UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE VETERINARIA

TESIS DOCTORAL

Relevancia de la ruta medioambiental de Toxoplasma gondii y desarrollo de métodos serológicos que permitan identificar las infecciones ocasionadas por ooquistes

Relevance of the environmental route of Toxoplasma gondii and development of serological assays to diagnose oocyst-driven infections

MEMORIA PARA OPTAR AL GRADO DE DOCTORA

PRESENTADA POR

Nadia María López Ureña

DIRECTORES

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MEMORY TO OBTAIN THE DEGREE OF DOCTOR

PRESENTED BY

Nadia María López Ureña

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SUPERVISORS' APPROVAL

We, Mr. Luis Miguel Ortega Mora, doctor in Veterinary Sciences and professor (equivalent to "catedrático" in the Spanish public system) at the Animal Health Department in the Complutense University of Madrid, Mrs. Gema Álvarez García, doctor in Veterinary Sciences and professor (equivalent to "catedrático" in the Spanish public system) at the Animal Health Department in the Complutense University of Madrid, and Mr. Rafael Calero Bernal, doctor in Veterinary Sciences and associated professor (equivalent to "profesor contratado doctor" in the Spanish public system) at the Animal Health Department in the Complutense University of Madrid,

CERTIFY that:

This doctoral thesis, titled "Relevance of the environmental route of *Toxoplasma gondii* and development of serological assays to diagnose oocyst-driven infections" (in Spanish: Relevancia de la ruta medioambiental de *Toxoplasma gondii* y desarrollo de métodos serológicos que permitan identificar las infecciones ocasionadas por ooquistes), and presented by Mrs. Nadia María López Ureña, who is graduated from a Veterinary degree, has been done in the Animal Health department at the Complutense University of Madrid, under our supervision, and fulfills all requirements stablished by the University to obtain the degree of doctor and the recognition of international doctorate.

In agreement with the current regulations, we sign this approval to authorize the presentation of this doctoral thesis.

Madrid, March 5th, 2024.

Dr. Luis Ortega Mora Dr. Gema Álvarez García Dr. Rafael Calero Bernal

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DOCTORAL THESIS WITH INTERNATIONAL MENTION

This doctoral thesis meets all requirements stablished by the Complutense University of Madrid to obtain the recognition of international doctorate. This included:

- A three-months internship in a prestigious research center outside Spain:
  - Place: Robert Koch Institute, Berlin, Germany.
  - Dates: from September 15th, 2022, until December 15th, 2022.
  - Supervisor: Dr. Frank Seeber.
- The doctoral thesis was drafted in English, which is a usual language for scientific communication in this field of knowledge.
- The doctoral thesis was evaluated by at least two doctors, experts in the field, from non-Spanish higher education or research institutions.
- At least one doctor, expert in the field, from non-Spanish higher education or research institutions, was part of the evaluating committee on the day of the doctoral thesis defense.

DOCTORAL THESIS PRESENTED IN PUBLICATIONS FORMAT

This doctoral thesis is a compendium of four studies that were published in indexed scientific journals and that systematically outlined the progression and outcomes of its defined research plan. In addition, the results from Mrs. López Ureña´s international internship will be published soon and were included herein as an article draft. The journals met the criterion established by authorities from the University and this presentation format has been authorized previously. The included published articles are the following:

1) Authors: Nadia-María López-Ureña, Umer Chaudhry, Rafael Calero-Bernal, Santiago Cano-Alsua, Davide Messina, Francisco Evangelista, Martha Betson, Marco Lalle, Pikka Jokelainen, Luis-Miguel Ortega-Mora, Gema Álvarez-García.

Title: Contamination of soil, water, fresh produce, and bivalve mollusks with *Toxoplasma gondii* oocysts: a systematic review.

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2) Authors: Nadia-María López-Ureña, Rafael Calero-Bernal, Nuria González-Fernández, Radu Blaga, Bretislav Koudela, Luis-Miguel Ortega-Mora, Gema Álvarez-García.

Title: Optimization of the most widely used serological tests for a harmonized diagnosis of *Toxoplasma gondii* infection in domestic pigs.

Journal, volume and pages: Veterinary Parasitology, 322/ 110024.

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3) Authors: Nadia-María López-Ureña, Rafael Calero-Bernal, Ángela Vázquez-Calvo, Roberto Sánchez-Sánchez, Luis-Miguel Ortega-Mora, Gema Álvarez-García.

Title: A comparative study of serological tests used in the diagnosis of *Toxoplasma gondii* infection in small ruminants evidenced the importance of cross-reactions for harmonizing their diagnostic performance.

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4) Authors: Nadia-María López-Ureña, Rafael Calero-Bernal, Bretislav Koudela, Simona Cherchi, Alessia Possenti, Fabio Tosini, Sandra Klein, Carmen San Juan-Casero, Silvia Jara-Herrera, Pikka Jokelainen, Javier Regidor-Cerrillo, Luis-Miguel Ortega-Mora, Furio Spano, Frank Seeber, Gema Álvarez-García.

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5) Authors: Nadia-María López-Ureña, Frank Seeber, Furio Spano, Bretislav Koudela, Rafael Calero-Bernal, Pikka Jokelainen, Luis-Miguel Ortega-Mora, Gema Álvarez-García.

Title: A thorough approach provides evidence for low diagnostic value of TgERP (TgLEA850) protein as a serological indicator for *Toxoplasma gondii* oocyst-derived infection.

Other data: manuscript not submitted (included as **Appendix 1**).

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Nadia María López Ureña

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ABSTRACT

Toxoplasma gondii, the etiological agent of toxoplasmosis, is a major foodborne parasite that can infect all homeothermic animals, including humans. *Toxoplasma gondii* typically leads to asymptomatic infections or mild flu-like symptoms in immunocompetent individuals, but it can cause reproductive failure in pregnant women, severe neurological and respiratory diseases in immunocompromised individuals, and it is a leading cause of posterior uveitis in immunocompetent patients. Moreover, it is a major cause of reproductive failure and economic losses in the livestock industry, especially in small ruminants.

Although the contribution of each route of transmission remains unknown, the environmental route is more significant than previously believed. In fact, until 2018 approximately 44.1% of worldwide documented human toxoplasmosis outbreaks were linked to oocyst contamination, with the largest outbreak involving more than 900 confirmed cases. However, this route has been poorly studied and, despite several attempts, no source-attributing serological tool is still available. This represents a relevant gap to determine the true extent of environmental transmission, hindering the development and implementation of effective intervention strategies.

Based on this, the objective of the present thesis was to enhance our comprehension of the role played by various environmental matrices in *T. gondii* transmission and to identify oocyst-specific proteins for developing and validating an oocyst-attributing serological test. To achieve this, first, the relevance of the environmental route and the potential limitation related to sampling strategies and detection methods employed in different environmental matrices was studied (objective 1) through a systematic review conducted on the direct detection of *T. gondii* oocysts in soil, water, fresh produce and bivalve mollusks worldwide (sub-objective 1.1). Data regarding sampling strategies, oocyst recovery and detection methods, and detection rates, among others, were collected and analyzed. A total of 102 out of 3201 articles were selected, with reported detection rates between 0.09% (1/1109) and 100% (8/8) based either on bioassay or molecular methods. *Toxoplasma gondii* was detected worldwide in all evaluated matrices, including a wide range of fresh produce. Most of the studies were conducted in America, mainly in Brazil. However, the absence of standardized methodologies led to significant heterogeneity among the published results. These outcomes highlight the

significant risk that environmental matrices may pose in the transmission of the parasite, emphasizing the need to differentiate between oocyst- and tissue cysts-driven infections.

Subsequently, a restrictive step-by-step workflow was followed herein to develop and validate the oocyst-attributing serological test (objective 2). Since no gold standard test is available for the serodiagnosis of *T. gondii*, the characterization of reference serum panels was performed through a comparative study of multiple serological tests that allowed their diagnostic validation (sub-objective 2.1). The serum panels characterized herein corresponded to pigs experimentally infected with oocysts and tissue cysts from type II and type III isolates, and sheep experimentally infected with oocysts from type II isolates. Blood samples were collected prior to the infection and up to six weeks post-infection (wpi) (42 pigs: n= 305, 20 sheep: n= 124). Sera from pigs (n= 244), sheep (n= 239) and goats (n= 434) naturally infected with *T. gondii* were also included in the comparative study to validate the tests with samples that might better mimic what happens in the field. Cross-reactivity with anti-*N. caninum* IgGs was also studied for small ruminants.

In the comparative study, first, a criterion of seropositivity for a tachyzoite based-Western blot test (WB) (TgSALUVET WB) was established. Later, TgSALUVET WB was regarded as reference to preliminarily validate a lyophilized tachyzoite-based enzyme-linked immunosorbent assay (ELISA) (TgSALUVET ELISA 2.0) and, in the case of pigs, also an immunofluorescence antibody test (TgSALUVET IFAT). Then, the in-house tests were subjected to a comparative study together with up to four commercial ELISA tests (PrioCHECK, IDScreen, Pigtype and IDEXX), using the results obtained by most of them as reference. The kinetics of anti-*T. gondii* IgGs and cross-reactivity with anti-*Neospora caninum* IgGs were evaluated. All tests showed good to excellent performance with all serum panels (sensitivity [Se] and specificity [Sp] equal to or above 93%), except for PrioCHECK with naturally and experimentally infected pigs (Sp= 72–73%) and for TgSALUVET WB in the case of naturally infected pigs (Se= 71%). However, a cutoff value readjustment allowed diagnostic performance improvement and data harmonization for all ELISA tests. A significant increase in anti-*T. gondii* IgG levels was recorded from two to three wpi with all techniques. Furthermore, cross-reactions between *T. gondii* antigens

and anti-*N. caninum* IgGs were recorded in all ELISA tests with sheep sera. Thus, additional cutoff readjustments were required to avoid false positive results during small ruminants testing.

Afterwards, previously characterized sera from pig and sheep experimentally infected with oocysts and tissue cysts were used to develop a source-attributing serological tool (sub-objective 2.2). A total of 32 proteins that had been found to be specific to *T. gondii* sporozoites or sporocysts/oocysts walls through an *in silico* approach based on *T. gondii* omics data were successfully expressed in their recombinant form. Among them, those proteins already described in the literature as oocyst-specific markers (TgERP, TgCCp5A, TgSporoSAG, TgOWP1, and TgOWP8), were included. The proteins that in the screening step with a limited set of sera induced seroconversion and discriminated between oocyst- and tissue cyst-derived infections based on WBs (TgCCp5A and TgSR1), were selected to develop source-attributing ELISAs for pigs. Unfortunately, when TgCCp5A and TgSR1-based ELISAs were validated with all pig serum samples from experimental infections conducted with oocysts and tissue cysts (n= 261, up to six wpi), using WB as reference, both proteins showed the same limitations of the previously discarded proteins: low antigenicity, lack of stage-specificity and unspecific reactivity in non-infected animals.

To conclude the search of valuable antigens for oocyst-attributing serological tools, additional efforts were dedicated to assessing the usefulness of TgERP, the unique protein used in several studies for identifying oocyst-derived infections in animals and humans. For this, specific antibodies produced in rabbits against TgERP (anti-rTgERP) or sporulated oocysts lysate (anti-TgOocyst) were used as controls. Batch-to-batch differences (n= 3) and various ELISA plate systems (in-house and commercial, with and without nickel-nitrilotriacetic acid [Ni-NTA]) aiming to improve the protein affinity and the orientation of the epitopes, were evaluated. The kinetics of IgG antibodies were also evaluated with pig sera. Anti-rTgERP showed positive reactivity with all TgERP batches and ELISA plate systems, while positive reactivity was recorded with anti-TgOocyst only with one TgERP batch and the conventional plate system (without Ni-NTA). No seroconversion was recorded in experimentally infected pigs.

In conclusion, environmental matrices (soil, water, fresh produce and bivalve mollusks) represent a relevant source of infection worldwide and, therefore, a concern for animal and public health. However, further harmonization of sampling strategies and diagnostic methodologies is required for future studies. Under this scenario, the development of a serological tool with source-attributing value was tackled. Unfortunately, despite the extensive work done herein in identifying and screening a wide panel of oocyst-specific proteins, no suitable marker for oocyst-attributing serological diagnosis was found and led us to also discard TgERP as a valuable marker of oocyst-derived infections. By contrast, this thesis allowed the validation of the most widely used conventional serological tests in the diagnosis of *T. gondii* infection in domestic pigs and small ruminants and provides practical recommendations to avoid false positive results. These findings are significant for advancing research on *T. gondii* environmental contamination and diagnostic serological tools development and validation.

Toxoplasma gondii, agente etiológico de la toxoplasmosis, es un parásito de transmisión alimentaria que infecta a todos los animales homeotermos, incluyendo a los seres humanos. Generalmente causa una infección asintomática o similar a la gripe, pero también produce problemas reproductivos en mujeres gestantes, cuadros neurológicos y respiratorios graves en individuos inmunodeprimidos y es una de las causas más frecuentes de uveítis posterior en pacientes inmunocompetentes. Además, la infección por *T. gondii* es una importante causa de fallo reproductivo en los pequeños rumiantes, lo que se traduce en relevantes pérdidas económicas para el sector.

Aunque se desconoce la contribución de cada ruta de transmisión, la ruta ambiental es más importante de lo que se pensaba anteriormente. Aproximadamente, un 44.1% de los brotes de toxoplasmosis humana documentados en el mundo hasta el 2018 fueron causados por ooquistes, con brotes con más de 900 casos confirmados. Sin embargo, este modo de transmisión ha sido poco estudiado y, a pesar de los intentos, aún no se dispone de una herramienta serológica que permita diferenciar entre rutas de transmisión. Esto impide determinar el verdadero impacto de la ruta medioambiental y el desarrollo y la implementación de estrategias adecuadas de intervención.

Basándonos en esto, el objetivo de esta tesis doctoral fue indagar sobre el papel desempeñado por diversas matrices ambientales en la transmisión de la infección por *T. gondii* e identificar proteínas específicas del ooquiste para desarrollar y validar una prueba serológica que permita identificar infecciones debidas a la ingestión de ooquistes. Para ello, primero se estudió la relevancia de la ruta medioambiental y las posibles limitaciones relacionadas a las estrategias de muestreo y los métodos de detección empleados en distintas matrices medioambientales (objetivo 1) a través de una revisión sistemática sobre la presencia de *T. gondii* en suelo, agua, productos frescos y moluscos bivalvos a nivel mundial (sub-objetivo 1.1). Se recopilaron y analizaron datos sobre estrategias de muestreo, métodos de concentración y detección, y tasas de detección, entre otros. Se seleccionaron un total de 102 de entre 3201 artículos, con tasas de detección entre 0,09% (1/1109) y 100% (8/8) basadas en métodos moleculares o bioensayo. *Toxoplasma gondii* se detectó en todas las matrices estudiadas a nivel mundial. La mayoría de los estudios se realizaron en América, principalmente en Brasil. Sin embargo, se observó una falta de protocolos estandarizados, lo que se tradujo en

una alta heterogeneidad entre artículos. Estos resultados resaltan el potencial riesgo que presentan las matrices medioambientales en la transmisión del parásito, destacando también la necesidad de diferenciar entre las infecciones debidas a la ingestión de ooquistes o de quistes tisulares.

Posteriormente, se siguió un exhaustivo flujo de trabajo para desarrollar y validar una prueba serológica que permitiera identificar infecciones causadas por ooquistes (objetivo 2). Dado que no hay una prueba de referencia para el serodiagnóstico de la infección por *T. gondii*, primero se hizo una caracterización de los sueros de referencia mediante un estudio comparativo empleando múltiples pruebas serológicas, estudio que también permitió la validación de las mismas (sub-objetivo 2.1). Los sueros caracterizados procedían de cerdos y ovejas infectados experimentalmente con ooquistes o quistes tisulares de genotipos II y III, cuyas muestras de sangre fueron tomadas antes de la infección y hasta seis semanas post-infección (spi) (42 cerdos: n= 305, 20 ovejas: n= 124). En este estudio también se incluyeron sueros de cerdos (n= 244), ovejas (n= 239) y cabras (n= 434) infectados naturalmente para validar las pruebas con muestras que mimetizan lo que sucede en la naturaleza. También se analizaron las reacciones cruzadas con IgGs anti-*N. caninum* en el caso de pequeños rumiantes.

En el estudio comparativo, primero se definió un criterio de seropositividad para un Western blot (WB) utilizando como antígeno taquizoítos del parásito (TgSALUVET WB). Posteriormente, se empleó TgSALUVET WB de referencia para desarrollar y preliminarmente validar un enzimoinmunoensayo (ELISA) empleando taquizoítos liofilizados como antígeno (TgSALUVET ELISA 2.0) y, en el caso de cerdos, también una prueba de inmunofluorescencia indirecta (TgSALUVET IFAT). A continuación, se estudiaron las características diagnósticas de estas tres pruebas junto a cuatro pruebas ELISA comerciales (PrioCHECK, IDScreen, Pigtype e IDEXX). Los resultados obtenidos por la mayoría de las técnicas se emplearon como referencia. Se evaluó la cinética de anticuerpos IgG anti-*T. gondii* y las reacciones cruzadas con IgGs de ovejas frente a *Neospora caninum*. Todas las pruebas mostraron un buen rendimiento diagnóstico con todos los sueros de referencia (sensibilidad [Se] y especificidad [Es] igual o superior al 93%), excepto PrioCHECK en cerdos infectados experimental o naturalmente (Es= 72–73%) y TgSALUVET WB en cerdos infectados naturalmente (Se= 71%). Sin embargo, un

ajuste del punto de corte permitió mejorar las características diagnósticas de las pruebas ELISA. Esta similitud en rendimiento se reflejó en las cinéticas de anticuerpos IgG anti-*T. gondii,* con un incremento significativo desde las dos-tres spi en todas las técnicas. Además, se registraron reacciones cruzadas con IgGs anti-*N. caninum*, por lo que, para evitar resultados falsos positivos, fueron necesarios ajustes adicionales de los puntos de corte en todas las pruebas de ELISA en los pequeños rumiantes.

Luego, los sueros previamente caracterizados de cerdos y ovejas infectados experimentalmente con ooquistes y quistes tisulares se utilizaron para desarrollar una herramienta serológica que identificara la ruta de transmisión (sub-objetivo 2.2). Se expresaron con éxito en su forma recombinante un total de 32 proteínas que habían resultado ser específicas de esporozoítos o paredes de esporoquistes/ooquistes de *T. gondii* en un estudio *in silico* de datos ómicos. Entre estas proteínas se incluyeron aquellas ya descritas en la literatura como marcadores específicos de ooquistes (TgERP, TgCCp5A, TgSporoSAG, TgOWP1 y TgOWP8). Las proteínas que, en la etapa de cribado con número reducido de sueros, demostraron inducir seroconversión y discriminar entre infecciones derivadas de ooquistes y quistes tisulares según las pruebas WB (TgCCp5A y TgSR1), fueron seleccionadas para desarrollar ELISAs que permitieran identificar rutas de transmisión en cerdos. Desafortunadamente, cuando los ELISAs basados en TgCCp5A y TgSR1 se validaron empleando todos los sueros de cerdos infectados experimentalmente con ooquistes y quistes tisulares (n= 261, de hasta seis spi), usando de referencia la prueba WB, ambas proteínas mostraron los mismos problemas que las proteínas previamente descartadas: baja antigenicidad, incapacidad para detectar exclusivamente infecciones inducidas por ooquistes y reactividad inespecífica en animales no infectados.

Para finalizar con la búsqueda de antígenos de valor para el desarrollo de un ELISA que permita detectar infecciones inducidas por ooquistes, se realizaron esfuerzos adicionales encaminados a reevaluar la utilidad de TgERP, la proteína específica de ooquistes más utilizada en estudios previos en humanos y animales. Para ello, se emplearon como controles anticuerpos específicos producidos en conejos frente a TgERP (anti-rTgERP) o frente al extracto total de ooquistes esporulados (anti-TgOocyst). Se evaluaron las variaciones entre lotes (n= 3) y distintos sistemas de placas de ELISA (“in-house” y comerciales, con y sin níquel-ácido nitrilotriacético [Ni-NTA]) con el objetivo de mejorar

la afinidad de la proteína y la orientación de los epítopos. También se evaluaron las cinéticas de los anticuerpos IgGs con sueros de cerdos. El suero anti-rTgERP mostró reactividad positiva con todos los lotes de TgERP y sistemas de placas ELISA, mientras que el anticuerpo anti-TgOocyst sólo la mostró con un lote de TgERP y el sistema de placa convencional (sin Ni-NTA). Utilizando este antígeno no se registró seroconversión en los cerdos infectados experimentalmente.

En conclusión, las matrices medioambientales (suelo, agua, productos frescos y moluscos bivalvos) representan una importante fuente de infección a nivel mundial y, por lo tanto, un problema en la salud pública y sanidad animal. Sin embargo, se necesita una mayor harmonización en las estrategias de muestreo y las metodologías diagnósticas para futuros estudios. Partiendo de este escenario, se abordó el desarrollo de una prueba serológica que permitiera identificar la ruta de transmisión. Desafortunadamente, a pesar del esfuerzo realizado en la identificación y selección de un amplio panel de proteínas específicas del ooquiste, ninguna de ellas resultó ser útil para la identificación serológica de infecciones causadas por ooquistes, descartando también la proteína TgERP como marcador de infecciones inducidas por ooquistes. Sin embargo, esta tesis doctoral permitió la validación de las técnicas serológicas convencionales más empleadas en el diagnóstico de la infección causada por *T. gondii* en cerdos y pequeños rumiantes domésticos, y proporciona recomendaciones prácticas para evitar resultados falsos positivos. Estos hallazgos son importantes para avanzar en la investigación sobre la contaminación ambiental por *T. gondii* y el desarrollo y la validación de herramientas serológicas diagnósticas.

CHAPTER II: INTRODUCTION

1. *Toxoplasma gondii* and toxoplasmosis, a paradigm of the One Health initiative

Toxoplasma gondii is a worldwide distributed foodborne zoonotic pathogen that causes toxoplasmosis, a disease with systemic affection that gives rise to a wide spectrum of clinical manifestations and responsible for remarkable clinical, social, and economic impacts (Dubey, 2021). Toxoplasmosis is usually self-limiting in most of immunocompetent humans (Djurković-Djaković et al., 2019), with the presentation of flu-like mild clinical signs/symptoms in 10% of them during the acute phase (Montoya and Liesenfeld, 2004). However, as it is an opportunistic pathogen, two risk groups deserve special attention, pregnant woman and immunocompromised people, who may develop reproductive failure and severe respiratory and neurological diseases, respectively (Barratt et al., 2010; Berger et al., 2009; Garweg et al., 2022). *Toxoplasma gondii* is a relevant pathogen within the veterinary field, not only owing to its zoonotic potential but also due to its substantial impact on the health and reproduction of several animal species (Dubey et al., 2020b, 2020a; Innes et al., 2009; Rodrigues Oliveira et al., 2022; Roe et al., 2013; Salas-Fajardo et al., 2023; Shapiro et al., 2019b).

