasmosis in Pregnant Cats. J Vet Med Sci, 55, 1005–1009. DOI 10.1292/jvms.55.1005.

Schares G, Vrhovec M G, Pantchev N, Herrmann D C, Conraths F J (2008): Occurrence of *Toxoplasma gondii* and *Hammondia hammondi* oocysts in the faeces of cats from Germany and other European countries. Vet Parasitol, 152, 34–45. DOI 10.1016/j.vetpar.2007.12.004.

Schlüter D, Däubener W, Schares G, Groß U, Pleyer U, Lüder C (2014): Animals are key to human toxoplasmosis. Int J Med Microbiol, 304, 917–929. DOI 10.1016/j.ijmm.2014.09.002.

Schreiner M, Liesenfeld O (2009): Small intestinal inflammation following oral infection with *Toxoplasma gondii* does not occur exclusively in C57BL/6 mice: review of 70 reports from the literature. Mem Inst Oswaldo Cruz, 104, 221–233. DOI 10.1590/s0074-02762009000200015.

Seers T, Myneni J, Chaudhry N L, Ugarte M (2021): Bilateral ocular toxoplasmosis in a returning traveller: age and route of infection as potential risk factors. BMJ Case Rep, 14, 1–4. DOI 10.1136/bcr-2020-237068.

Seipel D, Oliveira B C d L, Resende T L, Schuindt S H S, Pimentel P M d O, Kanashiro M M, Arnholdt A C V (2010): *Toxoplasma gondii* infection positively modulates the macrophages migratory molecular complex by increasing matrix metalloproteinases, CD44 and alphavbeta3 integrin. Vet Parasitol, 169, 312–319. DOI 10.1016/j.vetpar.2009.12.042.

Sheiner L, Vaidya A B, McFadden G I (2013): The metabolic roles of the endosymbiotic organelles of *Toxoplasma* and *Plasmodium* spp. Curr Opin Microbiol, 16, 452–458. DOI 10.1016/j.mib.2013.07.003.

Shwab E K, Zhu X-Q, Majumdar D, Pena H F J, Gennari S M, Dubey J P, Su C (2014): Geographical patterns of *Toxoplasma gondii* genetic diversity revealed by multilocus PCR-RFLP genotyping. Parasitology, 141, 453–461. DOI 10.1017/S0031182013001844.

Shwab E K, Saraf P, Zhu X-Q, Zhou D-H, McFerrin B M, Ajzenberg D, Schares G, Hammond-Aryee K, van Helden P, Higgins S A, Gerhold R W, Rosenthal B M, Zhao X, Dubey J P, Su C (2018): Human impact on the diversity and virulence of the ubiquitous zoonotic parasite *Toxoplasma gondii*. Proc Natl Acad Sci U S A, 115, E6956-E6963. DOI 10.1073/pnas.1722202115.

Sibley L D, Boothroyd J C (1992): Virulent strains of *Toxoplasma gondii* comprise a single clonal lineage. Nature, 359, 82–85. DOI 10.1038/359082a0.

Splendore A (1908): Un nuovo protozoa parassita deconigli incontrato nelle lesioni anatomiche d'une malattia che ricorda in molti punti il Kala-azar dell'uoma. Nota preliminare pel. Rev Soc Scient Sao Paulo, 3, 109–112.

Stelzer S, Basso W, Benavides Silván J, Ortega-Mora L M, Maksimov P, Gethmann J, Conraths F J, 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, Howe D K, Dubey J P, Ajioka J W, Sibley L D (2002): Identification of quantitative trait loci controlling acute virulence in *Toxoplasma gondii*. Proc Natl Acad Sci U S A, 99, 10753–10758. DOI 10.1073/pnas.172117099.

Su C, Evans D, Cole R H, Kissinger J C, Ajioka J W, Sibley L D (2003): Recent Expansion of *Toxoplasma* Through Enhanced Oral Transmission. Science, 299, 414–416. DOI 10.1126/science.1078035.

Su C, Zhang X, Dubey J P (2006): Genotyping of *Toxoplasma gondii* by multilocus PCR-RFLP markers: a high resolution and simple method for identification of parasites. Int J Parasitol, 36, 841–848. DOI 10.1016/j.ijpara.2006.03.003.

Su C, Shwab E K, Zhou P, Zhu X Q, Dubey J P (2010): Moving towards an integrated approach to molecular detection and identification of *Toxoplasma gondii*. Parasitology, 137, 1–11. DOI 10.1017/S0031182009991065.

Su C, Khan A, Zhou P, Majumdar D, Ajzenberg D, Dardé M-L, Zhu X-Q, Ajioka J W, Rosenthal B M, Dubey J P, Sibley L D (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, 5844–5849. DOI 10.1073/pnas.1203190109.

Verma S K, Ajzenberg D, Rivera-Sanchez A, Su C, Dubey J P (2015): Genetic characterization of *Toxoplasma gondii* isolates from Portugal, Austria and Israel reveals higher genetic variability within the type II lineage. Parasitology, 142, 948–957. DOI 10.1017/S0031182015000050.