Toxoplasma gondii is widely acknowledged as one of the most successful parasites (Djurković-Djaković et al., 2019). Its success is attributed to its cosmopolitan distribution, as it can infect a broad spectrum of susceptible hosts. It also exploits multiple transmission routes and maintains a triad of stages adapted to different spread strategies, predation, congenital transmission, and environmental resistance, in a given epidemiological scenario. As result, *T. gondii* stands as a paradigm of the One Health initiative (Aguirre et al., 2019; Djurković-Djaković et al., 2019), demanding multidisciplinary approaches for its study and the design of effective control and prevention strategies.

Toxoplasma gondii was discovered in 1908 by Nicolle and Manceaux in tissues from a North African rodent (*Ctenodactylus gundi*) used with research purposes at the Pasteur Institute in Tunisia and earned its name from its distinctive shape (Τοξο/toxo= bow and plasma= image/life in Greek) and the rodent host from where it was isolated. In the same year, Splendore also found *Toxoplasma* in a rabbit in Brazil, although he did not name it

(Dubey, 2008). The most important research milestones in the history of *T. gondii* and toxoplasmosis are summarized in **Table 1**.

Table 1. Summary of key milestones in the history of *Toxoplasma gondii* and toxoplasmosis (adapted from Dubey, 2008).

Finding	Reference
Etiologic agent	
Recognition in a rodent and in a rabbit	(Nicolle and Manceaux, 1908; Splendore, 1908)
Name assignment	(Nicolle and Manceaux, 1909)
Isolation from animals and humans	(Sabin and Olitsky, 1937; Wolf et al., 1939)
Identification of definitive and intermediate hosts	(Frenkel et al., 1970; Miller et al., 1972)
Parasite stages	
Tachyzoites	
Ultrastructure	(Gustafson et al., 1954; Sheffield and Melton, 1968)
Endodyogeny	(Goldman et al., 1958)
Term assignment	(Frenkel, 1973)
Tissue cysts and bradyzoites	
Recognition and cytologically description	(Frenkel, 1956; Frenkel and Friedlander, 1951; Levaditi et al., 1928)
Recognition of digestive enzyme resistance	(Jacobs et al., 1960)
Term assignment and description of tissue cyst	(Dubey and Beattie, 1988; Frenkel, 1973)
Ultrastructure	(Ferguson and Hutchison, 1987; Wanko et al., 1962)
Oocysts and sporozoites	
Coccidian phases	(Dubey and Frenkel, 1972; Frenkel et al., 1970)
Morphology	(Dubey et al., 1970)
Ultrastructure	(Sheffield, 1970; Speer and Dubey, 2005)
Transmission	
Congenital	
In humans	(Wolf et al., 1939)
In house mouse	(Beverley, 1959)
In large wild animals (white tailed deer)	(Dubey et al., 2008)
Meatborne	
Suspicion/suggestion	(Weinman and Chandler, 1954)
In humans	(Desmonts et al., 1965)
Environmental	
Demonstration	(Hutchison, 1965)
Genotypes and strains	
Grouped into three clonal types (I, II and III)	(Howe and Sibley, 1995; Sibley et al., 1992)
Genome	(Khan et al., 2005)
Toxoplasmosis	
In humans	
Clinical signs in congenital transmission	(Sabin, 1942)
Postnatal infection in a child	(Sabin, 1941)
Fatal course in adults	(Pinkerton and Weinman, 1940)
Susceptibility of acquired immunodeficiency syndrome (AIDS) patients	(Luft et al., 1983)
Chronic infections	(Kean and Grocott, 1947; Plaut, 1946)
In animals	
Domestic dog	(Mello, 1910)
Epidemic abortions in sheep	(Dubey and Beattie, 1988)
Marine mammal species	(Cole et al., 2000)

Finding	Reference
Immunity and protection	
Neutralizing antibodies	(Sabin and Ruchman, 1942)
Anti-*T. gondii* antibodies kill extracellular, but not intracellular parasites	(Sabin and Feldman, 1948)
Diagnosis	
Sabin–Feldman dye test	(Sabin and Feldman, 1948)
Direct and modified agglutination tests (DAT, MAT)	(Desmonts and Remington, 1980; Dubey and Desmonts, 1987)
Other serological tests	(Dubey, 1997; Dubey et al., 1995)
Polymerase chain reaction (PCR)	(Burg et al., 1989)

1.1. Taxonomy, morphology and life cycle

Toxoplasma gondii is an obligate intracellular parasite, member of the Sarcocystidae family, from which only one species has been documented. However, other parts of its taxonomy have suffered changes over the years, which are summarized in **Table 2.**

Table 2. *Toxoplasma gondii* taxonomy.

	Classical[1]	**Classical[2]**	**Modern[3,4]**
Phylum	Apicomplexa	Apicomplexa*	Myozoa
Infraphylum	-	-	Apicomplexa
Superclass	-	-	Sporozoa
Class	Conoidasida	Coccidia	Coccidiomorphea
Order	Eucoccidiorida	Eimeriida	Eimeriida
Family	Sarcocystidae	Sarcocystidae	Sarcocystidae
Genus	*Toxoplasma*	*Toxoplasma*	*Toxoplasma*
Species	*gondii*	*gondii*	*gondii*

[1]Taylor et al., 2016, [2]Deplazes et al., 2016, [3]Ruggiero et al., 2015, [4]Delgado et al., 2022.*Corresponds to the subphylum.

Toxoplasma gondii has a facultative heteroxenous life cycle in which members of the Felidae family are the only definitive hosts (DHs) and the rest of homoeothermic animals may serve as intermediate hosts (IHs). It presents three distinct infective stages: sporozoites (contained within sporulated oocysts), bradyzoites (contained within tissue cysts) and tachyzoites. Each of these stages exhibits distinctive elongated and arching/bow-like shapes and the presence of the typical apicomplexan apical complex where different organelles (rhoptries, conoid, micronemes, among others) are presented and related to parasite's invasion capabilities (Attias et al., 2020) **(Figure 1)**.

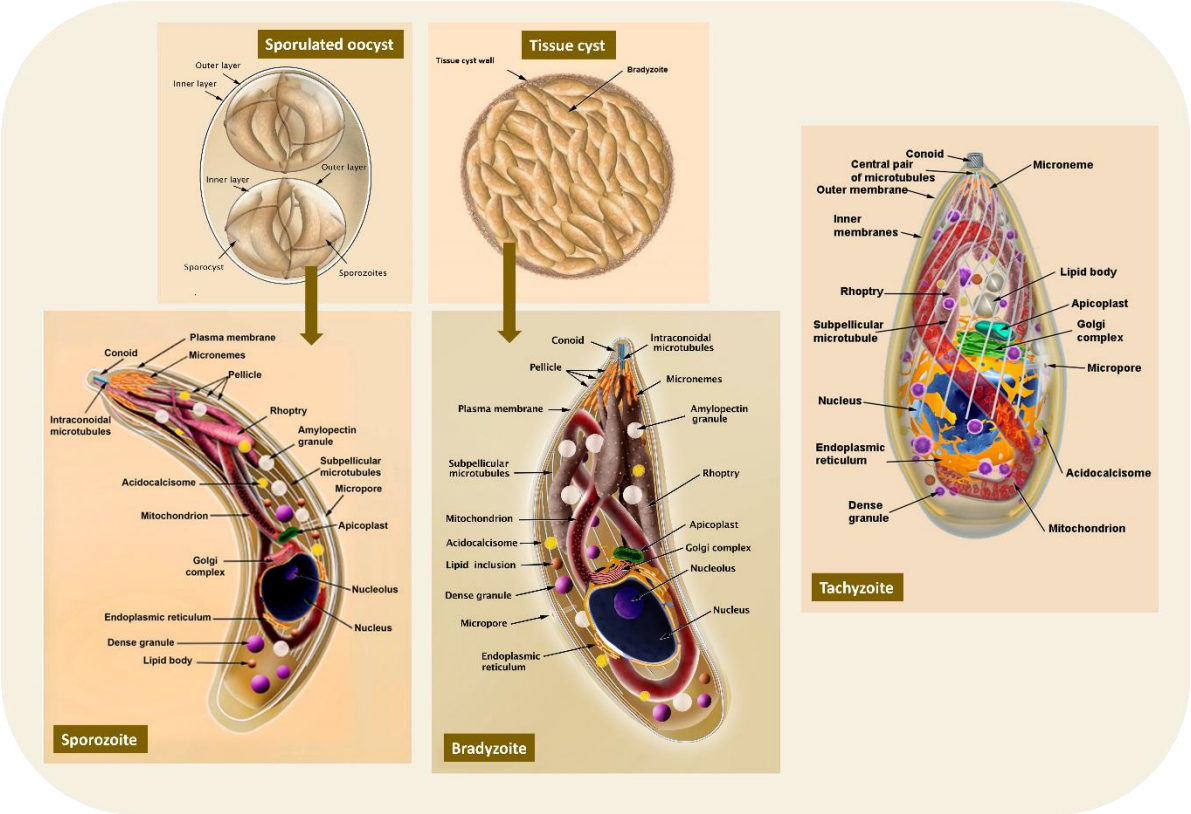

Figure 1. Infective stages of *Toxoplasma gondii* (adapted from Attias et al., 2020).

Oocysts result of the *T. gondii* sexual replication in the gut of the DHs. Recently shed and unsporulated oocysts are ovoid (10 × 12 µm) and contain a diploid cytoplasmatic mass protected by a bilayered wall. When they become mature (sporulated) in the environment, they take an ellipsoidal shape (11 × 13 µm) and two sporocysts are developed within, each containing four banana-shaped sporozoites (2–6 × 8 µm) (Dumètre et al., 2013; Freppel et al., 2019) **(Figure 1)**. Tachyzoites and bradyzoites arise during asexual replication, which could take place in any tissue of both the DHs and IHs. Tachyzoites (tachos= speed in Greek) (2 x 6 µm) are the rapid proliferation stage of *T. gondii,* responsible of the acute phase of the infection and the dissemination through host's organs **(Figure 1)**, while bradyzoites (brady= slow in Greek) (1.5 x 7 µm) are the slow-dormant replicating stage that develop during the establishment of a chronic infection within tissue cysts. Tissue cysts are particularly present in neural and muscle tissues, have an elastic and thin wall without internal septae and cluster up to hundreds of bradyzoites reaching different sizes depending on their maturity (5–100 µm) **(Figure 1)** (Dubey et al., 1998).

The life cycle of *T. gondii* typically starts when the DHs, domestic or wild felids, become infected by ingesting tissue cysts through contaminated raw meat from chronically infected IHs that serve as prey, such as small mammals (e.g., rodents) and birds, even though they can also get infected with tachyzoites (e.g., vertical transmission) or sporozoites, being both less efficient than bradyzoites in establishing the infection in the DHs (Dubey, 2010a). When bradyzoites are released from tissue cysts, they invade the epithelial cells of the DHs' intestines and undergo both asexual and sexual replication (Dubey et al., 1998). Unsporulated oocysts result from the gametocytes fusion and are later shed with felids' feces. Once in the environment, oocysts are spread with the aid of rainfalls, wind and other factors, and contaminate the soil, water bodies, fresh produce and shellfish (Shapiro et al., 2019a). Nevertheless, they only become infectious when sporulated, after a few days under certain conditions (around 7 days at 20–25 °C) (Freppel et al., 2019) **(Figure 2)**.

Intermediate hosts can become infected with tachyzoites during pregnancy, via placenta. Tachyzoites could be also transmitted in a transfused blood from an asymptomatic IH with parasitemia at the moment of the blood collection (Attias et al., 2020) or the ingestion of contaminated raw milk or cheese from infected food-producing animals (Dubey, 2021). In addition, IHs can get infected with sporulated oocysts when consuming contaminated food (e.g., vegetables, fruits, shellfish), feed (e.g., pastures and forages) and water, or with tissue cysts when consuming raw or undercooked meat from other chronically infected IHs (Attias et al., 2020; Montoya and Liesenfeld, 2004) **(Figure 2)**.

The success of each parasite stage at using different sources of transmission and at establishing the infection is, at some extend, related to their resistance characteristics. For example, oocysts, which are related to the exogenous life cycle of the parasite, are resistant to environmental conditions. Actually, numerous studies have demonstrated their resistance to commonly used disinfectants, such as bleach, gaseous chlorine, ozone, ethanol, and formalin, as well as some temperatures (freezing and up to 45°C), representing a challenge for the prevention of toxoplasmosis (Arranz-Solís et al., 2023; Freppel et al., 2019; Fritz et al., 2012a). Contrary, bradyzoites and tachyzoites are

susceptible to those conditions, and tachyzoites are less resistant to digestive enzymes than bradyzoites (Jacobs et al., 1960).

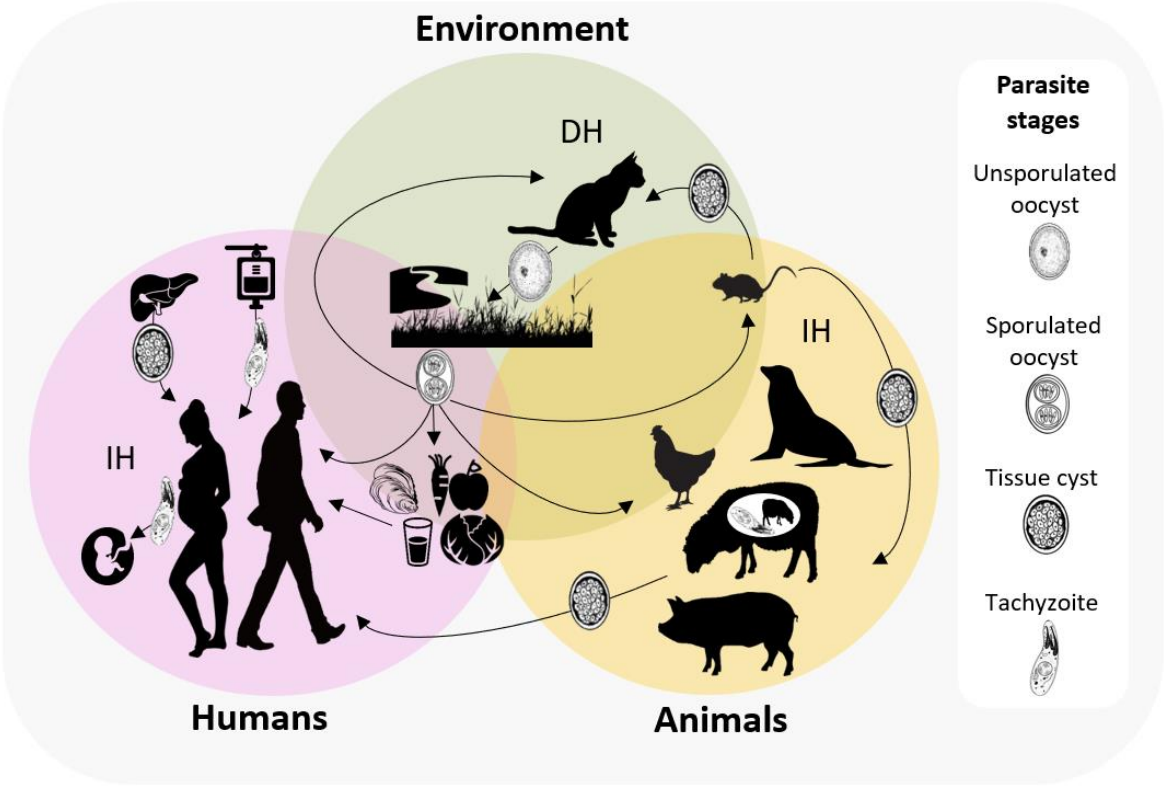

Figure 2. Life cycle of *Toxoplasma gondii*. DH: definitive host, IH: intermediate host.

1.2. Routes of transmission

The major transmission pathways of *T. gondii* can be categorized into the following: a) the environmental route, involving oocysts that contaminate different environmental matrices, b) meatborne route, through tissue cysts that are present in the meat or derivates of chronically infected animals, c) maternal-fetal route, through tachyzoites via placenta from the mother to the fetus/es. The individual contributions of these routes remain unclear, presenting a significant knowledge gap from a One Health perspective. This knowledge gap hampers the development and implementation of effective intervention strategies for preventing and controlling *T. gondii* infections.

In general terms, the maternal-fetal route of transmission is very important due to its impact on the fetus/es. Although the incidence rate of human congenital toxoplasmosis is difficult to estimate since just a few countries around the world have compulsory surveillance in pregnant woman, according to a review, the global estimated incidence

rate is 1.5:1000 live birth (Torgerson and Mastroiacovo, 2013), with marked differences between regions, from 3.8:1000 births in Panama to 4.5–5.1:100000 births in Greece (Dubey et al., 2021). In the European context, congenital toxoplasmosis is a communicable disease, but only France, Greece, Slovakia, Slovenia, Austria and Belgium have compulsory screening in pregnant women, with 133 confirmed cases (France accounting for 83% of them) and an incidence of 5.08:100000 live births for 2020 (ECDC, 2023). However, in terms of susceptible hosts that can get infected through one pathway, the maternal-fetal route could be considered as the less relevant since the transmission is limited to the infected mother and her fetus/es, which, based on a systematic review, could happen in 20% of infected mothers (Li et al., 2014). Actually, 42–61% of global toxoplasmosis cases are foodborne (Hald et al., 2016) and *T. gondii* is considered the third most important foodborne parasite worldwide (WHO and FAO, 2014), putting in evidence the relevance of the post-natal transmission through the meatborne and the environmental routes. Indeed, based on disability adjusted life years (DALYs), *T. gondii* significantly contributes to the burden of foodborne disease worldwide (≥ 10 DALYs per 100000 population), especially in Central and Southern America and the Caribbean (20 DALYs per 100000 population) (Havelaar et al., 2015).

According to a systematic and a meta-regression about the direct detection of *T. gondii* in meat, global pooled prevalence resulted in 2.6%, 12.3%, and 14.7% for cattle, pigs and sheep, respectively, with mice or cat parasite isolation in many cases, while in horse and goats the considered studies provided partial indications (Belluco et al., 2016). However, Belluco et al. (2016) remarked that this data could be overestimated due to potential bias, in addition to the reported high heterogeneity among articles and the significant influence of geographical areas, farming systems, animal age, among other variables. Respect to environmental matrices, PCR-*T. gondii* positive samples ranged from 0% (n= 120) in the United States to 49.82% in France in soil, 7.7% (n= 482) in France to 58.6% (n= 46) in Colombia in water, and 0.8% (n= 648) in Italy to 9.7% (n= 216) in Poland in fresh produce (Shapiro et al., 2019a).

There are some available studies about the contribution of different foods as source of human infections worldwide. In general, meat has been considered as a primary source

for *T. gondii*, whilst fresh produce, seafood and dairy products have been regarded as secondary sources (WHO and FAO, 2014). In agreement, Hoffmann et al. (2017), who took into consideration experts elicitations from 14 global subregions, estimated that red meats (e.g., from small ruminants, pork and beef) were the most important sources of toxoplasmosis cases in all subregions, causing between 50% and 64% of the infections, while vegetables contributed to a smaller number of cases, causing up to 23% of the infections and with variations between areas. Similarly, most of the worldwide human toxoplasmosis outbreaks have been attributed to the consumption of contaminated meat (Pinto-Ferreira et al., 2019a). However, as mentioned before, in terms of susceptible hosts, the environmental route results more relevant since *T. gondii* could reach a higher number of hosts in a shorter interval time (e.g., through contaminated water) compared to the meatborne (Balbino et al., 2022; Minuzzi et al., 2021) (see outbreaks information in section 1.3). In addition, the environmental route results more challenging than the meatborne route due to the difficulties to inactivate oocysts, which resist the common treatments applied in the food industry (Arranz-Solís et al., 2023; Freppel et al., 2019).

It is important to point out that there is no compulsory surveillance in foods and no standardized methods for the direct detection of *T. gondii* in different matrices. Thus, available data, even if they are based on published studies or professional experience and expertise, could be under or overestimated and might result not comparable. Furthermore, most of the accessible information of source attribution is related to outbreaks, which not necessarily represents the real scenario of what has happened in the global human population infected with *T. gondii*. On the other hand, it must be considered that consumption habits have a great influence on the pattern of foodborne *T. gondii* transmission.

1.3. Relevance of *Toxoplasma gondii* infections in human medicine and public health

As mentioned before, most of the infections are self-limiting in immunocompetent humans (Djurković-Djaković et al., 2019), but special consideration is warranted for two high-risk groups: pregnant women and immunocompromised individuals. Its

importance during pregnancy relies on its potential to cause congenital toxoplasmosis, with a higher probability of transmission the later the infection occurs, as well as reproductive failure, which is more pronounced during the early stages of gestation (Rico-Torres et al., 2016). Some of the reproductive failures induced by *T. gondii* are miscarriage, premature birth, stillbirth, malformation, chorioretinitis, calcifications, meningoencephalitis, and hydrocephaly, among others. Furthermore, *T. gondii* can cause hearing impairments, seizures, intellectual disabilities, development delays and ocular lesions at mid-long term in infected infants that did not show clinical signs at birth (Berger et al., 2009; Garweg et al., 2022). On the other hand, toxoplasmosis can also have life-threatening consequences in severely immunosuppressed people, in which *T. gondii* replicates and spreads rapidly either due to a recently acquired infection or a reactivation of a chronic infection. These individuals can suffer serious neurological, ocular, and respiratory diseases, which, in the absence of treatment, can lead to fatality (Barratt et al., 2010).

Around one third of the global human population has specific IgG antibodies against *T. gondii* (Dubey, 2021; Montoya and Liesenfeld, 2004; Rostami et al., 2021), indicating past exposure to the parasite. The seroprevalence varies among groups of individuals as well as geographical regions. According to a worldwide study on *T. gondii* seroprevalence in humans, which consisted on analyzing data from published systematic reviews and meta-analyses until the end of 2018, the highest pooled seroprevalence (considering IgG and IgM) was recorded in immunocompromised patients (42%) and general population (42%), followed by pregnant woman (40%), blood donors (33%), childbearing age woman (32%) and newborns and children (4%) (Rahmanian et al., 2020). Similar seroprevalence was recorded in a study focused on latent toxoplasmosis in pregnant women (33.8%), with significant higher ratios in low-income countries, the highest recorded in South America (56.2%) (Rostami et al., 2020). In relation to geographical association, based on a systematic review on human observational studies that apparently used healthy subjects, seroprevalence ranged from 0.5 to 87.7%, with the highest ratios documented in Africa (61.4%), followed by Oceania (38.5%), South America (31.2%), Europe (29.6%), the United States/Canada (17.5%) and Asia (16.4%) (Molan et al., 2019). However, the data between countries was not balanced and high

heterogeneity between studies within classified groups was reported in the majority of the cases by Rahmanian et al. (2020). In the European context, based on data from 30 countries, anti-*T. gondii* IgG overall prevalence in human population was estimated at 32.1%, with prevalence rates of 20.1%, 38.5%, 39.7% and 37.5% in the North, West, East and South regions, respectively (Calero-Bernal et al., 2023).

Considering the various methodologies employed across different studies, alongside the wide range of serological techniques utilized, it is essential to exercise caution when interpreting the data. In addition, it is important to point out that there is no compulsory surveillance for *T. gondii* infection in general population, and just a very few countries have established mandatory serological screening for the risk groups (including Brazil apart from the European countries mentioned above). Thus, published data is limited in terms of studied population segments (Peyron et al., 2017).

The earliest documented human toxoplasmosis outbreak can be traced back to 1965, a period during which environmental transmission had not been elucidated yet (Dubey, 2021). Since the ecology and epidemiological dynamic of *T. gondii* is influenced by multiple variables, including felids populations and their vicinity to urban areas, human population density, hosts susceptibility, *T. gondii* genotypes virulence, environmental conditions, food hygiene, sanitary conditions, and consumption habits, among others (Dubey, 2021; Shapiro et al., 2019a; Zhu et al., 2023), the characteristics of human toxoplasmosis outbreaks vary between geographical regions. For example, Brazil, which is a hotspot for outbreaks and where non-canonical strains are predominant and frequently associated to severe clinical signs and mortality even in immunocompetent hosts (Demar et al., 2012; Dubey et al., 2012; Vaudaux et al., 2010), it is more likely that the infection occurs through the environmental route (Balbino et al., 2022; Shapiro et al., 2019a) **(Figure 3)**. This could be attributable to poverty, underserved people, limited access to health care, the existence of precarious infrastructure for water and sewage treatment (Shapiro et al., 2019a), but it could be also remarkably influenced by consumption habits since meat is frequently consumed well-done by Brazilians. However, in Europe, where outbreaks occur infrequently and less virulent genotypes are predominant, such as type II strains (Fernández-Escobar et al., 2022), the meatborne route has been the most frequent one in followed-up outbreaks (Dubey, 2021), probably

because the meat is preferably consumed raw or undercooked **(Figure 3)**. Actually, 30–63% infections in pregnant women in Europe are attributable to the consumption of cured or undercooked meat and meat products (Cook et al., 2000).

Human toxoplasmosis outbreaks have been reported on every continent, except for the Antarctic: America (Brazil, French Guiana, Suriname, Panama, the United States and Canada), Europe (France and the United Kingdom), Africa (India), Asia (South Korea, Turkey) and Oceania (Australia). Among them, outbreaks predominated in America and Brazil counted with a greater number of recorded and investigated events (Dubey, 2021; Meireles et al., 2015; Pinto-Ferreira et al., 2019a) **(Figure 3)**.

The symptoms and clinical manifestations were very similar between outbreaks, including fever, headache, myalgia, arthralgia, stiff neck, sore throat, vomiting, eye pain, blurred vision, rash, sweats, reproductive failure, congenital transmission, and even the death of children and apparently non-immunocompromised adults. These symptoms and clinical manifestations have not remarkably varied between oocyst- and tissue-cysts driven infections (Dubey, 2021), although the incubation time seemed to be shorter in tissue cysts-driven infections (Meireles et al., 2015). Furthermore, differences due to parasite's genetic variants have been suggested since, e.g., some genotypes have shown significant tropism for the eye structures, inducing more severe ocular lesions (Dubey, 2021).

The reported (suspected) source of infection in documented outbreaks has temporarily changed; until early in the 90´s most of the outbreaks were attributed to the ingestion of meat and derivates that contained tissue cysts. Later, soil, sand and water contaminated with oocysts were proposed as the most important sources of infection up to 2000. However, recently, from 2010 onwards, many outbreaks have been attributed to vegetables and fruits contaminated with oocysts (Pinto-Ferreira et al., 2019a). This tendency aligns with the discovery of the complete cycle of *T. gondii* since the possibility of getting infected through oocysts intake was not suspected until 1970 (Dubey, 2021), as well as with the accessible information on *T. gondii* in different environmental matrices since the first studies were published relatively recently, in the last decade (Shapiro et al., 2019a). In addition, this temporary change might be also linked to variations in consumption habits, e.g., nowadays there is an increased interest

in healthy, but easy to prepare dishes, like ready-to-eat leafy greens, which, although convenient, can be susceptible to microbial contamination at various stages of the production chain (Castro-Ibáñez et al., 2017).

According to Pinto-Ferreira et al. (2019a), 47.1% (16/34) and 44.1% (15/34) of published worldwide outbreaks until early 2018 were tissue cyst- and oocyst-related, respectively. Among oocyst-related outbreaks, 46.7% (7/15), 40.0% (6/15), and 13.3% (2/15) were due to the consumption of contaminated water, soil/sand, and vegetables, respectively. Nevertheless, in the majority of the cases the source of infection was defined in retrospective and epidemiological studies since the detection and/or isolation of *T. gondii* from environmental samples failed. Even though most of the recorded outbreaks were tissue cyst-related, the highest number of affected people was counted in oocyst-related outbreaks (Dubey, 2021; Meireles et al., 2015; Pinto-Ferreira et al., 2019a).

In order to offer up-to-date information about global human toxoplasmosis outbreaks within this thesis, available data from three reviews (systematic or not) (Dubey, 2021; Meireles et al., 2015; Pinto-Ferreira et al., 2019a) was screened, analyzed and unified (duplicates were excluded) to create a map indicating the occurrence of outbreaks and the confirmed/presumed source of infection per country, as well as the interval time when they occurred and the scope of each source (number of confirmed cases) **(Figure 3)**. In summary, America continues to be a hotspot for toxoplasmosis outbreaks, where Brazil accumulates the highest number of cases (19/56). In addition, the environmental transmission route contributed in a great extend to the global occurrence of outbreaks. Furthermore, water was identified as the environmental matrix that caused the largest outbreak, where more than 900 laboratory-confirmed cases were recorded (Minuzzi et al., 2021) **(Figure 3)**. Anyhow, it is important to point out that the real scenario of human toxoplasmosis outbreaks is difficult to access since in some cases the information is only available as reports at regional or national level and therefore not accessible.

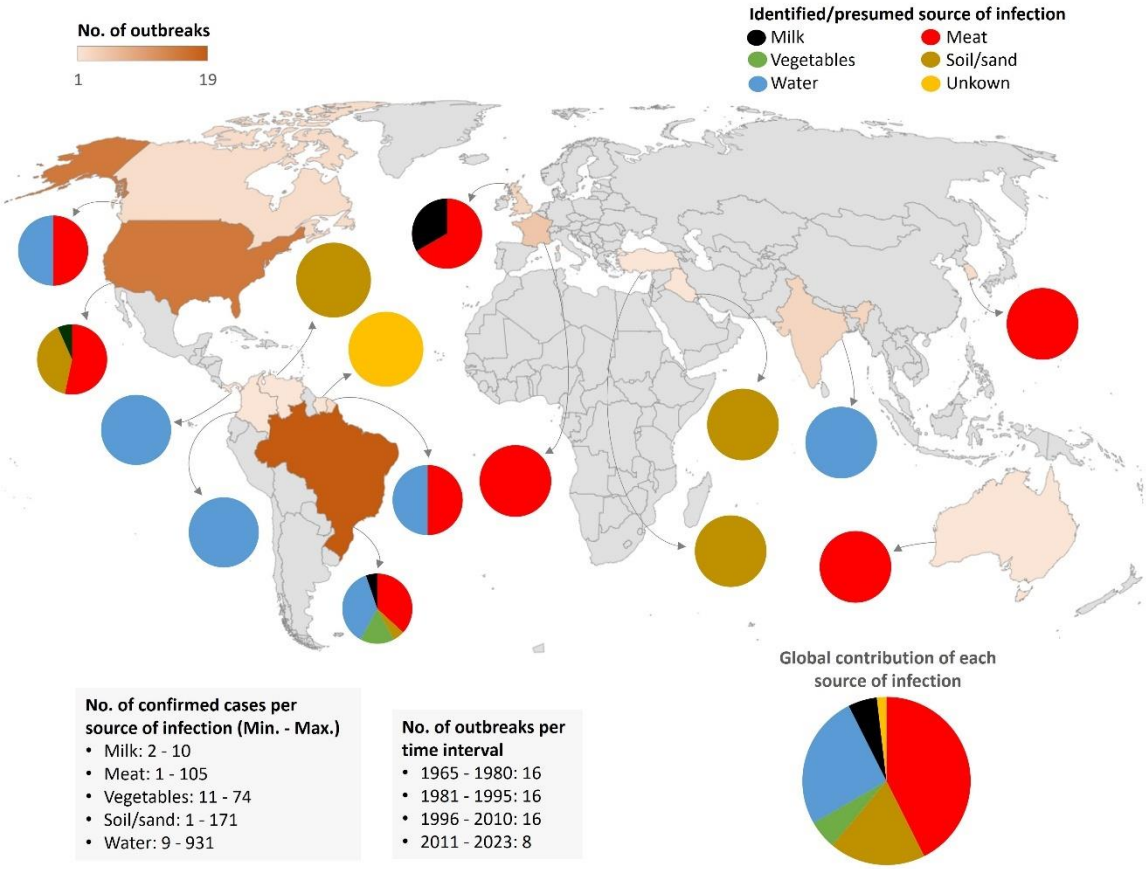

Figure 3. Worldwide human toxoplasmosis outbreaks occurred until August 2023. In the only study where the source of infection was labeled as "unknown" (circle in yellow), the environmental contamination with oocysts was hypothesized as source of infection.

1.4. Relevance of *Toxoplasma gondii* infections in animal health

In Veterinary Sciences, *T. gondii* is also a notable pathogen due to the following reasons: a) infected felids are responsible for the environmental contamination (Shapiro et al., 2019a; Zhu et al., 2023), b) the infection holds significant clinical relevance for animal pets such as cats and dogs (Calero-Bernal and Gennari, 2019), c) *T. gondii* has been identified as a major cause of reproductive failure in small ruminants and therefore a cause of great economic losses in the livestock industry (Dubey et al., 2020b, 2020a; Innes et al., 2009), d) the meat and meat derivates of infected food-producing animals is considered a primary food source of infection for humans (WHO and FAO, 2014), and e) *T. gondii* poses a risk for captive and free ranging wildlife conservation, especially for those species considered threatened and endangered (Barbieri et al., 2016; Landrau-

Giovannetti et al., 2022; Rodrigues Oliveira et al., 2022; Roe et al., 2013; Salas-Fajardo et al., 2023; Shapiro et al., 2019b).

One infected felid can shed millions of oocysts into the environment (Dubey, 1995), and the ingestion of a single oocyst may result enough to induce an infection in humans and animals (Rousseau et al., 2019a). Thus, the infection in both domestic and wild felids is of public health concern. Seroprevalence surveillance has been suggested as a monitoring method (Dámek et al., 2023), although seropositivity does not always imply oocyst excretion. Based on a systematic review and a meta-analysis, 37.5% (CI95%: 34.7–40.3) and 64% (CI95%: 60–67.9) of worldwide domestic and wild felids are *T. gondii*-seropositive, respectively (Hatam-Nahavandi et al., 2021). Anyhow, there is still much to do in understanding oocysts frequency and quantity shedding in different felids populations and regions, which results crucial to identify the load and distribution of oocysts in different scenarios and, consequently, the development of proper intervention strategies (Shapiro et al., 2019a).

In addition to the fact that felines contribute to the environmental contamination, at least in the case of domestic species, such as cats (as well as dogs), clinical manifestations have been described, which are more severe in the case of congenital toxoplasmosis, including hepatitis or cholangiohepatitis, pneumonia, encephalitis, ascites, lethargy, ocular disease, among others (Calero-Bernal and Gennari, 2019). Thus, *T. gondii* is also a pathogen of interest for veterinarians from a pet care point of view.

In terms of food-producing animals, *T. gondii* has been directly and indirectly detected in 28.3% of worldwide livestock and poultry based on studies published between 2000 and 2019 (Hajimohammadi et al., 2022). In relation to specific species of interest, it has been reported a pooled global seroprevalence of 19% in pigs (Foroutan et al., 2019), and of 33.86% and 31.78% in sheep and goats, respectively (Ahaduzzaman and Hasan, 2022). Although no pooled global seroprevalence data is available for chickens and cattle, high seropositivity have been documented in chickens, up to 100% (Dubey, 2010b), as well as in cattle, up to 91.8% (Dubey, 2010a). In the European context, and specifically related to pigs and small ruminants, which are the species of interest for this thesis, seroprevalence data from the last decade is summarized in **Table 3** and **Table 4.**

Table 3. *Toxoplasma gondii* seroprevalence in domestic pigs from studies conducted in the European countries in the last decade (adapted from Foroutan et al., 2019, and updated with additional studies).

Country	Serological test	No. positive pigs/ No. pigs tested	Prevalence (%)	Reference
Austria	ELISA	91/1368	6.7	(Steinparzer et al., 2015)
Czech Republic	ELISA	21/198	10.6	(Slany et al., 2016)
Denmark	ELISA	38/254	15.0	(Kofoed et al., 2017)
	ELISA	73/447	16.3	(Olsen et al., 2020)
Estonia	DAT	22/382	5.8	(Santoro et al., 2017a)
Finland	ELISA	43/1353	3.2	(Felin et al., 2015)
France	MAT	248/3595	6.9	(Djokic et al., 2016)
Greece	ELISA	26/609	4.3	(Papatsiros et al., 2016)
Ireland	LAT	15/317	4.7	(Halová et al., 2013)
Italy	ELISA	20/21	95.2	(Bacci et al., 2015)
	IFAT	36/498	7.2	(Santoro et al., 2017b)
	MAT	8/375	2.1	(Papini et al., 2017)
	ELISA	214/414	51.7	(Pipia et al., 2018)
	ELISA	14/370	3.8	(Gazzonis et al., 2018)
	ELISA	56/115	48.7	(Macaluso et al., 2019)
Latvia	ELISA	34/803	4.2	(Deksne and Kirjušina, 2013)
Poland	ELISA	193/760	25.4	(Puchalska et al., 2022)
Portugal	MAT	25/254	9.8	(Lopes et al., 2013)
	MAT	27/381	7.1	(Esteves et al., 2014)
Romania	IFAT	829/3595	23.1	(Paştiu et al., 2013)
	IFAT	44/94	46.8	(Paştiu et al., 2019)
Serbia	MAT	12/18	66.7	(Kuruca et al., 2016)
	MAT	31/182	17.0	(Kuruca et al., 2017)
Slovakia	ELISA	21/970	2.2	(Turčeková et al., 2013)
Spain	ELISA	192/709	27.1	(Hernández et al., 2014)
	IFAT	301/1200	24.5	(Herrero et al., 2016)
	DAT	222/2492	8.9	(Pablos-Tanarro et al., 2018)
	ELISA	79/361	21.9	(Fernández-Escobar et al., 2020b)
Sweden	ELISA	55/972	5.7	(Wallander et al., 2016)
United Kingdom	SFT	46/620	7.4	(Powell et al., 2016)
	MAT	75/2071	3.6	(Limon et al., 2017)

ELISA: enzyme-linked immunosorbent assay, DAT: direct agglutination test, MAT: modified agglutination test, LAT: latex agglutination test, IFAT: immunofluorescence antibody test, SFDT: Sabin-Feldman dye test.

Table 4. *Toxoplasma gondii* seroprevalence in domestic small ruminants from studies conducted in the European countries in the last decade (adapted from Ahaduzzaman and Hasan, 2022, and updated with additional studies).

Country	Species	Serological test	No. positive animals/ No. animals tested	Prevalence (%)	Reference
Belgium	Sheep	ELISA	2400/3170	75.7	(Verhelst et al., 2014)
Estonia	Sheep	DAT	667/1599	41.7	(Tagel et al., 2019)
Greece	Sheep	ELISA	246/458	53.7	(Anastasia et al., 2013)
	Goat	ELISA	230/375	61.3	(Anastasia et al., 2013)
	Sheep	ELISA	102/360	28.3	(Kantzoura et al., 2013)
	Goat	ELISA	30/179	16.8	(Kantzoura et al., 2013)
Denmark	Sheep	DAT	1/155	0.6	(Berg et al., 2021)
Ireland	Sheep	LAT	105/292	36.0	(Halová et al., 2013)
Italy	Goat	ELISA	19/30	63.3	(Gazzonis et al., 2019)
	Sheep	IFAT	298/502	59.4	(Gazzonis et al., 2015)
	Goat	IFAT	198/474	41.8	(Gazzonis et al., 2015)
	Sheep	IFAT	210/630	33.3	(Cenci-Goga et al., 2013)
	Goat	MAT	77/127	60.6	(Mancianti et al., 2013)
	Sheep	IFAT	214/630	34.0	(Sechi et al., 2013)
Latvia	Sheep	ELISA	179/1039	17.2	(Deksne et al., 2017)
Netherlands	Goat	ELISA	221/1664	13.3	(Deng et al., 2016)
Poland	Sheep	ELISA	30/64	46.9	(Moskwa et al., 2018)
	Goat	ELISA	8/39	20.5	(Moskwa et al., 2018)
	Goat	DAT	51/73	69.9	(Sroka et al., 2017)
Portugal	Sheep	MAT	40/119	33.6	(Lopes et al., 2013)
	Goat	MAT	34/184	18.5	(Lopes et al., 2013)
Russia	Goat	LAT	95/216	44.0	(Shuralev et al., 2018)
Serbia	Goat	DAT	316/431	73.3	(Djokić et al., 2014)
	Sheep	MAT	432/511	84.5	(Klun et al., 2006)
Spain	Sheep	MAT	464/998	46.5	(Jiménez-Martín et al., 2020)
	Goat	MAT	362/945	38.3	(Jiménez-Martín et al., 2020)
	Sheep	MAT	80/194	41.2	(Almería et al., 2018)
	Goat	MAT	5/89	5.6	(Almería et al., 2018)
	Goat	ELISA	43/552	7.8	(Rodríguez-Ponce et al., 2017)
	Goat	ELISA	299/638	46.9	(Díaz et al., 2016)
	Sheep	ELISA	915/2400	38.1	(Díaz et al., 2014)
	Sheep	ELISA	248/503	49.3	(García-Bocanegra et al., 2013)
	Goat	ELISA	124/494	25.1	(García-Bocanegra et al., 2013)
	Sheep	ELISA	213/342	62.3	(Fernández-Escobar et al., 2020a)

ELISA: enzyme-linked immunosorbent assay, DAT: direct agglutination test, LAT: latex agglutination test, IFAT: immunofluorescence antibody test, MAT: modified agglutination test.

The relevance of *T. gondii* infection in small ruminants extends beyond the potential threat their meat and derivates pose to public health since it also has a significant impact at reproductive level. In fact, *T. gondii* is widely acknowledged as one of the most important and prevalent reproductive transmissible agents in ewes and goats, causing reabsorption, abortion, neonatal death, delivery of stillborn or congenitally infected weak or clinically normal offspring, among others (Stelzer et al., 2019). Consequently, great economic losses have been estimated in livestock industry, with around 1.5 million lambs lost per year in Europe (Innes et al., 2009) and suggested annual cost of 5-15 million dollars in different countries (Stelzer et al., 2019).

In relation to wild species, data on *T. gondii* prevalence is limited and do not provide an overall overview about the real situation in this population, what could be influenced by the difficulties that sampling implies in these species and so the low number of tested samples included in many studies, some of them collected post-mortem. However, there is no doubt that *T. gondii* circulates in different wildlife populations. For example, according to a systematic review, *T. gondii* prevalence ranged from 6% to 100% in wild African herbivores, omnivores and carnivores based on direct or indirect detection methods (Bokaba et al., 2022). In America, 20.6% of carnivorous wild birds resulted *T. gondii*-seropositive in the United States, with successful parasite isolation in one case (Ammar et al., 2021), as well as 17.8% of wolves (*Canis lupus*), up to 25% of grizzly bears (*Ursus arctos*) and 43.4% of black bears (*Ursus americanus*) from Alaska (Elmore et al., 2012). In Europe, 36.5% of wild birds resulted seropositive in a study conducted in Portugal (Lopes et al., 2021) and similarly in other countries and continents. Fatal toxoplasmosis cases have been reported in captive squirrel monkey (*Saimiri boliviensis*) (Salas-Fajardo et al., 2023), free-ranging marmosets (*Callithrix* spp.) (Rodrigues Oliveira et al., 2022), dolphins (*Stenella longirostris* and *Cephalorhynchus hectori*) (Landrau-Giovannetti et al., 2022; Roe et al., 2013), sea otters (*Enhydra lutris nereis*) (Shapiro et al., 2019b) and monk seals (*Neomonachus schauinslandi*) (Barbieri et al., 2016), among other species, posing a risk for wildlife conservation. Furthermore, wildlife species infected with *T. gondii* whose meat is consumed by humans, such as wild boars, may also pose a risk to public health (Dubey, 2021).

1.5. Relevant risk factors associated with toxoplasmosis

Different factors related to food consumption, environment, contact with animals (including occupation), personal hygiene, and host status have been significantly associated with toxoplasmosis in humans and identified as risk factors. Based on a recent systematic review and meta-analysis of case-control, cohort, and cross-sectional studies on worldwide human sporadic toxoplasmosis diagnosticated by serological tests and published until the end of 2016 (n= 187), the consumption of raw/undercooked meat (pork, poultry, beef, processed meat, lamb, and game meat), raw milk, shellfish, unwashed vegetables and contaminated water, as well as the contact with soil and animals, in particular cats, and the lack of hygiene in food preparation, were identified as factors that significantly increase the probability of acquiring toxoplasmosis in adults, children and pregnant individuals (Thebault et al., 2021) **(Table 5)**. Furthermore, in the particular case of pregnant women, traveling abroad and blood transfusion were two additional risk factors significantly associated with toxoplasmosis, along with any condition that induce immunosuppression in any host population (Thebault et al., 2021) **(Table 5)**. Most of these risk factors coincided with those identified in studies focused on the European scenario (Cook et al., 2000; Friesema et al., 2023), although, controversially, in one of them no significant association with the consumption of raw pork meat, the presence of cats and kittens, cleaning litter tray and cat feed, was observed (Cook et al., 2000).

Regarding risk factors in animals (pets, livestock or wildlife), there is a lack of case-control studies, and no systematic review or meta-analysis is still available for having a global overview in this regard. However, some risk factors associated to toxoplasmosis in farm animals are summarized based on a review study (Stelzer et al., 2019): a) age, the older the longer the exposure to *T. gondii,* b) geographical characteristics, which influences the distribution and/or survival of *T. gondii,* c) production system, with presumably higher exposure to *T. gondii* in extensive systems, d) flock size, the smaller the higher the risk, probably due to the lower levels of biosecurity, e) presence of other animals, including small rodents and cats, with higher risk when their access to flocks is not controlled, f) feed and water, with higher risk with open/outdoor feed storage and non-potable/treated water.