Vilares A, Borges V, Sampaio D, Ferreira I, Martins S, Vieira L, Gargaté M J, Gomes J P (2020): Towards a rapid sequencing-based molecular surveillance and mosaicism investigation of *Toxoplasma gondii*. Parasitol Res, 119, 587–599. DOI 10.1007/s00436-019-06523-3.

Weiss L M, Dubey J P (2009): Toxoplasmosis: a history of clinical observations. Int J Parasitol, 39, 895–901. DOI 10.1016/j.ijpara.2009.02.004.

Wijburg S R, Montizaan M G E, Kik M J L, Joeres M, Cardron G, Luttermann C, Maas M, Maksimov P, Opsteegh M, Schares G (2024): Drivers of infection with *Toxoplasma gondii* genotype type II in Eurasian red squirrels (*Sciurus vulgaris*). Parasit Vectors, 17, 1–15. DOI 10.1186/s13071-023-06068-6.

Wolf A, Cowen D, Paige B (1939): Human toxoplasmosis: Occurence in infants as an encephalomyelitis verification by transmission to animals. Science, 89, 226–227. DOI 10.1126/science.89.2306.226.

World Health Organization, Food and Agriculture Organization of the United Nations (2014): Multicriteria-based ranking for risk management of food-borne parasites. Report of a joint FAO/WHO expert meeting, 3-7 September 2012, FAO Headquarters, Rome, Italy. Rome, Italy, Geneva, Switzerland: Food and Agriculture Organization of the United Nations; World Health Organization. ISBN: 9789241564700.

Xia J, Venkat A, Bainbridge R E, Reese M L, Le Roch K G, Ay F, Boyle J P (2021): Third-generation 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.

8 Publications and conference presentations

8.1 Peer-reviewed publications

Fernández-Escobar M, Schares G, Maksimov P, Joeres M, Ortega-Mora L M, Calero-Bernal R (2022): *Toxoplasma gondii* Genotyping: A Closer Look Into Europe
Frontiers in Cellular and Infection Microbiology 12:842595
DOI: 10.3389/fcimb.2022.842595

Joeres M, Cardron G, Passebosc-Faure K, Plault N, Fernández-Escobar M, Hamilton C M, O'Brien-Anderson L, Calero-Bernal R, Galal L, Luttermann C, Maksimov P, Conraths F J, Dardé M L, Ortega-Mora L M, Jokelainen P, Mercier A, Schares G (2023): A ring trial to harmonize *Toxoplasma gondii* microsatellite typing: comparative analysis of results and recommendations for optimization
European Journal of Clinical Microbiology & Infectious Diseases 42:803–818
DOI: 10.1007/s10096-023-04597-7

Joeres M, Maksimov P, Höper D, Calvelage S, Calero-Bernal R, Fernández-Escobar M, Koudela B, Blaga R, Globokar Vrhovec M, Stollberg K, Bier N, Sotiraki S, Sroka J, Piotrowska W, Kodym P, Basso W, Conraths F J, Mercier A, Galal L, Dardé M L, Balea A, Spano F, Schulze C, Peters M, Scuda N, Lundén A, Davidson R K, Terland R, Waap H, de Bruin E, Vatta P, Caccio S, Ortega-Mora L M, Jokelainen P, Schares G (2023): Genotyping of European *Toxoplasma gondii* strains by a new high-resolution next-generation sequencing-based method
European Journal of Clinical Microbiology & Infectious Diseases 43:355-371
DOI: 10.1007/s10096-023-04721-7

Wijburg S R, Montizaan M G E, Kik M J L, Joeres M, Cardron G, Luttermann C, Maas M, Maksimov P, Opsteegh M, Schares G (2023): Drivers of infection with *Toxoplasma gondii* genotype type II in Eurasian red squirrels (*Sciurus vulgaris*)
Parasites & Vectors 17:1-15
DOI: 10.1186/s13071-023-06068-6

8.2 Oral presentations

Joeres M, Maksimov P, Conraths F J, Höper D, Calvelage S, Koudela B, Blaga R, Caccio S, Fernández-Escobar M, Calero-Bernal R, Ortega-Mora L M, Jokelainen P, Schares G: Development of a next-generation sequencing-based typing method to detect within-genotype variation in *Toxoplasma gondii*

Junior Scientist Symposium of Friedrich-Loeffler-Institut: Animal Welfare – Challenges and solutions for science and agriculture, online, 20th – 21st of October 2021

Joeres M, Maksimov P, Höper D, Calvelage S, Calero-Bernal R, Fernández-Escobar M, Koudela B, Blaga R, Globokar Vrhovec M, Stollberg K, Bier N, Sotiraki S, Sroka S, Piotrowska W, Kodym P, Basso W, Conraths F J, Dardé M L, Spano F, Caccio S, Ortega-Mora L M, Jokelainen P, Schares G: A novel Ion AmpliSeq-based typing method of *Toxoplasma gondii* reveals and excellent typing resolution among European type II strains
Tagung der DVG-Fachgruppe „Parasitologie und parasitäre Krankheiten“ 2022, Berlin, Germany, and online, 23th – 25th of May 2022

Joeres M, Maksimov P, Höper D, Calero-Bernal R, Fernández-Escobar M, Koudela B, Blaga R, Globokar Vorhovec M, Stollberg K, Bier N, Sotiraki S, Sroka J, Piotrowska W, Kodym P, Basso W, Conraths F J, Dardé M J, Caccio S, Ortega-Mora L M, Jokelainen P, Schares G: A novel Ion AmpliSeq-based typing method of *Toxoplasma gondii* reveals a higher typing resolution among type II isolates than microsatellite typing
15th International Congress of Parasitology, Copenhagen, Denmark 21st – 26th of August 2022

Joeres M, Maksimov P, Höper D, Calvelage S, Calero-Bernal R, Fernández-Escobar M, Koudela B, Blaga R, Globokar Vrhovec M, Stollberg K, Bier N, Sotiraki S, Sroka J, Piotrowska W, Kodym P, Basso W, Conraths F J, Mercier A, Galal L, Dardé M L, Balea A, Spano F, Caccio S, Ortega-Mora L M, Jokelainen P, Schares G: Typing of European *Toxoplasma gondii* type II strains by a novel Ion AmpliSeq-based method
30th Annual Meeting of the German Society for Parasitology, Gießen, Germany, 15th – 17th of March 2023

Wijburg S R, Montizaan M G E, Kik M J L, Joeres M, Cardron G, Maksimov P, Luttermann C, Opsteegh M, Dijkstra V, Maas M, Schares G: Various genotypes of *Toxoplasma gondii* type II frequently detected in Eurasian red squirrels (*Sciurus vulgaris*) found dead in the Netherlands Tagung der DVG-Fachgruppe „Parasitologie und parasitäre Krankheiten“ 2023, Munich, Germany, 15th – 17th of May 2023

8.3 Poster presentations

Joeres M, Maksimov P, Tuschy M, Bärwald A, Conraths F J, Koudela B, Blaga R, Caccio S, Fernández-Escobar M, Calero-Bernal R, Ortega-Mora L M, Jokelainen P, Schares G: Unprecedented whole genome sequencing effort reveals highly polymorphic regions in the genome of European *Toxoplasma gondii* strains

One Health EJP Annual Scientific Meeting 2021 (OHEJP ASM2021), Copenhagen, Denmark, and online, 9th – 11th of June 2021

Joeres M, Maksimov P, Tuschy M, Bärwald A, Conraths F J, Koudela B, Blaga R, Caccio S, Fernández-Escobar M, Calero-Bernal R, Ortega-Mora L M, Jokelainen P, Schares G: Development of a next-generation sequencing-based typing method to detect within-genotype variation in *Toxoplasma gondii*
Tagung der DVG-Fachgruppe „Parasitologie und parasitäre Krankheiten“ 2021, online, 28th – 30th of June 2021

Joeres M, Cardron G, Passebosc-Faure K, Plault N, Fernández-Escobar M, Hamilton C, O'Brien-Anderson L, Calero-Bernal R, Maksimov P, Conraths F J, Dardé M L, Nielsen V, Ortega-Mora L M, Jokelainen P, Mercier A, Schares G: A ring trial to harmonize *Toxoplasma gondii* microsatellite typing provides the basis for a comparative analysis of results obtained in five European research laboratories
Tagung der DVG-Fachgruppe „Parasitologie und parasitäre Krankheiten“ 2022, Berlin, Germany, and online, 23th – 25th of May 2022

Joeres M, Maksimov P, Höper D, Calvelage S, Calero-Bernal R, Fernández-Escobar M, Koudela B, Blaga R, Globokar Vrhovec M, Stollberg K, Bier N, Sotiraki S, Sroka J, Piotrowska W, Kodym P, Basso W, Conraths F J, Mercier A, Galal L, Dardé M L, Balea A, Spano F, Caccio S, Ortega-Mora L M, Jokelainen P, Schares G: Typing of European *Toxoplasma gondii* type II strains by a novel Ion AmpliSeq-based technique
ApicoWplexa 2022, Bern, Switzerland, 5th – 7th of October 2022

Joeres M, Maksimov P, Conraths F J, Höper D, Calvelage S, Calero-Bernal R, Fernández-Escobar M, Ortega-Mora L M, Koudela B, Caccio S, Jokelainen P, Schares G: A novel Ion AmpliSeq-based typing method of *Toxoplasma gondii* reveals an excellent typing resolution among type II strains
Junior Scientist Symposium of Friedrich-Loeffler-Institut: Past, Present and Future – Science in Context, Greifswald, Germany, 14th – 16th of November 2022

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11 Conflict of interest

In the context of this work, there are no conflicts of interest due to contributions from third parties.

12 Declaration of independence

I hereby certify that I have prepared this thesis independently. I certify that I have used only the sources and aids indicated.

This dissertation has not yet been presented to any other examination authority in the same or a similar form and has not yet been published.

Duisburg, 04.07.2024

Maike Joeres