Table 5. Risk factors significantly associated with sporadic *Toxoplasma gondii* infections in humans, based on case-control, cohort, and cross-sectional worldwide studies diagnosticated by serological tests and reported until the end of 2016. Data presented for stratified populations: mixed (age not defined) and pregnant women (adapted from Thebault et al., 2021).

Population	Risk factor	Pooled odds ratio ($CI_{95\%}$)	Studies included/ total of studies	*P* value	Heterogeneity analysis (I^2)
		Food			
Mixed*	Dairy	1.56 (1.30–1.88)	18/27	<.0001	76.8
	Meat	1.76 (1.57–1.97)	66/287	<.0001	
	Produce**	1.87 (1.54–2.28)	37/58	<.0001	
	Seafood	1.70 (1.33–2.18)	4/12	<.0001	
Pregnant	Dairy	1.52 (1.12–2.07)	28/44	0.008	69.5
	Meat	1.96 (1.47–2.61)	65/241	<.0001	
	Produce**	1.65 (1.27–2.15)	34/64	0.001	
All	Poor handing***	2.00 (1.60–2.50)	19/35	<.0001	43.6
		Environment			
Mixed****	Untreated drinking water	1.43 (1.22–1.68)	32/64	<.0001	62.0
	Farm environment	1.36 (1.15–1.62)	37/62	0.001	
	Playground	1.66 (1.40–1.95)	41/66	<.0001	
	Wastewater	1.52 (1.05–2.21)	4/6	0.028	
Pregnant	Untreated drinking water	1.49 (1.28–1.73)	34/54	<.0001	66.5
	Farm environment	1.80 (1.47–2.22)	32/52	<.0001	
	Playground	1.46 (1.32–1.62)	47/74	<.0001	
	Wastewater	1.86 (1.16–2.99)	07/11	0.01	
		Animals			
Mixed	Farm animals	1.48 (1.10–2.00)	6/8	0.009	59.1
	Occupational	2.04 (1.64–2.52)	20/65	<.0001	
	Pets	1.76 (1.50–2.07)	67/137	<.0001	
	Flies/rodents	1.53 (1.25–1.88)	8/13	<.0001	
Pregnant	Occupational	1.56 (1.25–1.95)	8/9	0.0001	22.8
	Pets	1.54 (1.37–1.72)	71/176	<.0001	
	Flies/rodents	1.47 (1.13–1.91)	4/6	0.004	
		Personal hygiene			
All	Poor personal hygiene	2.02 (1.69–2.42)	3/9	<.0001	0.0
		Travelling			
Pregnant	Abroad	1.88 (1.28–2.75)	6/7	0.001	17.9
		Host-specific			
Mixed	Immunosuppression	2.41 (1.48–3.91)	14/ 32	0.001	15.5
Pregnant	Blood transfusion	1.79 (1.03–3.09)	6/10	0.039	0.4

*Oceania excluded (odds ratio of 1 excluded). **Only vegetables were identified as a significant risk factor within this category, with a pooled odds ratio of 1.87 ($IC_{95\%}$: 1.49–2.34) for mixed population and of 1.37 ($IC_{95\%}$: 1.20–1.57) for pregnant women. ***Indicates no handwashing before eating or cooking and no knife washing. ****Africa removed (odds ratio of 22).

The identification of risk factors is crucial for both humans and livestock as it forms the foundation for the effective development and implementation of prevention strategies. Nevertheless, there are still relevant limitations in identifying these risk factors,

remarking the fact that most of the studies rely solely on serological diagnosis, employing tests that lack validation or are not validated for the target hosts.

2. Diagnostic approaches under a One Health perspective

At the very beginning of *T. gondii* discovery most of the diagnoses were performed post-mortem, based on bioassay, until 1948 when Sabin and Feldman described the first procedure to detect antibodies in living individuals, today known as the Sabin-Feldman dye test. This test consists of a complement-mediated cytolysis of tachyzoites coated with anti-*T. gondii* antibodies, what prevents tachyzoites to take up the methylene blue stain, thus, being the absence of color indicative of the presence of specific antibodies (Sabin and Feldman, 1948). Since this test requires a high amount of live tachyzoites, representing a potential risk for operators, special efforts have been done to develop accurate serological tests based on inactivated tachyzoites (lysate or soluble extract) or recombinant/chimeric proteins, accomplishment that has been notably greater in human medicine compared to veterinary medicine (Wyrosdick and Schaefer, 2015). On the other hand, the direct detection of the parasite is still of relevance, especially for food safety purposes and epidemiological investigations. Although bioassay is usually regarded as reference test, it is expensive, time consuming and of ethical concern, thus, as with serology, continue efforts have been done to develop sensitive and specific techniques to detect *T. gondii* in different matrices, most of them based on the recognition of parasite's nucleic acids.

2.1. Direct detection of *Toxoplasma gondii* in environmental matrices

Unlike other foodborne pathogens such as *Cryptosporidium* spp. and *Giardia* spp., there is no established method from the International Organization for Standardization (ISO) to detect *T. gondii* in environmental matrices. This, in addition to the heterogeneous distribution of oocysts in the environment, the low expected level of contamination and so the large samples size that needs to be tested (e.g., water), still make the detection of *T. gondii* in environmental samples a difficult task (Dumètre and Dardé, 2003; Slana et al., 2021). Based on previous studies, general procedures aiming to detect oocysts in environmental matrices comprises three key points: i) sampling strategy, ii) oocysts

concentration/recovery, and iii) specific detection, that can be interestingly complemented with assays targeting oocyst viability and genetic characterization (Dumètre and Dardé, 2003; Slana et al., 2021). Each key point is described below:

a) Sampling strategy: this is a crucial step and should guarantee that the right sample was collected under the ideal conditions at the most opportune time and place. Some of the variables to be considered in this first step are the presence of felids, temperature, humidity, rainfalls, water turbidity, soil/sand moisture, depth of sampling collection, number, and size samples, among others (Dumètre and Dardé, 2003). Pooled sample collection has been considered on multiple occasions for different environmental matrices to enhance the likelihood of detecting oocysts due to a more comprehensive representation of samples/areas and the advantages this offers in terms of management and cost-effectiveness (Caradonna et al., 2017; Ribeiro et al., 2015), although it could entail the dilution of oocysts.

b) Oocyst concentration/recovery: since the required sample size is usually large due to the low expected level of oocysts contamination (load), and some samples contain a high level of detritus that may interfere with the detection, the next step consists in concentrating (sometimes purifying) and recovering the oocysts. Flocculation, filtration, flotation, centrifugation and immunomagnetic separation are some examples of the methods used for these purposes (da Silva and Langoni, 2016; Marchioro et al., 2016; Ortiz- Pineda et al., 2020; Wang et al., 2014). Nonetheless, not all of them are effective/efficient for all type of matrices or even within the same type of matrix (Dumètre and Dardé, 2003). Anyhow, although this step is recommended, some researchers skip it, as is the case of one study carried out on oysters (Silva et al., 2020).

c) Oocyst detection: although isolating *T. gondii* through bioassay involving laboratory animals is still regarded as the reference method for detecting *T. gondii*, most of the studies are nowadays based on microscopy and/or different polymerase chain reaction (PCR) methods (Shapiro et al., 2019a; Slana et al., 2021) due to the limitations mentioned above and its unsuitability for large-scale studies. The most frequently molecular methods used are listed in **Table 6**.

Among the detection techniques, microscopy is the less reliable one since it depends on the expertise of the operator, it is time consuming and can lead to misdiagnosis due to the structural similarity between oocysts from different members of the Sarcocystidae family (Smith, 1981). In the case of molecular methods, an additional issue is the presence of PCR inhibitors, something that up to date is rarely considered by operators/researchers (Slana et al., 2021).

d) Oocyst viability: given that microscopy and commonly employed molecular techniques fail to offer insights into the infectivity or viability of detected oocysts, bioassay methods remain indispensable and irreplaceable. Nevertheless, new procedures to determine oocysts viability without requiring bioassay are under development, such as propidium monoazide coupled with quantitative PCR (qPCR), staining with propidium iodide, and reverse transcription quantitative PCR (RT-qPCR) (Kim et al., 2021; Rousseau et al., 2019b; Ware et al., 2010). However, further studies are still required as, e.g., some of these techniques has not been tested in different matrices and, in the case of RT-qPCR, it has been shown that it could successfully discriminate between viable and non-viable oocysts in seawater and hemolymph, but it did not perform well in other oyster matrices (Kim et al., 2023).

e) Genetic characterization: different methods have been developed and improved since the 1980s for this purpose (Dubey, 2008) **(Table 6)**. In environmental matrices, this data is rarely documented (Shapiro et al., 2019a) and presents relevant challenges due to the quality and quantity of the recovered genomic material. In the European context, restriction fragment length polymorphism (RFLP) and microsatellite (MS) have been the most frequently used techniques in environmental matrices (Fernández-Escobar et al., 2022). To date, the effect of the genotype in the transportation and persistence of oocysts in the environment remains unknown (Shapiro et al., 2019a). Furthermore, the lack of data related to circulating genotypes in environmental matrices hampers the clear understanding of the epidemiological dynamics of *T. gondii* oocysts and toxoplasmosis cases in humans and animals within and between geographical regions.

Table 6. Molecular methods frequently used for the detection and genetic characterization of *Toxoplasma gondii* (adapted from Liu et al., 2015).

Molecular methods	Main purposes	DNA target regions	Reference
Conventional PCR	Species detection	B1 gene, 529 bp repeat element, 18S rDNA gene, SAG1, SAG2, GRA1, ITS1	(Burg et al., 1989; Hurtado et al., 2001)
Real-time PCR	Species detection	B1 gene, 529 bp repeat element, 18S rDNA gene, SAG1	(Homan et al., 2000)
Loop-mediated isothermal amplification (LAMP)	Species detection	529 bp repetitive element, B1, SAG1, SAG2, GRA1, MIC3, oocyst wall protein genes	(Cao et al., 2014)
Microsatellite analysis (MS)	Genotyping	TUB2, W35, TgM-A, B18, B17, M33, IV.1, XI.1, M48, M102, N60, N82, AA, N61, and N83	(Ajzenberg et al., 2010)
Multilocus sequence typing (MLST)	Genotyping	BTUB, SAG2, GRA6, GRA7 and SAG3	(Bertranpetit et al., 2017)
PCR restriction fragment length polymorphism (PCR-RFLP)	Genotyping	SAG1, SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1 and Apico	(Howe and Sibley, 1995; Su et al., 2010)
Random amplified polymorphic DNA-PCR (RAPD-PCR)	Genotyping	Genomic DNA	(Ferreira et al., 2004)
High-resolution melting (HRM)	Genotyping	B1 gene	(Costa et al., 2011)

PCR: polymerase chain reaction.

2.2. Serological diagnosis of *Toxoplasma gondii* infections in humans and animals

Although the diagnosis of *T. gondii* infections could be established by multiple methods, including the amplification of DNA, parasite isolation, histopathology, serology, among others (Montoya, 2002), serological tests are the most frequently used techniques to initially identify *T. gondii* infections in both humans and animals (Huertas-López et al., 2023; Villard et al., 2016). An accurate diagnosis of toxoplasmosis is crucial to implement intervention strategies on time, e.g., pharmacological treatment implementation in infected pregnant women. However, there is still an absence of multidisciplinary integrative research in this field from a One health perspective (Huertas-López et al., 2023).

Indirect serological techniques allow the detection of specific antibodies produced against *T. gondii.* IgM antibody isotype appears early, one week after the infection, and usually declines in less than a year, similarly to IgA and IgE, although these two last isotypes could be also detected in congenitally infected infants and IgE depletion is

shorter. Thus, the presence of anti-*T. gondii* IgM, IgA and/or IgE is usually interpreted as an acute infection (Montoya, 2002). However, IgM and IgA results must be analyzed with caution since they have been steadily detected years after the acute infection (Bobić et al., 1991; Bortoletti Filho et al., 2013; Montoya, 2002). On the other hand, IgG isotype is detectable in one to two weeks post-infection on average, and even though it decreases after a few months, it remains detectable lifelong (in most of species), indicating previous infection (Villard et al., 2016). In contrast, a significant increase of IgG level in serial sampling and/or low avidity values are compatible with an acute infection (Villard et al., 2016). Based on this, many human diagnostic laboratories suggest analyzing different isotypes at the same time with or without an avidity test and serial samplings for a better discrimination (Montoya, 2002).

Apart from the Sabin-Feldman dye test, there are currently a wide variety of serological assays used in the diagnosis of toxoplasmosis in humans and animals, listed in **Table 7** together with their most relevant characteristics. Among them, enzyme-linked immunosorbent assays (ELISA) are one of the most used ones (Huertas-López et al., 2023) **(Table 7)**.

Most of in-house and commercial serological tests are based on tachyzoites lysate or soluble tachyzoite extract, which are characterized by a complex mixture of surface and/or cytosolic native antigens. However, special effort has been done in the recent years in order to improve the performance of the tests and make them more affordable by reducing the cost and time of production. In this regard, a high number of proteins from different specific parts/structures of the parasite, but also from different parasite stages, have been identified and recombinantly or chimerically produced by employing different systems (e.g., bacterial: *Escherichia coli*, yeast: *Pichia pastoris* or insect cells) with His tag, TRX, CKS and GST domains (Ferra et al., 2020; Huertas-López et al., 2021; Ybañez et al., 2020) **(Table 8)**. Purified recombinant or chimeric proteins have been used single or in combination, offering a higher specificity (Liyanage et al., 2021), avoiding in some instances the issue of cross-reactivity, e.g., with closely related parasites such as *Neospora caninum* (Holec-Gąsior et al., 2014).

Although there are many in-house and commercially available serological tests, a still scientific and health care community concern is the fact that there is no consensus on

the validation process of the tests and the data interpretation (e.g., positivity criterion). Thus, many of the available techniques are not standardized and if they are, scarce data is available in relation to their validation process and performance, hampering data comparison and reproducibility.

2.2.1. Specific considerations for the serodiagnosis of toxoplasmosis in humans

Different serological approaches have been suggested for the serodiagnosis of toxoplasmosis in humans based on the conditions of the individuals, especially focused on pregnant woman and immunocompromised people. Nevertheless, as most of the countries lack mandatory surveillance for the diagnosis of toxoplasmosis, significant variations on serological screening approaches could be found both among and within regions.

In general, non-detectable levels of anti-*T. gondii* IgG and IgM are interpreted as the absence of both acute or chronic infection and usually no further analysis is required at least in general population that does not present symptoms and clinical signs. However, taking France as an example, one of the few countries with a mandatory surveillance program for preventing toxoplasmosis, serological screening is suggested monthly or every six months for seronegative pregnant woman and immunocompromised individuals, respectively (Villard et al., 2016). **Figure 4** and **Figure 5** summarize the serological screening and result interpretation suggested by the French National Reference Center for Toxoplasmosis for these two groups of risk under different scenarios.

In addition, some authors have suggested that an increase in avidity values corresponds to a decrease in IgA levels and that pregnant women who exhibit detectable IgA alongside IgG and IgM are more likely to have an acute infection compared to those with undetectable IgA, suggesting therefore the detection of IgA together with IgG and IgM for discriminating between acute and chronic infections (Olariu et al., 2019).

Table 7. Summary of the main characteristics of commonly employed serological tests for the detection of anti-*Toxoplasma gondii* antibodies in humans and animals.

Test	Antigen	Antibody isotype/s	Basis	Advantage	Disadvantage	Reference
Sabin-Feldman dye test (SFDT)	Live tachyzoites	IgM/IgG/IgA	Staining	Does not need species-specific conjugate, highly sensitive and specific	Needs live tachyzoites, requires experienced operators and a microscope, does not identify the source of infection	(Sabin and Feldman, 1948)
Direct agglutination test (DAT)	Formalin-fixed tachyzoites	IgG	Agglutination	Does not need live tachyzoites, species-specific conjugate or special equipment, easy to perform, cheap, highly sensitive	Requires large antigen amount, does not identify the source of infection, the presence of IgM can induce non-specific agglutination	(Desmonts and Remington, 1980)
Modified agglutination test (MAT)	Formalin-fixed tachyzoites	IgG	Agglutination	Does not need live tachyzoites and species-specific conjugate, easy to perform, cheap, highly specific and sensitive	Not commercially available in some countries, does not identify the source of infection, the presence of IgM can induce non-specific agglutination	(Desmonts and Remington, 1980; Dubey and Desmonts, 1987)
Indirect hemagglutination assay (IHA)	Tachyzoites lysate	IgG	Agglutination	Does not need live tachyzoites and species-specific conjugate, easy to perform, cheap, highly specific and sensitive	Less sensitive and specific than MAT, not longer commercially available in many countries, does not identify the source of infection, the presence of IgM can induce non-specific agglutination	(Dubey and Thulliez, 1989)
Latex agglutination test (LAT)	Tachyzoites lysate	IgM/IgG	Agglutination	Does not need live tachyzoites and species-specific conjugate, commercially available	Less sensitive and specific than MAT, does not identify the source of infection, the presence of IgM can induce non-specific agglutination	(Dubey and Thulliez, 1989)
Indirect enzyme-linked immunosorbent assay (ELISA)	Tachyzoites lysate, lyophilized tachyzoites, recombinant/chimeric proteins	IgM/IgG/IgA/ IgE	Colorimetric	Does not need live tachyzoites, commercially available worldwide, cheap, not time-consuming, automated reading, could be based on different	Unknown performance in some cases, influence of reagents on performance, needs species-specific conjugate, requires a	(Álvarez-García et al., 2021; Huertas-López et al., 2023; Robert-

Test	Antigen	Antibody isotype/s	Basis	Advantage	Disadvantage	Reference
				antigens and sample types, could be quantitative, usually highly sensitive and specific, some in-house tests have been developed to discriminate between oocysts *vs.* tissue cysts-driven infections	spectrophotometer, can present cross-reactivity	Gangneux and Guegan, 2021)
Immunofluorescence antibody test (IFAT)	Formalin-fixed tachyzoites	IgM/IgG	Fluorescence	Does not need live tachyzoites, easy to perform, cheap, can be used to define titers	Subjective interpretation of results, needs species-specific conjugate and a fluorescence microscope, not commercially available, does not identify the source of infection, can present cross-reactivity	(Basso et al., 2013; Huertas-López et al., 2023; Sánchez-Sánchez et al., 2019)
Western blot (WB)	Tachyzoites lysate, recombinant/chimeric proteins	IgM/IgG	Colorimetric	Does not need live tachyzoites, allows the identification of the molecular weight of the identified antigen, highly specificity. In-house tests have been developed to discriminate between oocysts *vs*. tissue cysts-driven infections	Time consuming, requires expertise, influence of reagents on performance, it is expensive, needs species-specific conjugate and special equipment, can present cross-reactivity	(Álvarez-García et al., 2021; Basso et al., 2013; Huertas-López et al., 2023)
Immunochromatography (ICT)	Tachyzoites lysate, recombinant proteins	IgM/IgG	Colorimetric	Does not need live tachyzoites or skilled people or special infrastructure or equipment or reagents, could be based with different sample types. It is cheap, rapid, and easy to transport and preserve, commercially available	Does not identify the source of infection, can present cross-reactivity	(Khan and Noordin, 2020)

Other techniques such as the microarray and the time-resolved fluorescence immunoassay have been employed.

Table 8. Summary of recombinant *Toxoplasma gondii* antigens used in serological tests developed for humans and/or animals without the intent to differentiate the route of transmission (adapted from Ferra et al., 2020; Liyanage et al., 2021; Ybañez et al., 2020).

Groups	Antigen name (other identification)	Parasite stage	Serological test (antibody isotype if specified)
Dense granule antigens (GRA)	GRA1 (P24), GRA2 (P28), GRA3, GRA4 (P41), GRA5, GRA6 (P32), GRA7 (P29), GRA8 (P35), GRA14, GRA15	Most of them expressed in tachyzoite, bradyzoite and/or sporozoite	ELISA (IgM/IgG), avidity (IgG), WB (IgG), ICT (IgG)
Microneme antigens (MIC)	MIC1, MIC2, MIC3, MIC10	Expressed in tachyzoite, bradyzoite and/or sporozoite	ELISA (IgM/IgG), avidity (IgG)
Rhoptry antigens (ROP)	ROP1 (P66), ROP2 (P54), ROP5, ROP8, ROP9, ROP18	Most of them expressed in tachyzoite, bradyzoite and/or sporozoite	ELISA (IgM/IgG), avidity (IgG), WB (IgG)
Surface antigens (SAG)	SAG1 (P30), SAG2 (P22), SAG2 A	Tachyzoite	ELISA (IgM/IgG), avidity (IgG), WB (IgG), ICT (IgG)
	SAG2 D, BSR4	Bradyzoite	ELISA (IgG)
Enzymes	LDH1	Tachyzoite	ELISA (IgG)
	LDH2	Bradyzoite	ELISA (IgG)
Other antigens	H4, H11	Tachyzoite	ELISA (IgG)
	M2AP	Tachyzoite	ELISA (IgG)
	MAG1	Tachyzoite, bradyzoite (matrix)	ELISA (IgG)
	AMA1	Tachyzoite (apical membrane)	ELISA (IgG)
	BAG1	Bradyzoite (cytosol)	ELISA (IgG), WB (IgG)

ELISA: enzyme-linked immunosorbent assay, WB: Western blot test; ICT: immunochromatography.

Serology is considered a valuable tool for the management of toxoplasmosis and remains the first-line tests to identity patients at risk due to either reactivation or primary infections **(Figure 5)** and, furthermore, it is probably the only accessible diagnostic resource in low-income countries (Dupont et al., 2021). However, there are some limitations when used in immunocompromised people, mainly related to the immune deficiency itself and therapeutic treatments, such as those containing anti-*T gondii* antibodies. Consequently, techniques like PCRs of blood samples are high recommended (Martino et al., 2005).

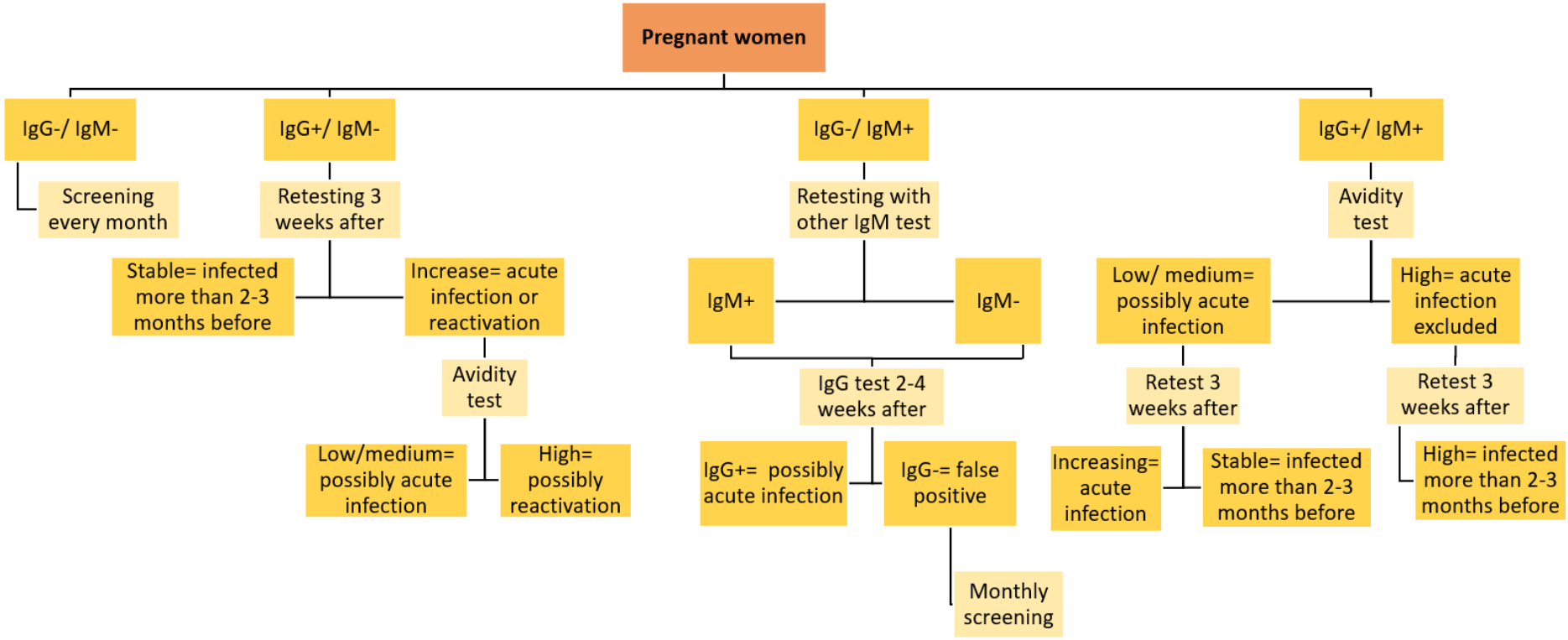

Figure 4. Serological screening and result interpretation for different immunological scenarios in pregnant women based on the recommendations from the French National Reference Center for Toxoplasmosis (adapted from Villard et al., 2016).

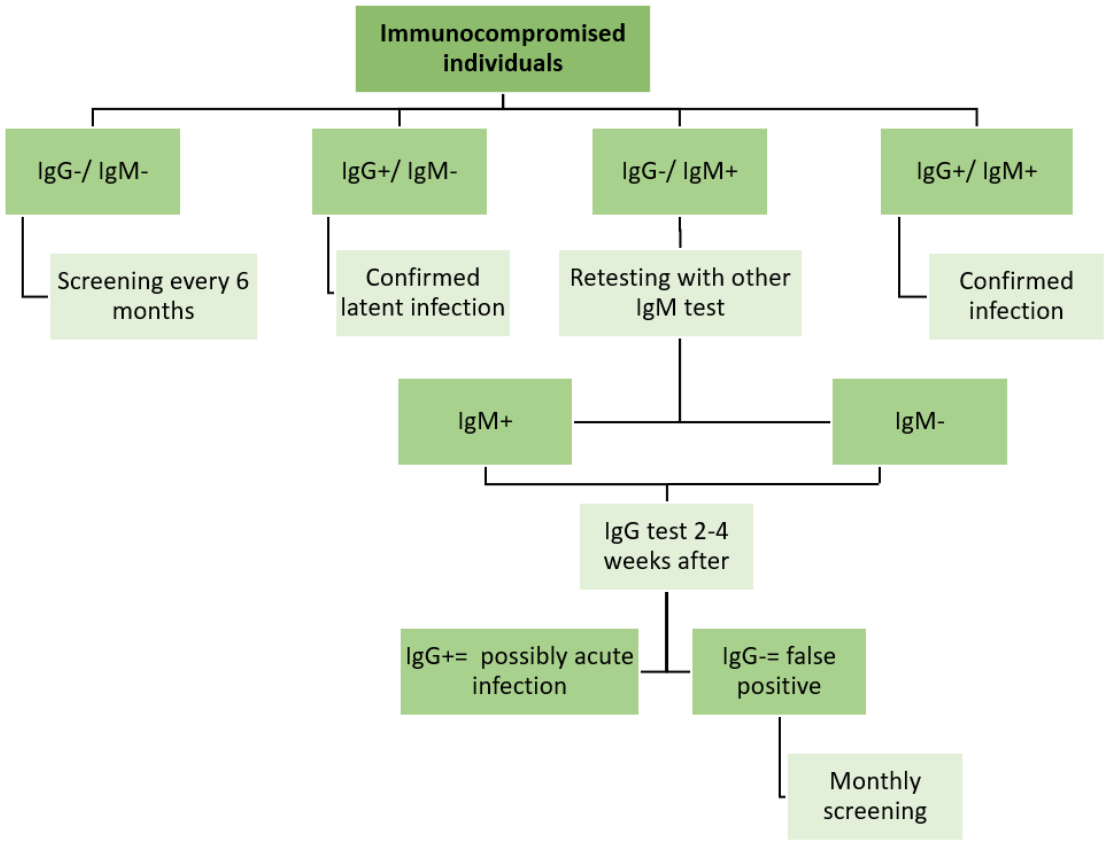

Figure 5. Serological screening and result interpretation for different immunological scenarios in immunocompromised individuals based on the recommendations from the French National Reference Center for Toxoplasmosis (adapted from Villard et al., 2016).

In the case of congenital toxoplasmosis, prenatal serodiagnosis has been abandoned due to the risk that implies periumbilical fetal blood sampling. Thus, nowadays, the prenatal diagnosis is mainly based on ultrasonography and amniocentesis for the direct detection of *T. gondii*. In the case of abortion, tissue and fluid samples could be used for the isolation or molecular detection of *T. gondii.* In newborns, the diagnosis is mainly based on the detection of specific IgA and IgM, and preferably of IgA since its detection is more sensitive than of IgM, and confirmation 10 days later is highly recommended. The solely detection of specific IgG is not useful since they are expected to be present in the case of acute or chronic infection in the mother, unless newborns are retested a year later and turned IgG-negative, ruling out a congenital toxoplasmosis, together with negative IgA and IgM. Seronegative newborns from confirmed infected mothers should be followed up for at least a few months and additional evaluations are suggested, such as ophthalmologic examination, and tomography, among others (Montoya, 2002).

While it is currently feasible to distinguish between acute and chronic infections using conventional serological methods, as previously mentioned, serological discrimination between oocyst- *vs.* tissue cyst-driven infections remains a challenging task in the field. In addition to the fact that the type and severity of the clinical manifestations in oocyst *vs.* tissue cyst-related infections are not different (Dubey, 2021), the incubation period of time of toxoplasmosis is between 7 and 30 days, which means that when the symptoms and clinical signs show up, the source of infection could no longer exists or could no longer be contaminated, and so the direct detection of *T. gondii* in suspected sources frequently fails, as happened in different toxoplasmosis outbreaks (Dubey, 2021). Hence, a source-attributing serological test could result extremely useful to design and implement efficient prevention and control strategies.

Creating serological tests capable of pinpointing oocysts as the infection source presents a significant hurdle. This is primarily because the exposure time of sporozoites/sporocysts/oocysts antigens to the immune system is exceedingly brief. Sporozoites initially invade interepithelial cells but rapidly transform into tachyzoites in 24 hours or less to establish the infection and disseminate throughout the organism (Dubey et al., 1998). Moreover, the kinetics of oocyst-specific antibodies remain unknown, the immunological response could vary based on the host species, age, the parasite dose and strain, among other factors, including cross-reactivity with other closely related parasites or even within different stages of *T. gondii*.

Anyhow, there have been some attempts to develop oocyst-attributing serological tests, extensively reviewed by Álvarez-García et al. (2021). In summary, the first test developed was an ELISA based on TgSporoSAG, a predicted sporozoite-specific protein. Anti-SporoSAG IgM and IgG were detectable from one to 15 and from 40 to 120 days post-infection (dpi) in mice inoculated with oocysts, respectively (Döşkaya et al., 2014). However, this data is not conclusive since the authors did not provide information about its recognition by mice infected with tissue cysts. Contrary to these results, no reactivity to TgSporoSAG was found when using sera from mice experimentally infected with oocysts and tissue cysts, rabbits infected with oocysts or naturally infected humans (Crawford et al., 2010) **(Table 9)**.

Later, another protein predicted to be sporozoite-specific was tested, TgERP, also known as TgLEA850 (Arranz-Solís et al., 2023). For the validation of the tests based on TgERP, the authors used sera from pigs and mice experimentally infected with oocysts and tissue cysts and found reactivity only in oocysts-driven infections up to 8 months after the inoculation, as well as in humans who were, confirmed or not, infected with oocysts (Hill et al., 2011). Similar conclusions respect to the discriminatory capacity of TgERP were drawn by other research groups based on serum (Vieira et al., 2015) or salivary (Mangiavacchi et al., 2016) samples from humans naturally infected with *T. gondii,* although no clear conclusions were reached by others (Burrells et al., 2016) **(Table 9)**.

After that, Santana et al. (2015) tested a sporozoite-specific and an oocyst wall-specific antigens, TgCCp5A and TgOWP1, respectively, and observed positive reactivity to TgCCp5A only in pigs (from 7 to 28 dpi) and mice experimentally infected with oocysts compared to those infected with tachyzoites and tissue cysts, as well as in naturally infected chickens and pigs, while no reactivity to TgOWP1 was recorded with sera from pigs experimentally infected with oocysts. More recently, Liu et al. (2019) corroborated the oocyst-attribution usefulness of TgCCp5A and another oocyst-specific protein, TgOWP8, but using only sera from natural infections **(Table 9)**.

Despite the progress made in source-attributing serology, relevant limitations were found among these studies: a) just a few of them used sera from animals experimentally infected with oocysts and tissue cysts, which are ideal to determine stage-specificity, b) some of them only used serum samples from humans, who could be reinfected with different stages through their lifetime, c) reference serum samples were characterized using one or two in-house or commercial tests with unknown diagnostic performance, d) no reference test was defined in most of the cases to develop and standardize the new oocyst-attributing serological tests, e) no consensus criteria nor exhaustive workflow were followed. Hence, further studies are required to confirm the usefulness of already employed antigens under different conditions and to find new candidates following a step-by-step pipeline as suggested by Álvarez-García et al. (2021).

Table 9. Oocyst-attributing serological tests described in the literature (adapted from Álvarez-García et al., 2021).

Protein (gene ID)	**Protein location**	**Technique/s developed**	**Reference sera host** (N: natural infection, E: experimental infection)	**Tests used to characterize the reference sera**	**Anti-protein antibody kinetics**	**Results: useful to identify oocyst-driven infections?**	**Reference**
TgOWP1 (TGME49_204420)	Oocyst wall	WB, ELISA	Pig (N, E[a]), chicken (N, E[a])	STAg WB/ ELISA	Not specified	Yes (except for pigs)	(Santana et al., 2015)
TgOWP8 (TGME49_271590)	Oocyst wall	WB, ELISA	Humans (N), pig (N, E[b]), chicken (N)	GRA7 WB/ ELISA	N/A	Yes	(Liu et al., 2019)
TgERP/TgLEA850 (TGME49_276850)	Sporozoite cytoplasm	WB, ELISA	Humans (N), pig (E[c]), mice (E[c])	MAT/ DAT/ SFDT/ Diff. ELISA tests*	Pigs: up to 8 mpi (presumably IgG) Mice: not specified	Yes	(Hill et al., 2011)
		ELISA	Humans (N)	MAT/ commercial ELISA/ CMIA	N/A	Yes	(Vieira et al., 2015)
		ELISA	Humans (N)	STAg ELISA	N/A	Not clearly stated**	(Burrells et al., 2016)
		ELISA	Humans (N)	Commercial ELISA/ CMIA	N/A	Yes	(Mangiavacchi et al., 2016)
TgCCp5A (TGME49_258400)	Sporozoite cytoplasm	WB, ELISA	Humans (N), pig (N, E[b]), chicken (N)	GRA7 WB/ ELISA	N/A	Yes	(Liu et al., 2019)
		WB, ELISA	Humans (N), pig (N, E[a]), chicken (N, E[a]), mice (E[a])	STAg WB/ ELISA	Pig: 7-28 dpi (IgG) Mice: 15-30 dpi (IgM), 15-60 dpi (IgG)	Yes	(Santana et al., 2015)
TgSporoSAG (TGME49_258550)	Sporozoite surface	ELISA	Humans (N), mice (E[d]), rabbits (E[d])	SAG1 and SRS2 ELISA	No reactivity	No	(Crawford et al., 2010)

Protein (gene ID)	**Protein location**	**Technique/s developed**	**Reference sera host** (N: natural infection, E: experimental infection)	**Tests used to characterize the reference sera**	**Anti-protein antibody kinetics**	**Results: useful to identify oocyst-driven infections?**	**Reference**
		ELISA	Mice (E[e])	GRA1 ELISA	1-15 dpi (IgM), 40-120 dpi (IgG)	Not conclusive***	(Döşkaya et al., 2014)

STAg: soluble tachyzoite antigen, WB: Western blot test, ELISA: indirect enzyme-linked immunosorbent assay, MAT: modified agglutination test, DAT: direct agglutination test, SFDT: Sabin-Feldman dye test, Diff.: different, CMIA: chemiluminescent microparticle immunoassay, N/A: do not apply, mpi: months post-infection, dpi: days post-infection. [a]Chickens infected with STAg and tissue cysts (sampling days not specified), pigs infected with tachyzoites and oocysts (serum samples collected at 0, 7, 14, 21, and 28 days post-infection), mice infected with tissue cysts or oocysts (serum samples collected at 0, 15, 30, 45, and 60 days post-infection). [b]Pigs infected with tachyzoites (no reactivity with TgOWP8 or TgCCp5A) (sampling days not specified). [c]Pigs infected with oocysts and tissue cysts (sampled weekly up to 9 months post-infection), mice infected with oocysts and tissue cysts (sampling days not specified). [d]Mice infected with oocysts and tissue cysts, rabbits infected with oocysts (sampling days not specified). [e]Mice infected with oocysts and tissue cysts (samples collected at 0, 1, 2, 3, 6, 10, 15, 40, and 120 days post-infection). *Based on different antigens. **One positive sample that tested negative in the second sampling. ***TgSporoSAG was tested only with sera from oocyst-infected mice.

2.2.2. Specific considerations for the serodiagnosis of toxoplasmosis in animals

Unlike the diagnosis in humans, where established guidelines exist for some countries and different antibody isotypes are frequently subjected to analysis, no guidelines are available for the serodiagnosis in animals and most of the serological tests already employed with diagnostic purposes have been developed and/or validated to detect only anti-*T. gondii* IgGs. Another noteworthy limitation relies on the necessity of species-specific secondary antibodies (as outlined in **Table 7** above), which restricts the number of tests developed/validated for species of interest, if available.

The serodiagnosis in animals serves epidemiological purposes, aiding in the detection of the cause of clinical signs, such as in cases of abortions in small ruminants, which can be complemented by the direct detection of the parasite. Furthermore, the application of serodiagnosis in animals has proven to be useful for assessing the potential risks associated with the consumption of specific food-producing animals. Although the correlation between the presence of antibodies against *T. gondii* and the direct detection of the parasite in the tissue of infected animals varied between studies, from poor to moderate in naturally infected small ruminants, chickens, and pigs, to the absence or poor correlation in horses and cattle, it is more likely to directly detect the parasite in meat of seropositive animals. For example, *T. gondii* tissue cysts were detected by bioassay or PCR methods in the meat of 39.4% (395/1,002), 53.4% (897/1,679) and 58.8% (348/529) *T. gondii*-seropositive small ruminants, chickens, and pigs, respectively (Opsteegh et al., 2017). Based on this, animal serology has been suggested by authorities for public health commitments, especially at slaughterhouses for pigs and small ruminants (EFSA, 2013, 2011).

CHAPTER III: JUSTIFICATION AND OBJECTIVES

Toxoplasma gondii is a widespread protozoan parasite of importance in public and veterinary health. Around one third of the global human population might be infected by *T. gondii* (WHO and FAO, 2014), although flu-like symptoms/ clinical signs are developed just by 10–20% of immunocompetent people (Montoya and Liesenfeld, 2004). However, it poses a major risk for pregnant women due to reproductive failure and congenital infections, as well as for immunocompromised people by causing severe neurological and respiratory diseases (Innes et al., 2009; Koutsoumanis et al., 2018; Lindsay and Dubey, 2020; Rostami et al., 2020; Stelzer et al., 2019). Indeed, *T. gondii* has ranked as the third most important foodborne parasite worldwide (WHO and FAO, 2014). Also, it should be noted that it causes frequent reproductive failure in small ruminants (Stelzer et al., 2019), what is translated to significant economic losses to the livestock industry (Innes et al., 2009).

Toxoplasma gondii is a paradigm of the One Health approach due to its zoonotic relevance, its wide range of susceptible hosts (any homoeothermic species) and the multiple potential sources of infection (Djurković-Djaković et al., 2019). The meat route through the intake of tissue cysts present in raw or undercooked meat from chronically infected food-producing animals or their derivates, the environmental route through the ingestion of sporulated oocysts that may contaminate the soil, water, vegetables, fruits and bivalve mollusks, and the maternal-fetal route by the transplacental transmission of tachyzoites from the mother to the fetus(es), have been reported to predominate in humans (Attias et al., 2020). Although the maternal-fetal route tends to cause severe consequences, the number of susceptible hosts that the parasite could reach through this pathway is limited (fetuses) compared to the meat and environmental routes. Until now, the relative contribution of each route of transmission remains unknown. However, according to a systematic review, 44.1% (15/34) of worldwide human toxoplasmosis outbreaks reported up to 2018 were attributable to the ingestion of oocysts (Pinto-Ferreira et al., 2019a).

An increase of the relative importance of the environmental route has been recently acknowledged, probably because it has been identified as the cause of the largest human outbreaks where around 400–910 toxoplasmosis cases were confirmed (De Moura et al., 2006; Minuzzi et al., 2021). Nevertheless, relevant gaps related to the identification of

oocysts-driven infections are recognized: a) clinical signs show up a few days after the exposition and therefore the environmental source might not contain the parasite anymore (e.g. contaminated water), b) the majority of diagnostic techniques used to detect oocysts in the environment have not been validated for such matrices, c) there is a lack of studies on *T. gondii* detection in environmental samples worldwide, making it difficult to assess the risk that each matrix poses, d) there is no available serological technique that could discriminate between oocyst- and tissue cyst-driven infections. Thus, in many cases, the identification of the source of infection relies on conventional serological tests and epidemiological questionnaires.

The availability of a serological assay capable of identifying oocyst-driven infections is of major interest to design and implement prevention and/or control strategies, mainly in places with endemic cases or recurrent outbreaks. In this sense, some efforts using sporozoite-specific proteins have been carried out, to note TgERP, also known as TgLEA850 (Burrells et al., 2016; Hill et al., 2011; Mangiavacchi et al., 2016; Vieira et al., 2015), TgCCp5A (Liu et al., 2019; Santana et al., 2015) and TgSporoSAG (Crawford et al., 2010; Döşkaya et al., 2014); or oocyst-specific proteins, such as TgOWP1 (Santana et al., 2015) and TgOWP8 (Liu et al., 2019). Most of these studies reported promising results, although not fully consistent. In fact, relevant limitations have been found in these investigations, including the absence of a reference test and appropriate reference serum panels, the differences in the experimental design followed (e.g. host species, natural *vs.* experimental infections, parasite stages, doses and strains), and the limited number of proteins/antigens tested, among others, what led some researchers to propose a workflow for studies aiming to identify stage-specific antigens and develop source-attributing serological tests (Álvarez-García et al., 2021).

Within this scenario, the overall aim of this doctoral thesis was to contribute for a better understanding of the relevance of different environmental matrices in the transmission of *T. gondii* and to identify a wide set of sporozoite- or sporocyst/oocyst wall-specific antigens to further develop and validate a serological assay specifically for diagnosing oocyst-driven infections. For the proof-of-concept study, sera from experimental infections conducted in sheep and pigs with oocysts and tissue cysts were employed, adhering to the recommendations outlined by Álvarez-García et al. (2021) regarding the

control serum panels and the reference animal sera. To accomplish this, the following specific objectives were pursued:

Objective 1: To determine the relevance of the environmental route in *T. gondii* transmission and the potential limitations related to the sampling strategies and detection methods employed with different environmental matrices.

- Sub-objective 1.1: To perform a comprehensive systematic review about the presence of *T. gondii* oocysts in soil, water, vegetables, fruits, and bivalve mollusks worldwide and the different sampling strategies and detection methods employed.

Objective 2: To develop and validate a serological method that discriminate between *T. gondii* oocyst- *versus* tissue cyst-driven infections.

- Sub-objective 2.1: To develop and harmonize conventional serological tests frequently used in the diagnosis of *T. gondii* infections in three species whose meat consumption supposes a burden of infection for humans: pigs, sheep and goats, and to properly characterize the serum panels for sub-objective 2.2.
- Sub-objective 2.2: To develop and validate an enzyme-linked immunosorbent assay (ELISA) based on *T. gondii* sporozoite- or sporocyst/oocyst wall-specific proteins to differentiate between oocyst- *versus* tissue cyst-driven infections.

CHAPTER IV: WORKPLAN AND METHODOLOGY

Adhering to the doctoral regulations regarding thesis presented in publication/article format, the methodology followed to accomplish each objective is summarized below. For a more comprehensive understanding, detailed information can be found in the respective articles.

Objective 1: To determine the relevance of the environmental route in *T. gondii* transmission and the potential limitations related to the sampling strategies and detection methods employed with different environmental matrices.

- Sub-objective 1.1: To perform a comprehensive systematic review about the presence of *T. gondii* oocysts in soil, water, vegetables, fruits, and bivalve mollusks worldwide and the different sampling strategies and detection methods employed.

With this sub-objective we attempted to provide a worldwide overview about the presence of *T. gondii* in environmental matrices, as well as the different sampling strategies and detection methods followed in order to determine the relevance of this route of transmission and the possible limitations related to the detection of oocysts in the environment. For this, we followed the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines and searched existing literature that reported the direct detection of oocysts in soil, water, vegetables, fruits, and bivalve mollusks worldwide until the end of 2020, using three widely used databases and more than seventeen combinations of search terms. We collected detailed data related to the sampling strategy, the recovery and detection methods and detection rates. After the data analyses, experimental design improvement opportunities were suggested for further studies on *T. gondii* oocysts environmental contamination to obtained robust and comparable results.

Objective 2: To develop and validate a serological method that discriminate between *T. gondii* oocyst- *versus* tissue cyst-driven infections.

- Sub-objective 2.1: To develop and harmonize conventional serological tests frequently used in the diagnosis of *T. gondii* infections in three species whose meat consumption supposes a burden of infection for humans: pigs, sheep and goats, and to properly characterize the serum panels for sub-objective 2.2.

With this sub-objective we aimed to offer an updated diagnostic performance of a wide panel of serological tests routinely used for the indirect diagnosis of *T. gondii* infections in pigs, sheep and goats and to recommend cutoff readjustments to improve their performance and/or avoid cross-reactivity with *N. caninum,* a closely related parasite, based on the purpose of the serological study and the epidemiological scenario. For this, we developed and validated three in-house conventional tests (Western Blot [WB], enzyme-linked immunosorbent assay [ELISA], and immunofluorescence antibody test [IFAT]) using serum panels from pigs, sheep and goats experimentally or naturally infected with different stages and strains of *T. gondii* and included them in a comparative study together with four commercial ELISA tests (IDScreen, PrioCHECK, Pigtype and IDEXX). In the absence of a gold standard test for *T. gondii* serodiagnosis, with this comparative study we were able to properly characterize the serum panels that were used in the next sub-objective, 2.2.

- Sub-objective 2.2: To develop and validate an enzyme-linked immunosorbent assay (ELISA) based on *T. gondii* sporozoite- or sporocyst/oocyst wall-specific proteins to differentiate between oocyst- *versus* tissue cyst-driven infections.

The purpose of this sub-objective was to identify *T. gondii* sporozoite- or sporocyst/oocyst wall-specific proteins (proteins of interest [POIs]) with source-attribution capacity using a genome-wide *in silico* prediction approach and to later develop and validate an ELISA test that could differentiate oocyst-driven infections from tissue cyst-driven infections. For this, we applied a suggested workflow to screen a wide panel of proteins or hypothetical proteins that were predicted to be oocyst-specific based on *T. gondii* omics data. The screening of candidates was conducted using POI-WBs. This involved analyzing a few selected well-characterized serum samples collected before and after the infection from wide serum panels of pigs experimentally infected with oocysts and tissue cysts (two panels) or sheep experimentally infected with oocysts (one panel) from different genotypes. Those POIs that induced seroconversion and were stage-specific, with concordance in the case of pig serum panels, were used for the development of an ELISA. Then, all samples from selected serum panels were analyzed by selected POI-WB and POI-ELISA to reach the validation step. Cross-reactivity with anti-*N. caninum* IgGs was also tested for selected POIs. Further effort was done to provide

evidence of the low value of non-selected POIs that were previously described in the literature as valuable source-attributing proteins for serological diagnosis, with special focus on TgERP, which was the most frequently cited protein, and for which we included two additional serum controls and studied multiple variables, such as batch-to-batch variation and ELISA plate systems.

CHAPTER V: RESULTS (PUBLICATIONS)

Objective 1: To determine the relevance of the environmental route in *T. gondii* transmission and the potential limitations related to the sampling strategies and detection methods employed with different environmental matrices.

- Sub-objective 1.1: To perform a comprehensive systematic review about the presence of *T. gondii* oocysts in soil, water, vegetables, fruits, and bivalve mollusks worldwide and the different sampling strategies and detection methods employed.

The outcomes from this sub-objective were published in the following open access article **(paper No. 1)**: López-Ureña, N.M., Chaudhry, U., Calero-Bernal, R., Cano-Alsua, S., Messina, D., Evangelista, F., Betson, M., Lalle, M., Jokelainen, P., Ortega-Mora, L.M., Álvarez-García, G., 2022. **Contamination of Soil, Water, Fresh Produce, and Bivalve Mollusks with *Toxoplasma gondii* Oocysts: A Systematic Review**. Microorganisms 10, 517. https://doi.org/10.3390/MICROORGANISMS10030517/S1. Microorganisms journal had an impact factor of 4.5 and was classified as Q2 in 2022.

Abstract: *Toxoplasma gondii*, a significant foodborne pathogen, has been associated with oocyst-induced toxoplasmosis outbreaks or endemic cases. Despite this, the environmental transmission route's relevance has not been thoroughly explored. In response, a comprehensive systematic review was conducted on *T. gondii* oocyst contamination in soil, water, fresh produce, and bivalve mollusks, adhering to PRISMA guidelines. A total of 102 out of 3201 articles were selected, with 34 focusing on soil, 40 on water, 23 on fresh produce, and 21 on bivalve mollusks. *Toxoplasma gondii* was detected in all matrices, with detection rates that ranged between 0.09% and 100% based bioassay or PCR-based methods. However, there was a high heterogeneity (I^2 = 98.9%), influenced by diverse sampling strategies and diagnostic methodologies. The study underscores the need for harmonized approaches in detecting *T. gondii* in environmental matrices to ensure robust and comparable results.

These findings were presented as an online oral communication in the 13th European Multicolloquium of Parasitology (October 2021) and the Apicowplexa virtual meeting (June 2022), as well as an online poster communication in the One Health European Joint Programme Annual Scientific Meeting (April 2022).

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Review

Contamination of Soil, Water, Fresh Produce, and Bivalve Mollusks with *Toxoplasma gondii* Oocysts: A Systematic Review

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Abstract: *Toxoplasma gondii* is a major foodborne pathogen capable of infecting all warm-blooded animals, including humans. Although oocyst-associated toxoplasmosis outbreaks have been documented, the relevance of the environmental transmission route remains poorly investigated. Thus, we carried out an extensive systematic review on *T. gondii* oocyst contamination of soil, water, fresh produce, and mollusk bivalves, following the PRISMA guidelines. Studies published up to the end of 2020 were searched for in public databases and screened. The reference sections of the selected articles were examined to identify additional studies. A total of 102 out of 3201 articles were selected: 34 articles focused on soil, 40 focused on water, 23 focused on fresh produce (vegetables/fruits), and 21 focused on bivalve mollusks. *Toxoplasma gondii* oocysts were found in all matrices worldwide, with detection rates ranging from 0.09% (1/1109) to 100% (8/8) using bioassay or PCR-based detection methods. There was a high heterogeneity ($I^2 = 98.9\%$), which was influenced by both the sampling strategy (e.g., sampling site and sample type, sample composition, sample origin, season, number of samples, cat presence) and methodology (recovery and detection methods). Harmonized approaches are needed for the detection of *T. gondii* in different environmental matrices in order to obtain robust and comparable results.

Keywords: *Toxoplasma gondii*; oocysts; environment; soil; water; fresh produce; fruit; bivalve mollusk; sampling strategy; methodology

1. Introduction

Toxoplasmosis is one of the most important opportunistic parasitic diseases affecting humans and animals worldwide and is caused by the obligate intracellular protist *Toxoplasma gondii*. Clinical manifestations associated with toxoplasmosis are various, and they include ocular disease [1,2], pneumonia [3,4], and encephalitis in immunocompromised

patients [1,5]. *Toxoplasma gondii* infection can also cause spontaneous abortion, congenital malformations, and stillbirth in both humans and animals [6,7].

Domestic and wild felids are the specific definitive hosts of *T. gondii*, whereas warm-blooded vertebrates, including humans, are intermediate hosts [8]. Up to 70% of the cat population is infected with *T. gondii* [9], and the infected cats can shed millions of oocysts in their feces. The subsequent development of sporulated oocysts in the environment depends on temperature and humidity [10,11].

Humans, as well as animals, can become infected with *T. gondii* through the consumption of raw or undercooked meat of infected animals harboring the tissue-dwelling stages of the parasite (bradyzoites contained within tissue cysts) [12] as well as via congenital transmission and blood transfusion by the active replicative stages of the parasite (tachyzoites) [10]. Another important route of human and animal infection is through the ingestion of sporulated *T. gondii* oocysts present in the environment, contaminating soil, water, and feed and food, including fresh produce and seafood [13]. According to a systematic review of studies carried out up to March 2018, 44.1% (15/34) of documented worldwide outbreaks were oocyst-related [14].

Soil contamination is a significant source of human infection, with soil of public parks, schools, gardens, and farms considered particularly important. Oocysts can be distributed within the soil by arthropods, earthworms, wind, and rain [7], and the sporulated oocysts are highly resistant and can persist infective in soil for up to two years [11].

Waterborne infections associated with *T. gondii* oocysts are nowadays considered increasingly significant due to evidence of large-scale outbreaks [7,13]. Water in irrigation systems, rivers, lakes, beaches, and coasts, as well as wastewater and groundwater can be contaminated with the environmentally resistant oocysts. Moreover, oocysts can survive various inactivation procedures using chemical reagents, including sodium hypochlorite and chlorine [15,16]. Oocysts remain viable in water for 18 months at 4 °C after exposure to 2% sulfuric acid [7,17], for 15 and 54 months at 20–25 °C and 4 °C in fresh water, respectively, and around 6 months in artificial seawater (15 ppt) at the same temperatures [18].

In recent years, *T. gondii* infection cases linked to fresh vegetable consumption have been on the increase [14]. Oocyst contamination of fresh vegetables may occur through cultivation in contaminated soil or using contaminated water for irrigation or washing. As testing for parasite contamination in fresh produce is neither regulated nor mandatory, the increased popularity of consumption of raw and ready-to-eat vegetables may pose a new potential risk for consumers who could be accidentally exposed to oocysts, since most post-harvest processing measures do not guarantee the complete removal of oocysts or their effective inactivation [16,19].

Toxoplasma gondii oocysts can also enter the marine environment through improper disposal of sewage, inefficient treatment plants, water discharge, and water runoff [20], and they can cause infections in marine animals and the contamination of marine fauna [21,22]. Consistently, oocysts have been detected in wild and commercial bivalve mollusks in several countries. Bivalves continuously filter large volumes of water and concentrate microorganisms [23]. They can retain viable *T. gondii* oocysts for 85 days following uptake [24]. Thus, they are considered good biological indicators of parasitic contamination of aquatic environments and could pose another risk for consumers when consumed undercooked or raw [18,25].

Environmental contamination with *T. gondii* oocysts is understudied and likely underestimated, which is partly due to the lack of suitable harmonized sampling approaches and detection methods. Studies on cat feces or susceptible intermediate hosts have been used as a substitute to predict the level of environmental contamination [26,27], but they may have inadequate power to accurately assess contamination. Due to limited baseline data on oocyst occurrence in environmental samples, accurate estimation of the contamination in the environment requires large sample sizes and sample volumes, which may contain small quantities of oocysts of different ages [7]. Limitations in oocyst recovery and detection methods, in combination with various sampling strategies, have made it

difficult to ascertain the contribution of environmental contamination with *T. gondii* oocysts to human infections. Indirect methods for discriminating between infections caused by oocysts *vs.* tissue-dwelling stages of *T. gondii* have been developed but have not been widely applied [28].

Another important challenge to full evaluation of the relevance of *T. gondii* oocyst infection route is the assessment and quantification of oocyst viability and therefore infectivity for humans and animals. So far, the only reliable method is based on mouse bioassay, i.e., experimental administration of oocysts to mice and detection of infection in tissues [8], although new approaches based on molecular methods have been proposed and are under evaluation for their applicability [29–31].

To date, reviews on *T. gondii* environmental contamination of one [18,32] or more matrices [7], and systematic reviews covering one matrix exist [9,33–35]; however, they mainly focused on detection rates or analytical methods. Thus, this article aims to provide a more complete, comprehensive systematic review of the existing literature on environmental contamination with *T. gondii* oocysts, including available data on sampling strategies, and identifying relevant knowledge gaps and limitations in relation to sampling strategies and methods for the recovery and detection of *T. gondii* oocysts in soil, water, fresh produce, and bivalve mollusks. Finally, based on the observations, recommendations are suggested for future studies on environmental contamination with *T. gondii* oocysts.

2. Materials and Methods

2.1. Literature Search and Eligibility Criteria

A systematic review of the prevalence of *T. gondii* oocysts in soil, water, fresh produce (vegetables and fruits), and bivalve mollusks worldwide was performed; all papers published, with no restriction on language, until the end of 2020 were included, following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) [36].

The databases used were PubMed, Web of Science, and Scopus. In all cases, a combination of three search terms was employed and included "*Toxoplasma*" and "oocysts" or "oocyst" and "vegetables" or "fruits" or "ready to eat" or "salads" or "greenery" or "water" or "soil" or "bivalves" or "mussels" or "clams" or "oysters" or "abalone". In view of the diversity of terms yielding eligible studies, an additional search was performed using related terms such as "food" or "products" or "otters". Additionally, the bibliographies of the selected articles were screened to identify studies to include (Supplementary Table S1).

The articles were selected using the following inclusion criteria: worldwide studies reporting direct detection of *T. gondii* oocysts in one of the matrices of interest (soil, water, fresh produce, and bivalves) with full text available. Exclusion criteria were the following: methodological studies aiming only to the development or improvement of oocyst recovery or detection methods (i.e., using artificial spiking of matrices), studies performed on other matrices, studies without available full text, studies published after 2020, and duplicates.

2.2. Selection Process and Data Extraction

Three investigators carried out the initial screening focusing on title and abstract, and based on this, eligible articles were preselected and subjected to an in-depth review to confirm if they met the selection criteria. Subsequently, data extraction was carried out by two co-authors, and a third co-author resolved discrepancies.

For data extraction, one data sheet per matrix was created in Microsoft Excel 2013. For all matrices, the data sheet included sample type/details and origin, sampling year and season, period, country and continent, samples by categories (n), total number of samples, sample units, presence of cats in the sampling area, association with human *T. gondii* infection or toxoplasmosis (outbreaks or sporadic cases), positive samples by categories

(number and percentage), total number of positive samples (number and percentage), sample collection and preparation, oocyst recovery and detection methods, DNA extraction method and molecular markers used, oocyst quantification (mean, median, and range), analytical sensitivity (Se), additional molecular methods used, source of information, journal subject area, and other parasites investigated (Supplementary Tables S2–S5).

Specific columns were also included in the spreadsheets according to the type of matrix. For soil, there were columns related to sampling site, sample size, and depth of sample collection (cm). Columns related to the type of aquifer, the uses, and the treatment received were added for water, and matrix composition, product type (organic, conventional or both), and product presentation (bulk, packaged, or ready-to-eat (RTE)) for fresh produce. Finally, for bivalves, columns related to sampled species, sampling site, specimen length (cm), depth of collection (cm), and type of tissue or material analyzed were added.

When a study analyzed two or more matrices, data were extracted and considered separately for each matrix. The data extracted were limited to the information provided in the articles.

2.3. Data Analyses

Several studies reported oocyst detection by light microscopy or direct visualization of *T. gondii* oocysts by autofluorescence using an epifluorescent microscope as the only or initial screening method. However, since these techniques cannot prove the identity of *T. gondii* oocysts due to their shape and size similarity with several genera and species of the Sarcocystidae family, and because oocyst wall autofluorescence is not an exclusive feature of *T. gondii*, data based on microscopy findings were extracted and included in Supplementary Tables S2–S5, but they were not considered for data analyses. Accordingly, only data based on molecular and bioassay methods were included in the Results and Discussion sections. Moreover, only data from individual experimental samples were included in the analysis, not data from pooled samples. Regarding fresh produce, it was not always clear whether pooled samples were analyzed. Thus, if the mass of the sample analyzed was greater than the sample unit mass, it was considered to be a pool and was consequently excluded (e.g., sample units of 3600 g of lettuce [37] or 1000 g of strawberries [38]).

For the evaluation of heterogeneity and pooled estimates, detection rates reported in each study were combined per matrix (soil, water, fresh produce, bivalve mollusks), using STATA 15.0 software (StataCorp, Texas, the United States) and a restricted maximum likelihood method with a random effects model. A Forest plot was created for easy data deviation within matrix type (Supplementary Figures S1–S4). The inverse variance index (I^2) was used to quantify heterogeneity [39,40]. In addition, study bias and heterogeneities at the study level were calculated by Egger's test, funnel plots (Supplementary Figure S5), and Cochran's Q test, respectively [41].

3. Results and Discussion

3.1. Literature Search and Article Selection

A total of 3201 articles were obtained from the search process, and 321 were preselected based on their titles or abstract and removal of duplicates. Finally, 102 articles were included for data extraction (Figure 1; Supplementary Table S1). Among them, 13 articles focused on the analysis of two or more matrices and 34 articles reported data on soil, 40 reported data on water, 23 reported data on fresh produce (vegetables and fruit), and 21 reported data on bivalve mollusks. An attempt to gather more data on *T. gondii* oocyst prevalence was undertaken by collecting gray literature (e.g., unpublished scientific information, including reports from governmental agencies, thesis dissertations, conference proceedings) using an online survey administered to experts in the field. The search

yielded seven reports not published in English-language peer-reviewed journals with very limited information on the sampling strategies and methodologies employed [42].

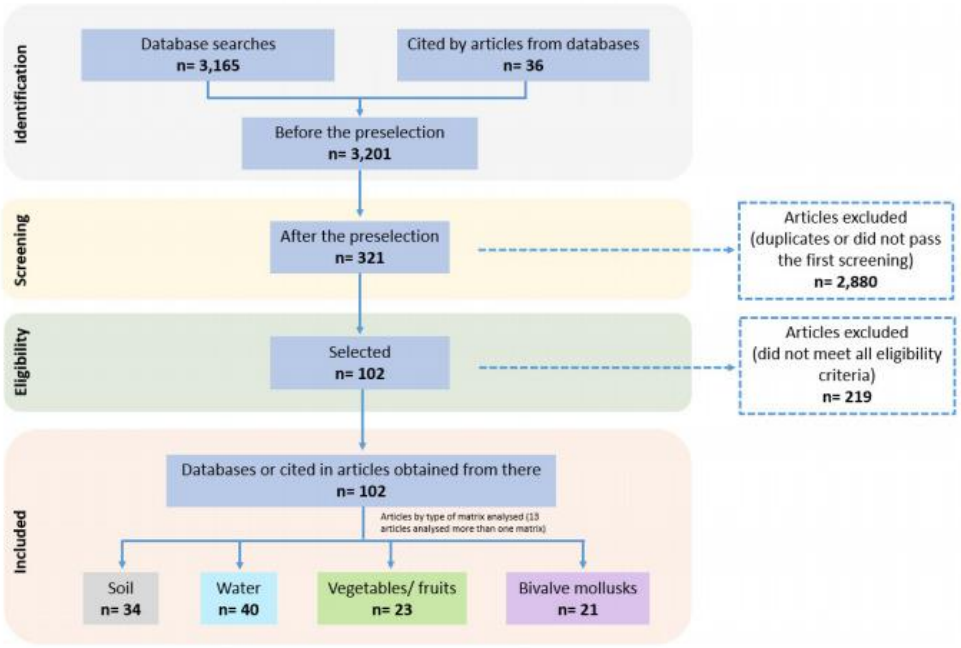

Figure 1. Four-step flow diagram of the systematic review of the presence of *Toxoplasma gondii* oocysts in soil, water, vegetables, fruit, and bivalve mollusks worldwide until the end of 2020.

3.2. *Toxoplasma gondii* Oocyst Detection in Environmental Matrices

Different environmental matrices have received increasing attention over the past 50 years. The studies included were conducted on soil ($n = 34$) between 1971 and 2019, water ($n = 40$) between 1992 and 2019, fresh produce ($n = 23$) between 2006 and 2019, and bivalves ($n = 21$) between 2002 and 2018. Soil was first investigated early in the 1970s immediately after the full life cycle of *T. gondii* was described and the environmentally resistant stage, the oocyst, was discovered [43]. Later, in the 1990s, the first reports of the presence of *T. gondii* in water were published. More recently, in the 2000s, studies have been conducted in bivalve mollusks and fresh produce.

The timeline of the studies included here appears to be in accordance with our increased understanding of the importance of other food and waterborne zoonotic protists (particularly *Cryptosporidium* spp., but also *Giardia duodenalis* and *Cyclospora cayetanensis*) and the detection of outbreaks. Indeed, from the 1990s onwards, numerous studies demonstrated the presence of *Cryptosporidium* spp. in public water supplies and recreational and river water sources [44], together with two massive outbreaks of cryptosporidiosis in humans associated with water supplies in Georgia and Milwaukee in the United States [45,46], among others. Moreover, water-related toxoplasmosis outbreaks were documented earlier than fresh produce-related outbreaks [14]. Finally, the first studies conducted on mollusks and fresh produce from 2002 or 2006 onwards coincide with similar investigations carried out in other food and waterborne protists. Late in the 1990s, it was reported that bivalves could act as mechanical vectors of *Cryptosporidium* spp. oocysts due

to their survival in estuarine waters for several weeks [47], which led to further studies on different bivalve species. Since 2000, both *Cryptosporidium* spp. and *T. gondii* have been more extensively studied in fresh vegetables and fruit [33]. A recent review stated that 5.9% (2/34) of oocyst-related outbreaks were attributable to fresh produce consumption, with both types of fresh produce, vegetables and fruit, as sources of oocysts in outbreaks occurring since 2009 [14].

Toxoplasma gondii was detected in different environmental matrices worldwide using molecular methods (e.g., PCR and loop-mediated isothermal AMPlification, LAMP) or bioassays, which are sensitive and specific methods, as shown in Tables 1–4 and Figure 2.

The presence of *T. gondii* oocysts in soil was detected in 28 out of 34 studies in the following countries: Brazil (n = 5), China (n = 7), Costa Rica (n = 1), France (n = 3), French Guiana (n = 1), Iran (n = 3), Iraq (n = 1), Mexico (n = 1), Panama (n = 1), Pakistan (n = 1), Poland (n = 1), the Netherlands (n = 1), and the United States (n = 2) with overall detection rates ranging from 1.0% (7/700) [48] to 100% (5/5) [49], both from China (Table 1 and Figure 2; Supplementary Table S2).

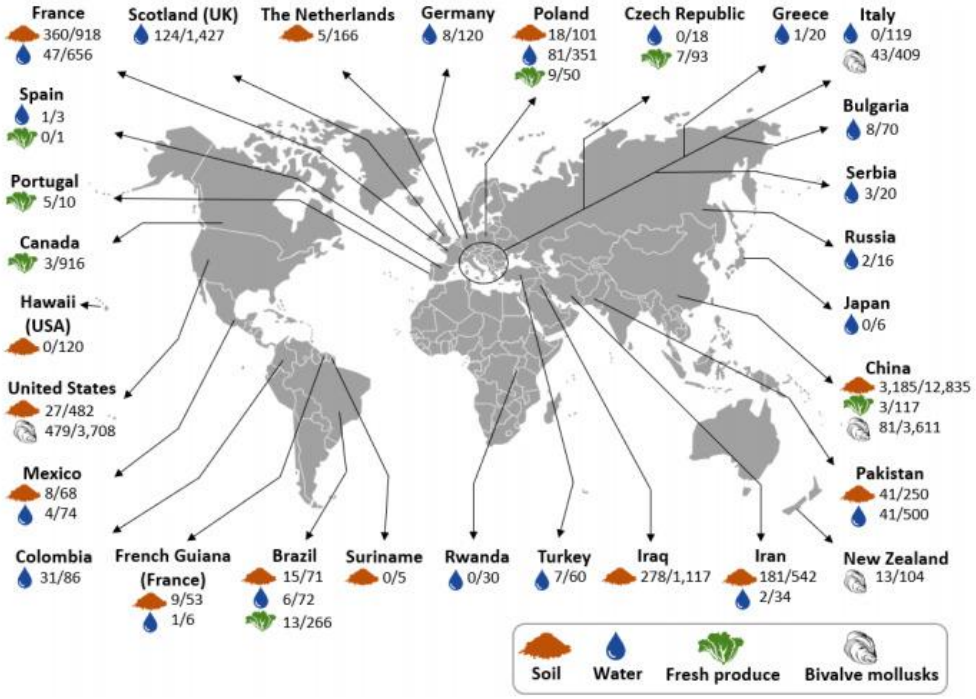

Figure 2. Worldwide detection of *Toxoplasma gondii* oocysts in environmental matrices based on molecular methods (PCR, qPCR, and LAMP) in studies published by the end of 2020. Results are presented as positive samples/total of samples collected. Articles that analyzed pooled samples and did not specify how the number of positive individual samples was estimated were excluded.

Water was the environmental matrix most extensively studied worldwide with 25 out of 40 articles reporting *T. gondii*-positive samples in Brazil (n = 6), Colombia (n = 2), Egypt (n = 1), France (n = 2), French Guiana (France) (n = 1), Germany (n = 1), Greece and Bulgaria (n = 1), Iran (n = 1), Mexico (n = 1), Pakistan (n = 1), Poland (n = 3), Russia and

Bulgaria (n = 1), Scotland (n = 1), Serbia (n = 1), Spain (n = 1), and Turkey (n = 1). Overall, detection rates ranged from 5% (1/20) in Greece [50] to 100% (8/8) in Brazil [51], and most studies reported a detection rate below 20% (Table 2 and Figure 2; Supplementary Table S3).

Altogether, twenty-three studies were conducted on fresh produce matrices that were classified as leafy greens, non-leafy vegetables (including root crops), herbs, and fruit. Positive samples were reported in all fresh produce matrices in 13 articles from Brazil (n = 2), Canada (n = 1), China (n = 1), Colombia (n = 2), the Czech Republic (n = 1), Egypt (n = 1), Italy (n = 1), Pakistan (n = 1), Poland (n = 1), Spain and Portugal (n = 1), and Switzerland (n = 1) (Table 3 and Figure 2). Detection rates in fresh produce ranged from 0.3% (3/1171) in Canada [52] to 50.0% (13/26) in Portugal [37], and in the majority of studies, detection rates were below 10% (Table 3 and Figure 2, Supplementary Table S4).

Finally, the presence of *T. gondii* oocysts in bivalves was reported in 19 out of 22 studies in Brazil (n = 4), China (n = 2), France (n = 1), Italy (n = 3), New Zealand (n = 1), Tunisia (n = 1), Turkey (n = 2), and the United States (n = 5), with detection rates that varied from 0.1% (1/1109) [21] to 46.3% (19/41) [53], both in the United States, and from 1.3% (2/160) to 31.0% (19/60) in pooled samples from Brazil [54] and the United States [55], respectively. In most studies, the detection rates were below 7% (Table 4 and Figure 2, Supplementary Table S5).

Table 1. Worldwide detection of *Toxoplasma gondii* oocysts in soil based on molecular and bioassay methods in articles published up to the end of 2020.

Sampling Strategy						Methods Used		Results	Sources
Sampling Location (Country)	Sample Origin	No. of Samples Collected	Sample Amount Collected/Sample Size Analyzed (Depth)	Presence of Cats	Link with Human Toxoplasmosis [a]	Oocyst Recovery Method [†]	Detection Methods (Molecular Target)	Positive Samples (%)	
Brazil	Dairy farm	5	500 g/500 g (no data)	Yes	Yes	Wash, filtration, centrifugation, flotation, centrifugation, wash, and centrifugation	Mouse bioassay: Sabin Feldman dye test and brain smear confirmed by bioassay in cats	1 (20.0) [b]	[56]
Brazil	Paddocks from ostrich farms	40	250 g/25 g (5–10 cm)	No data *	No	Wash, filtration, centrifugation, flotation, centrifugation, wash, and centrifugation	PCR, qPCR (529 RE and 18S rRNA)	13 (32.5) [b]	[57]
Brazil	Elementary public schools	31	1000 g/no data (5 cm)	No data	No	Flotation and centrifugation	Mouse bioassay: squash Mouse bioassay: histopathology Mouse bioassay: immunohistochemistry Mouse bioassay: indirect fluorescent antibody test (IFAT)	7 (22.6) 0 10 (32.3) 8 (25.8)	[58]
Brazil	Sheep farms	10, each inoculated in 5 mice	1 g/1 g (no data)	Yes	No	Wash, flotation, and centrifugation	PCR (529 RE) Mouse bioassay IP/PO-PCR (529 RE) Mouse bioassay IP/PO-IFAT	0 IP: 6 (30.0), PO: 7 (23.3) IP: 14 (70.0), PO: 19 (63.3)	[59]
Brazil	Sludge from a cistern, and soil from greenhouses and vegetable gardens	11	500 mL and 100 g/no data (no data)	Yes	Yes	Centrifugation and flotation	PCR (529 RE)	0	[60]
Brazil	Horticultural properties	10	10 g/10 g (from surface)	Yes	No	Wash and centrifugation	PCR (529 RE)	2 (20)[b]	[61]

Sampling Strategy						Methods Used		Results	Sources
Sampling Location (Country)	Sample Origin	No. of Samples Collected	Sample Amount Collected/Sample Size Analyzed (Depth)	Presence of Cats	Link with Human Toxoplasmosis [a]	Oocyst Recovery Method [f]	Detection Methods (Molecular Target)	Positive Samples (%)	
China	Schools, parks, farms, and coastal beaches	2100	20 g/no data (5 cm)	No data	No	Wash, flotation, centrifugation, wash, and centrifugation	PCR, Semi-nPCR, nPCR (529 RE, B1, and ITS-1)	230 (10.9) [d]	[62]
China	Public parks	252	No data/0.5 g (5 cm)	Yes	No	No data	PCR (B1 and 529 RE) LAMP (MIC3, F3, B3, BIP, FIP, LD, BF)	41 (16.3) [d] 58 (23.0)	[63]
China	Pig farms	95	No data/0.5 g (5 cm)	Yes	No	No data	PCR (B1 and 529 RE) LAMP (MIC3, F3, B3, BIP, FIP, LD, BF)	20 (21.1) 36 (37.9)	[64]
China	Urban areas (foci of human habitation, gravel, sand, industrial and commercial land, woodland, grassland)	9420	20 g/4 replicates of 5 g (10 cm)	Yes	No	Wash, flotation, centrifugation, wash, and centrifugation	qPCR (529 RE)	2853 (30.3)	[65]
China	Swine hoggery	5	No data/0.5–5 g (no data)	No data	No	Ultrasonic treatment and sugar flotation	Mouse bioassay: Sabin Feldman dye test and kitten bioassay	5 (100) [b]	[49]
China	Schools, parks, and grazing area	268	No data/5 g (no data)	No data	No	Wash, filtration, centrifugation, flotation, wash, and centrifugation, presumably	Semi-nested PCR (529 RE)	34 (12.7) [d]	[66]
China	Chicken farms (free-range and scale farms)	700	10–15 g/10–15 g (from surface)	No data	No	No data	PCR (ITS-1)	7 (1) [d]	[48]
Costa Rica	Yard and coffee plantation	15	10 g/10 g (from surface or 5–7 cm)	Yes	No	Wash, centrifugation, flotation, centrifugation	Mouse bioassay: Dye test and squash	4 (26.7) [b]	[67]
France	Areas around a hospital where cats defecate	117	200–300 g/10 g (2 cm)	Yes	No	Wash, filtration, centrifugation, flotation, and centrifugation	qPCR (529 RE)	11 (9.4) [b]	[68]

Sampling Strategy						Methods Used		Results	Sources
Sampling Location (Country)	Sample Origin	No. of Samples Collected	Sample Amount Collected/Sample Size Analyzed (Depth)	Presence of Cats	Link with Human Toxoplasmosis [a]	Oocyst Recovery Method [†]	Detection Methods (Molecular Target)	Positive Samples (%)	
France	Village areas, crop field, grassland, forest	243	20 g/4 replicates of 5 g (up to 2 cm)	Yes	No	Wash, flotation, centrifugation, wash, and centrifugation	qPCR (529 RE)	71 (29.2)	[69]
France	Dairy farms	558	20 g/5 g (2 cm)	Yes	No	Wash, centrifugation, flotation, wash, and centrifugation	qPCR (529 RE)	278 (49.8)	[12]
French Guiana (France)	Areas around houses and random sites	53	No data/20 g (no data)	Yes	Yes	Wash and centrifugation	PCR (529 RE)	9 (17.0) [b,d]	[70]
Hawaii (USA)	University campus and a natural area reserve	120	No data/20 g (10 cm)	Yes	No	Wash, centrifugation, flotation, centrifugation, wash, and centrifugation	PCR (GRA6)	0	[71]
Iran	Urban and rural areas	192	300–500 g/7 g (no data)	Yes	No	Wash, centrifugation, flotation, centrifugation, wash, and centrifugation	nPCR (529 RE)	150 (78.1)	[72]
Iran	Sand pits, playgrounds, public parks, and areas around rubbish dumps	200	400 g/40 g (2–5 cm)	Yes	No	Wash, filtration, centrifugation, flotation, and centrifugation	PCR (GRA6)	18 (9) [d]	[73]
Iran	Rubbish dumps, children`s playground, parks and public places	150	300 g/no data (3 cm)	No data	No	Wash, centrifugation, flotation, and centrifugation	PCR (B1)	13 (8.7) [d]	[74]
Iraq	Private gardens, schools, agricultural lands, territory of waste dumps, abandoned lands where children sometimes play, playgrounds, and parks	1117	300 g/40 g (2–5 cm)	Yes	No	No data	nPCR (B1)	278 (24.9) [b]	[75]

Sampling Strategy						Methods Used		Results	Sources
Sampling Location (Country)	Sample Origin	No. of Samples Collected	Sample Amount Collected/Sample Size Analyzed (Depth)	Presence of Cats	Link with Human Toxoplasmosis [a]	Oocyst Recovery Method [†]	Detection Methods (Molecular Target)	Positive Samples (%)	
Mexico	Playground boxes	68	10 g/10 g (<2 cm, 2–10 cm or until reaching rock bottom)	Yes	No	Wash, centrifugation, flotation, wash, and centrifugation	nPCR (SAG1)	8 (11.8)	[76]
Panama	Outdoor children's play areas	924	30 g/30 g (no data)	Yes	Yes	Wash, centrifugation, flotation, and centrifugation	Mouse bioassay: direct agglutination test	10 (1.1)	[77]
Pakistan	Homes, gardens, public enclosures, and backyards from urban and rural areas	250 [c]	300 g/no data (2-5 cm)	Yes	No	No data	PCR (B1, 529 RE)	B1 = 41 (16.4) [b] 529 RE = 41 (16.4)[b]	[78]
Poland	Sand pits, rubbish dumps and sand heaps	101	300 g/40 g (2-5 cm)	Yes	No	Wash, centrifugation, flotation with centrifugation, attachment to a glass slide and wash of the glass slide	PCR (B1 and 200–300 REP)	18 (17.8) [d]	[79]
Suriname	Different areas from a village	5	200 g/50 g (no data)	Yes	Yes	Flotation (no more information is given)	qPCR (B1)	0	[80]
The Netherlands	Residential gardens and a limited number of playgrounds	166 [e]	100 g/25 g (5 cm)	No data	No	Magnetic capture	qPCR (529 RE)	5 (3.0)	[81]
The United States	Cities, state parks, public playgrounds, and community gardens	482 [f]	20–50 g/replicates of 5 g (2–5 cm)	Yes	No	Wash, flotation, centrifugation, wash, and centrifugation	nPCR (ITS1)	27 (5.6) [d]	[82]
The United States	Pig farms	79	250 g/250 g (no data)	Yes	No	Wash, filtration, centrifugation, flotation, wash, and centrifugation	Mouse bioassay-squash and serology	1 (1.3)	[83]

* Cats were observed near the feed tanks, but no information is provided about their presence in the paddocks, and it is not clear how far the feed tanks were from the sampling area. [†] Oocyst recovery method specified step by step. [a] Investigations linked with human toxoplasmosis: outbreaks, endemic or sporadic cases (IgG and/or IgM tested and/or clinical signs/symptoms documented). [b] Detection rate not given, calculated based on the data provided. [c] Stated that 500 soil samples were collected, but results corresponded to 250 samples. [d] Positive samples were sequenced and/or genotyped. [e] A total of 148 out of 166 samples collected yielded interpretable results by qPCR, but the results were based on the 166 samples collected. [f] According to Table 3 of the original manuscript, 501 samples were collected, but 482 samples were considered in the text. Mouse bioassay IP: inoculated intraperitoneally, mouse bioassay PO: peroral. IFAT: indirect immunofluorescence test. LAMP: loop-mediated isothermal amplification. Articles with results only or also based on microscopy assay or other methods that were not specified: [49,57,60,80,84–86].

Table 2. Worldwide detection of *Toxoplasma gondii* oocysts in water based on molecular and bioassay methods in articles published up to the end of 2020.

Sampling Strategy							Methods Used		Results	Reference
Sampling Location (Country)	Sample Details	No. of Samples Collected	Sample Volume Collected (Sample Volume Analyzed) (Liters—L)	Water Treatment	Presence of Cats	Link with Human Toxoplasmosis [a]	Oocyst Recovery Method [a]	Detection Methods (Molecular Target)	Positive Samples (%)	
Brazil	Water from wells	1750 L filtered through 17 membranes and inoculated into 8 chickens	50 per well	No data	No data	Yes (endemic toxoplasmosis area)	Filtration	Chicken bioassay: MAT Molecular (no data)	3 (37.5) [b] 0	[87]
Brazil	Irrigation and municipal water	3	10	No data	Yes	Yes	Filtration, wash, and centrifugation	PCR (529 RE)	1 (33.3) [b]	[88]
Brazil	Water from cisterns	3	10–20	No data	Yes	Yes	Filtration, wash, and centrifugation	PCR (529 RE)	0	[60]
Brazil	Irrigation water	10	0.01	No data	Yes	No	Filtration, wash, and centrifugation	PCR (529 RE)	2 (20.0)	[61]
Brazil	Drinking water	4650 L filtered through 56 membranes	No data	Untreated	Yes	Yes	Filtration and centrifugation	PCR (B1) Mouse, chicken, pig and cat bioassays	Positive by at least 1 assay [c]	[89]
Brazil	Surface water used to produce drinking water	39	20	No data	No data	No	Filtration, wash, and centrifugation	qPCR (B1)	3 (7.7)	[90]
Brazil	Drinking water	8	Given ad libitum to the piglets	Treated (process not specified)	No data	Yes	Directly	Piglet bioassay: IFAT Piglet bioassay: tissue PCR (529 RE) Piglet bioassay: tissue mouse bioassay and PCR (529 RE)	8 (100) 5 (62.5) 5 (62.5) [b]	[51]

Sampling Strategy							Methods Used		Results	Reference
Sampling Location (Country)	Sample Details	No. of Samples Collected	Sample Volume Collected (Sample Volume Analyzed) (Liters—L)	Water Treatment	Presence of Cats	Link with Human Toxoplasmosis [a]	Oocyst Recovery Method [a]	Detection Methods (Molecular Target)	Positive Samples (%)	
Brazil	Farm water	No data (0.003)	No data	No data	Yes	No	Flotation and centrifugation	PCR (529 RE) Mouse bioassay	No data	[59]
Canada	Untreated water that supplied municipal drinking water treatment plants	11	Mean of 1051	Untreated	No data	Yes	Filtration, wash, centrifugation, flotation, wash, and centrifugation	Mouse bioassay: microscopy from tissue and MAT	0	[91]
Colombia	Water	40	0.2 or 4	Boiled and others not specified	No data	Yes	Sedimentation by centrifugation with formalin-ether	nPCR (B1)	4 (10.0) [b,c]	[92]
Colombia	Surface water before and during treatment, in the treatment plant network and from homes	46	10	Untreated and treated: coagulation, flocculation, sedimentation, filtration, and chlorination	No data	No	Sedimentation by centrifugation with formalin-ether	nPCR (B1)	27 (58.6) [c]	[93]
Czech Republic	Irrigation and vegetables	18	10	No data	Not data	No	Filtration, wash, and centrifugation	qPCR (B1 and 529 RE)	0	[94]

Sampling Strategy							Methods Used		Results	Reference
Sampling Location (Country)	Sample Details	No. of Samples Collected	Sample Volume Collected (Sample Volume Analyzed) (Liters—L)	Water Treatment	Presence of Cats	Link with Human Toxoplasmosis [a]	Oocyst Recovery Method [b]	Detection Methods (Molecular Target)	Positive Samples (%)	
	washing water									
Egypt	Irrigation water	54	No data	No data	No data	No	Filtration and centrifugation	Mouse bioassay-smears and MAT	9 (16.7)	[95]
France	Wastewater	35	20	Treated and untreated (process not specified)	No data	No	Filtration, wash, centrifugation, immunomagnetic separation of *Cryptosporidium* spp. and *G. duodenalis*, centrifugation, and flotation	PCR (529 RE)	0	[96]
France	Untreated surface, ground, and public drinking water	139	100 (7–100)	No data	No data	No	Filtration, wash, centrifugation, immunomagnetic separation of *Cryptosporidium* spp. and *G. duodenalis*, flotation, centrifugation, wash, and centrifugation	qPCR (B1) Mouse bioassay- agglutination test and smear	10 (8.0) [d] 0	[97]
France	Untreated surface, ground, and public drinking water	482	5–100	No data	No data	No	Filtration, wash, centrifugation, immunomagnetic separation of *Cryptosporidium* spp. and *G. duodenalis*, centrifugation, flotation, and centrifugation	PCR (B1 and 529 RE)	37 (7.7) [e]	[98]
French Guiana (France)	Water from cisterns, little streams,	6	10	No data	Yes	Yes	Filtration and presumably wash, centrifugation, flotation, centrifugation, wash, and centrifugation	PCR (529 RE)	1 (16.7) [b,c]	[70]

Sampling Strategy							Methods Used		Results	Reference
Sampling Location (Country)	Sample Details	No. of Samples Collected	Sample Volume Collected (Sample Volume Analyzed) (Liters—L)	Water Treatment	Presence of Cats	Link with Human Toxoplasmosis [a]	Oocyst Recovery Method [a]	Detection Methods (Molecular Target)	Positive Samples (%)	
	and brooks									
Germany	Wastewater	25	1	Untreated and treated: mechanical and biological treatments	No data	No	Filtration (sieve and cellulose filters), wash, and centrifugation	PCR (B1)	0	[99]
Germany	Variable: drinking water and others not specified	95	5–2500	Treated and untreated (process not specified)	No data	No	Flocculation for WWTPs, filtration for drinking, groundwater and surface water, then centrifugation and flotation for samples	LAMP (B1)	8 (8.4)	[100]
Greece Bulgaria Japan	River, reservoir, well, spring, tap, sewage, and recreational water	20 34 6	10	No data	No data	No	Flocculation, centrifugation, discontinuous sucrose gradients, wash, and centrifugation	nPCR (18S rRNA)	1 (5) [b] 3 (8.8) [b] 0	[50]
Iran	Natural water	34	5	No data	No data	No	Filtration, wash, centrifugation, and flotation	LAMP (B1)	2 (5.8)	[101]
Italy	Wastewater	119	10–20	Sand, membrane-bioreactor, plug-flow reactor,	No data	No	Filtration, wash, centrifugation, and flotation	qPCR (B1-multiplex)	0	[102]

Sampling Strategy							Methods Used		Results	Reference
Sampling Location (Country)	Sample Details	No. of Samples Collected	Sample Volume Collected (Sample Volume Analyzed) (Liters—L)	Water Treatment	Presence of Cats	Link with Human Toxoplasmosis [a]	Oocyst Recovery Method [a]	Detection Methods (Molecular Target)	Positive Samples (%)	
				and membrane ultrafiltration						
Mexico	Public drinking water	74	5	Chlorination	No data	No	Filtration, wash, centrifugation, flotation, centrifugation, wash, and centrifugation	nPCR (SAG1)	4 (5.4)	[103]
Pakistan	Drinking, recreational, and irrigation water	500	No data	No data	No data	No	Flocculation or filtration	PCR (B1 and 529 RE)	41 (8.2) [b]	[78]
Poland	Drinking water	114	5	No data	Yes	Yes	Filtration, wash, centrifugation, flotation with centrifugation, wash, and centrifugation	PCR (no data)	31 (27.2)	[104]
Poland	Drinking and natural water	201	5	No data	Yes	Yes	Filtration, wash, centrifugation, flotation with centrifugation, wash, and centrifugation	PCR (B1) Mouse bioassay of 14 PCR positive samples-tissue PCR or agglutination test	43 (21.4) [b,c] Tissue PCR: 9 (64.3), agglutination test: 3 (21.4) [b]	[105]
Poland	Bathing and drinking water	36	50	No data	No data	No	Filtration, wash, and centrifugation	nPCR (B1)	7 (19.4) [c]	[106]
Russia Bulgaria	Natural water	16 36	No data	No data	No data	No	Flocculation, wash, and discontinuous sucrose gradient	nPCR (18S rRNA)	2 (12.5) [f] 5 (13.9) [f]	[107]

Sampling Strategy							Methods Used		Results	Reference
Sampling Location (Country)	Sample Details	No. of Samples Collected	Sample Volume Collected (Sample Volume Analyzed) (Liters—L)	Water Treatment	Presence of Cats	Link with Human Toxoplasmosis [a]	Oocyst Recovery Method [†]	Detection Methods (Molecular Target)	Positive Samples (%)	
								LAMP (B1)	9 (56.3) [f] 16 (44.4) [f]	
Rwanda	Irrigation and post-harvest washing water	30	1	Untreated those from rivers, lagoons, marshlands, and lakes	No data	No	No data	PCR (529 RE)	0	[108]
Scotland	Public water supply	1427	Up to 1000	No data	No data	No	Filtration, centrifugation, immunomagnetic separation of *Cryptosporidium* spp. and centrifugation	qPCR (529 RE)	124 (8.8) [c,g]	[109]
Serbia	Surface water from rivers	20	10	No data	No data	No	Filtration, wash, and centrifugation	PCR (529 RE)	3 (15.0) [c]	[110]
Spain presumably	Irrigation water	3	1.5	No data	No data	No	Centrifugation	qPCR (18S rRNA)	1 (33.3) [b,c]	[111]
Turkey	Natural water	60	10	No data	No data	No	Flocculation, centrifugation, wash, and discontinuous sucrose gradient	nPCR (18S rRNA) LAMP (B1)	7 (11.7) [c,h] 15 (25.0)	[112]
The United States	Presumably drinking water for animals	No data	0.05	No data	Yes	No	Centrifugation	Mouse bioassay-agglutination test and examination	No data	[83]

[†] Oocyst recovery method specified step by step. [a] Investigations linked with human toxoplasmosis: outbreaks, endemic, or sporadic cases (IgG and/or IgM tested and/or clinical signs/symptoms documented). [b] Detection rate not given, calculated based on the data provided. [c] Positive samples were sequenced and/or genotyped. [d] A total of 125 out of 139 samples collected yielded interpretable results. The detection rate was based on the interpretable results. [e] A total of 480 out of 482 samples collected yielded interpretable results. The detection rate was based on the interpretable results. [f] Reported one detection rate for both countries: nPCR

= 7/52 (13.5%), LAMP = 25/52 (48.0%). Detection rates for each country based on the data provided. [g] A total of 1411 out of 1427 samples collected yielded interpretable results. The detection rate was based on the interpretable results. [h] Six samples were positive by nPCR (mentioned in the abstract); however, detection rates were recalculated according to the results included in the text and tables (7 positive samples). LAMP: loop-mediated isothermal amplification. Articles with results only or also based on microscopy assay or other methods that were not specified: [78,86,96,99,104,105,107,113–117].

Table 3. Worldwide detection of *Toxoplasma gondii* oocysts in fresh produce (vegetables and fruit) based on molecular and bioassay methods in articles published up to the end of 2020.

Sampling Strategy								Methods Used		Results	Reference
Sampling Location (Country)	Matrix	Production Type (Organic and/or Conventional)	Product Presentation (Bulk, Packaged or Ready to Eat—RTE)	No. of Samples Collected	Sample Mass Collected (Sample Mass Analyzed)	Presence of Cats	Linked with Human Toxoplasmosis [a]	Oocyst Recovery Method [†]	Detection Methods (Molecular Target)	Positive Samples (%)	
Brazil	Lettuce	No data	No data	4	No data	Yes	Yes	Wash, scraping, and centrifugation	PCR (529 RE)	0	[88]
Brazil	Crisp lettuce, regular lettuce, chicory, rocket, and parsley	Organic and conventional	No data	220 [c]	50 g	No data	No	Wash, filtration, and centrifugation	PCR (B1 and 529 RE)	9 (3.8)	[118]
Brazil	Vegetable clumps (no more details given)	No data	No data	11	50 g	Yes	Yes	Wash, filtration, and centrifugation	PCR (529 RE)	0	[60]
Brazil	Crisp lettuce, arugula, chicory, chives, purple lettuce, spinach, and chard	Organic	No data	42	50 g	Yes	No	Wash, filtration, and centrifugation	PCR (529 RE)	4 (9.5) [e]	[61]
Canada	Variable [‡]	Organic and conventional	Bulk and packaged	1171	35 ± 0.5 g	No data	No	Wash, centrifugation, and flotation	qPCR (18S rDNA)	3 (0.3) [b]	[52]
China	Lettuce, pak choi, Chinese cabbage, rape, asparagus, *Chrysanthemum coronarium*, endive, Chinese chives, cabbage, red cabbage, and spinach	No data	No data	279	No data	No data	No	Wash, flocculation, and centrifugation	qPCR (B1)	10 (3.6) [b]	[19]

Sampling Strategy								Methods Used		Results	Reference
Sampling Location (Country)	Matrix	Production Type (Organic and/or Conventional)	Product Presentation (Bulk, Packaged or Ready to Eat—RTE)	No. of Samples Collected	Sample Mass Collected (Sample Mass Analyzed)	Presence of Cats	Linked with Human Toxoplasmosis [a]	Oocyst Recovery Method [a]	Detection Methods (Molecular Target)	Positive Samples (%)	
Colombia	Lettuce, cabbage, cucumber, carrot, and tomato	No data	No data	30	200 g	No data	Yes	Wash, sedimentation/centrifugation with formalin ether	nPCR (B1)	1 (3.3) [b,e]	[92]
Colombia	Strawberries	No data	Bulk and packaged	120	250 g (3 replicates of 30 g)	No data	No	Wash and centrifugation	qPCR (529 RE-multiplex)	6 (5.0)[b]	[119]
Czech Republic	Carrot, cucumber, lettuce (butterhead lettuce, iceberg lettuce, little gem, and lollo lettuce)	No data	Bulk and packaged (just for lettuce)	292	100 g	No data	No	Wash and centrifugation	qPCR (B1 and 529 RE)	28 (9.6) [b]	[94]
Egypt	Lettuce, carrot, and cucumber	No data	No data	54	150 g	No data	No	Wash, filtration, and centrifugation	Mouse bioassay: smears + MAT	7 (13.0)	[95]
Italy	Mix salad: curly and escarole lettuce, red radish, rocket salad, and carrots	No data	RTE	648 (72 pools)	100 g	No data	No	Wash and centrifugation	qPCR (B1)	5 (0.8) [b]	[120]
Pakistan	Apple, banana, guava, cabbage, brinjal, and tomato	No data	No data	250	No data	No data	No	No data	PCR (B1 and 529 RE)	12 (4.8) [e]	[78]
Poland	Strawberries, radish, carrot, and lettuce	No data	No data	216	1–20 units, 500–1000 g	Yes (in farms-home gardens)	No	Wash, flocculation, and centrifugation	qPCR (B1)	21 (9.7)[b]	[38]

Sampling Strategy								Methods Used		Results	Reference
Sampling Location (Country)	Matrix	Production Type (Organic and/or Conventional)	Product Presentation (Bulk, Packaged or Ready to Eat—RTE)	No. of Samples Collected	Sample Mass Collected (Sample Mass Analyzed)	Presence of Cats	Linked with Human Toxoplasmosis [a]	Oocyst Recovery Method [†]	Detection Methods (Molecular Target)	Positive Samples (%)	
Spain Portugal	Lettuce, carrot, parsley, watercress, coriander, mix salad, arugula, strawberries, raspberries, and blueberries	Organic and conventional	Bulk, packaged, and RTE	9 26	64–3600 g	No data	No	Wash, centrifugation, immunomagnetic separation of *Cryptosporidium* spp. and *G. duodenalis*	PCR (529 RE)	2 (22.2) [b,d] 13 (50.0) [b,d]	[37]
Switzerland	Lettuce (different types, but not specified)	No data	No data	100	900–1800 g (pools of 9 lettuce)	No data	No	Wash, filtration, and centrifugation	PCR (B1)	6 (6.0) [b,e]	[121]

[†] Oocyst recovery method specified step by step. [‡] Types of fresh produce analyzed: arugula/baby arugula, kale, spinach/baby spinach, romaine, chard, leaf lettuce (green and red), spring mix, leafy green mixes (mix of 2 or more leafy green types), any dandelion, collards, rapini, escarole and marche. [a] Investigations linked with human toxoplasmosis: outbreaks, endemic, or sporadic cases (IgG and/or IgM tested and/or clinical signs/symptoms documented). [b] Positive samples were sequenced and/or genotyped. [c] According to the abstract, a total of 238 samples were collected, but the sum of each type of vegetable collected corresponded to 220 samples. [d] Fourteen positive samples were reported in the text, but there were 15 positive samples in the tables, and detection rates by country were not given. [e] Detection rate not given, calculated based on the data reported. Articles with results only or also based on microscopy assay or other methods that were not specified: [120,122–129].

Table 4. Worldwide detection of *Toxoplasma gondii* oocysts in bivalve mollusks based on molecular and bioassay methods in articles published up to the end of 2020.

Sampling Strategy					Methods Used		Results	Reference
Sampling Location (Country)	Sample Type (Scientific Names)	Samples Collected	Sample Units per Pool or Sample Mass (Length)	Type of Tissue or Material Analyzed	Oocyst Recovery Method [†]	Detection Methods (Molecular Target if Apply)	Positive Samples (%)	
Brazil	Oysters (*Crassostrea rhizophorae*), mussels (*Mytella guyanensis*)	80 pools	5–15 units/pool	Whole oyster or mussel	Wash, filtration, centrifugation, wash, and centrifugation	nPCR (B1) Mouse bioassay-smear + IFAT	2 (2.5) [a,b] 0	[130]
Brazil	Oysters (*Crassostrea rhizophorae*)	208 pools of each tissue	3 units/pool (no data)	Gills and digestive glands	Not performed *	PCR (529 RE) nPCR (SAG1)	0 17 (8.1) [b]	[131]
Brazil	Oysters (*Crassostrea* spp.)	120 pools	10 units/pool (no data)	Gills, gastrointestinal tract, and intervalvular liquid	Not performed *	nPCR (B1)	7 (5.8) [b]	[132]
Brazil	Oysters (*Crassostrea* spp.)	80 pools of each tissue	5 units/pool (no data)	Gills and digestive glands (visceral mass)	Not performed*	nPCR (SAG1)	2 (2.5) [b]	[54]
China	Oysters (not specified)	998	1 unit (no data)	Hemolymph, digestive glands and gills	Centrifugation	Semi nPCR (B1)	26 (2.6) [b]	[133]
China	Mussels (*Mytilus edulis*)	2215	1 unit (no data)	Gills, digestive glands and hemolymph	Not performed *	Semi nPCR (B1)	55 (2.5) [b]	[134]
China	Oysters (*Concha ostreae*)	398	1 g/sample (no data)	Digestive tract tissues	Not performed *	PCR (ITS1)	0	[135]
France	Mussels (*Dreissena polymorpha*)	96 pools	9 units/pool (18–25 mm)	Whole mussel	Enzyme digestion, centrifugation	qPCR (529 RE)	3 (3.1)	[136]
Italy	Mussels (*Mytilus galloprovincialis*)	409	25 mg (>5 cm)	Digestive gland	Not performed *	qPCR (B1)	43 (10.5) [b]	[137]
Italy	Mussel (*Mytilus galloprovincialis, Mytilus edulis*)	135 pools	10 g (no data	Intestinal tissues	Wash, filtration, centrifugation, wash, and centrifugation	End-point PCRs (B1 and 529 RE)	10 (7.4) [b]	[138]
Italy	Oysters (*Crassostrea gigas*), mussels (*Mytilus galloprovincialis*), clams	62 pools of each tissue	11–30 units/pool (no data)	Digestive glands, gills and hemolymph	For hemolymph: flotation, centrifugation, wash, and	nPCR and FLAG-qPCR (B1)	2 (3.2)	[139]

Sampling Strategy					Methods Used		Results	Reference
Sampling Location (Country)	Sample Type (Scientific Names)	Samples Collected	Sample Units per Pool or Sample Mass (Length)	Type of Tissue or Material Analyzed	Oocyst Recovery Method [†]	Detection Methods (Molecular Target if Apply)	Positive Samples (%)	
	(*Tapes philippinarum, Tapes decussatus*)				centrifugation. Not specified for digestive glands and gills			
New Zealand	Mussels (*Perna canaliculus*)	104	1 unit (no data)	Hemolymph	Centrifugation	nPCR (dhps)	13 (12.5)[b]	[23]
Tunisia	Clams (*Ruditapes decussatus*), oysters (*Pinctada radiata*), mussels (*Mytilus galloprovincialis, Perna perna*)	87 pools	9–18 units/pool (no data)	No data	Wash, filtration, centrifugation, wash, and centrifugation	qPCR (B1)	4 (4.6) [a,b]	[140]
Turkey	Mussels (*Mytilus galloprovincialis*)	53 pools	15 units/pool (5–8 cm)	Gills and digestive system	Filtration and centrifugation	qPCR (B1) + HRM	5 (9.4) [b]	[141]
Turkey	Mussels (*Mytilus galloprovincialis*)	53 pools	15 units/pool (no data)	Gills and digestive system	Flotation or filtration and centrifugation	qPCR + HRM (B1)	7 (13.2)	[102]
Italy		60 pools	500 g (no data)	Hemolymph, gills and digestive glands			0	
The United States	Oysters (*Crassostrea virginica*)	1440	50–100 mg wet weight of total tissue (no data)	Mantle, gills and rectum	Not performed *	qPCR (ITS1)	446 [a] (31.0)	[55]
The United States [‡]	Mussels (*M. californianus*), gaper clams (*Tresus nuttallii*), pismo clams (*Tivela stultorum*)	1109	50 mg of digestive tissue or 50–100 µL of pelleted hemolymph (no data)	Hemocytes and digestive gland	Not performed *	qPCR (18S rRNA)	1 (0.1) [a,b]	[21]
The United States [‡]	Mussels (*Mytilus californianus*)	959	1 unit (no data)	Hemolymph	Centrifugation	nPCR (ITS1 and B1)	13 (1.4) [b]	[22]
The United States	Mussels (*Mytilus* spp.)	41	1 unit (no data)	Hemolymph, gills and digestive glands	Filtration and centrifugation	qPCR and end-point PCR (529 RE)	19 (46.3) [a,b]	[53]
The United States	Clams (*Mya arenaria*), mussels (*Geukensia demissa, Mytilus edulis*), oysters (*Crassostrea virginica*)	159	1 unit (no data)	Digestive gland, mantle, gills, foot, and siphon	Not performed *	PCR (GRA6)	0	[142]
The United States	Mussel (*Mytilus californianus*)	Analyzed pools, but the exact number was not	30 units/pool (≥3 cm)	Hemolymph	Not performed *	PCR (ITS1, 529 bp and B1)	13 (1.5) [b,c]	[117]

Sampling Strategy					Methods Used		Results	Reference
Sampling Location (Country)	Sample Type (Scientific Names)	Samples Collected	Sample Units per Pool or Sample Mass (Length)	Type of Tissue or Material Analyzed	Oocyst Recovery Method †	Detection Methods (Molecular Target if Apply)	Positive Samples (%)	
		Specified (total of units = 959)						

† Oocyst recovery method specified step by step. ‡ Presence of cats in the sampling area reported by previous studies. * Samples were analyzed without a preceding oocyst recovery/concentration process. [a] Positive samples or detection rates not specified, calculated based on the data provided. [b] Positive samples were sequenced and/or genotyped. [c] Detection rate based on the total of individual samples collected, not based on analyzed pools. HRM: high-resolution melt curve. FLAG: fluorescent amplicon generation. IFAT: indirect immunofluorescence test. None of the articles were linked to human toxoplasmosis.

Most studies were focused on a few countries, so data cannot be extrapolated to other areas. The fact that most of the studies included in this systematic review were performed in North and South America could be linked to the frequency of oocyst-associated toxoplasmosis outbreaks, which were documented as early as 1966 in these regions [143]. Brazil is the country most represented in the studies, which is likely because it is a hotspot for outbreaks and the presence of a wide variety of strains that appear more virulent [7].

It is noticeable that very few studies addressed *T. gondii* infection using a multisectoral and transdisciplinary approach, according to the One Health concept. Indeed, only 13 of the selected articles studied the association between oocyst detection in environmental matrices with human *T. gondii* infection and toxoplasmosis (outbreaks, endemic, or sporadic cases), most of them from North and South America. Five of these studies focused on soil [56,60,70,77,80], with three of them reporting positive samples; 10 were in water [51,60,70,87–89,91,92,104,105], with eight reporting positive samples; and three were on fresh produce [60,88,92], with one reporting positive samples and the other reporting negative samples, but suggesting that the occurrence of toxoplasmosis was connected with vegetable consumption in a restaurant [60] (Tables 1–4).

3.3. Sampling Strategies

The studies selected were not comparable due to the large differences between them. When analyzing pooled detection rates by matrix type, a high degree of heterogeneity was observed ($I^2 = 98.9\%$, $p < 0.001$) due to the different sampling and methodological approaches adopted among the 64 studies included here (Table 5). Fresh produce stood out as the least heterogeneous matrix ($I^2 = 78.2\%$, $p < 0.001$). Nevertheless, this might be a consequence of the small number of studies selected (n= 8) because most of the available surveys analyzed pooled samples and were excluded. A larger number of studies (n = 28) were considered for water. However, high heterogenicity was obtained ($I^2 = 85.4\%$, $p < 0.001$) even though sampling strategies were adopted from standardized protocols for other waterborne parasites such as *Cryptosporidium* spp. and *G. duodenalis* [144]. As expected, similar results were found when analyzing heterogeneity by Cochran's chi-squared (Q = 6679.21 (d.f. = 74), $P< 0.001$). In addition, the first approach to estimate the sampling bias showed a significant influence (Egger's test = 4.41, $p < 0.001$), which provides additional statistical evidence of heterogeneous sampling strategies and methodologies [41,145] (Tables 1–4; Supplementary Figures S1–S4). Such bias was also evident in the funnel plots constructed for each of the matrices (Supplementary Figure S5). Nevertheless, we did not exclude any of the studies aiming to show a detailed overview of the investigations carried out up to date. Thus, harmonized procedures should be implemented in future studies.

Table 5. Subgroup analysis for comparison of the occurrence of *Toxoplasma gondii* oocysts detected by molecular methods in each matrix.

Matrix	No. of Studies Included	Pooled Detection Rates (95% CI)	Heterogeneity Test				Egger's Test	
			I^2 (%)	*Q* (X^2)	Q/df	Q-p (*P*)	*t*	*p*
Soil	25	17.3 (11.0–23.7)	99.3	3388.03	24	<0.001	1.08	0.292
Water	28 [a]	9.2 (6.3–12.0)	85.4	205.09	23	<0.001	2.33	0.030
Fresh produce	8 [b]	5.2 (1.7–8.8)	78.2	36.76	8	<0.001	9.09	<0.001
Bivalve mollusks	10 [c]	6.8 (4.4–9.2)	98.8	757.99	9	<0.001	2.82	0.030
Total	71 *	12.0 (10.0–14.0)	98.9	6679.21	74	<0.001	4.41	<0.001

I^2, inverse variance index; Q, Cochran's X^2; Q-P p-value of Q-tests. * Few articles analyzed samples from more than one country. [a] Excluded: [59,89] (the number of positive samples was not specified). [b] Excluded: [60,78,92,119–121] (analyzed pooled samples). [c] Excluded: [54,102,117,130–132,136,138–140,141] (analyzed pooled samples).

3.3.1. Soil

Soil samples were grouped into different categories according to their origin, which was mainly based on their proximity to urban areas and the presence of domestic and wild felids: public parks and playgrounds, schools, gardens, backyards, and houses (including vegetable gardens/orchards), livestock farms, crop fields and grasslands, and forests (Table 1, Supplementary Table S2). In general, the detection rates in soil near urban areas were between 1.1% (10/924) in playgrounds [77] and 94.1% (16/17) in vegetable gardens [72]. On livestock farms, detection rates ranged from 1.0% (7/700) [48] to 100% (5/5) [49], in crop fields and grasslands from 20.0% (2/10) [61] to 32.4% (274/845) [65], and in forests from 32.1% (26/81) [69] to 85.7% (6/7) [72] (Supplementary Table S2).

The higher detection rates reported in livestock farms, vegetable gardens, and forests may be explained by the presence of felines, since 20 out of 23 articles that documented the presence of cats near the sampling area also reported positive samples. There is evidence that *T. gondii* oocyst contamination is more common at known cat defecation sites than at other sites [68], and in farms with higher cat densities [63]. In a study in eastern France, soil contamination with oocysts decreased as the distance from core areas of cat home ranges increased [69]. In studies reporting the presence of cats, the detection rates ranged from 1.1% (10/924) [77] to 78.1% (150/192) [72], whereas they ranged from 1.0% (7/700) [48] to 100% (5/5) [49] in studies where no information was provided. For further studies that aim to determine the risk that the presence of cats poses to *T. gondii* environmental contamination, quantitative data on cat colonies would help to better interpret the results obtained.

The prevalence of oocyst-shedding cats may vary with seasonal reproductive patterns, and the likelihood of exposure to *T. gondii* may be influenced by climatic conditions [62]. There is evidence that season and extreme weather events are variables that influence *T. gondii* contamination. *T. gondii* oocysts remain viable for a long period of time in moist soil conditions and mild temperatures. For example, significant levels of rainfall may lead to humidity, precipitation, and excess runoff, and thus, exposure to *T. gondii* oocyst is increased [12,21]. In contrast, drier conditions and hot temperatures reduce the persistence (and viability) of *T. gondii* oocysts in the soil [82,146]. A handful of studies have investigated the effects of climate conditions and season on soil contamination with *T. gondii*

oocysts. Soil, temperature, and humidity were found to be associated with oocyst contamination in Harbin, China [65]. In another study from China, soil contamination was more common in a sub-tropical climate [62]. In three studies, oocyst positive soil samples were found more frequently in autumn [48,62,82]. In contrast, a gradual decrease in soil detection rates from spring to winter was reported in Wuhan, China [63], and levels of soil contamination were higher in the summer season than in the spring in Mazandaran Province, Iran [72]. Local variations in climate may explain the seasonal differences observed, and this highlights the importance of recording climatic conditions when undertaking environmental sampling.

Other sampling variables to be considered are the number of samples collected that ranged from 5 [56] to 9420 [65], the mass of soil sampled that varied from 1 g [59] to 1000 g [58] and the sampling depth that ranged between 2 and 10 cm (Table 1). However, this information was not provided in some articles, and thus, comparison between articles was not possible.

Currently, there is a lack of knowledge on the nature or extent of any effect of soil type on *T. gondii* oocyst survival. The biological, chemical, and physical parameters of soil may vary with soil type and sampling season and therefore affect oocysts' viability, recovery, and detection. An experimental study conducted with *T. gondii* oocysts and different types of artificial and natural soil matrices demonstrated that the efficiency of oocyst recovery is affected by the soil characteristics, with significantly higher efficiency from samples that had the lowest sand content [147], which was probably due to the structural damage caused by mixing before and during the flotation procedure, as documented previously for *Cryptosporidium* spp. [148]. Therefore, all these parameters should be documented to facilitate the development of risk assessment and management strategies aimed at detecting *T. gondii* oocysts, estimating the environmental contamination burden, and reducing public health risks [62].

3.3.2. Water

Regarding investigations on the occurrence of *T. gondii* oocysts in water, specific sampling variables were considered: the water origin (groundwater: wells; surface: rivers, beaches, lakes, pools; wastewater; piped water: from homes or public drinking water), uses (recreational: swimming and/or playing sports; irrigation/washing; potable water) and water treatment (boiling, chlorination, filtration) (Table 2, Supplementary Table S3).

Toxoplasma gondii detection rates ranged from 5.4% (4/74) [103] to 37.5% (30/80) [104] in groundwater water, 5.0% (5/100) [98] to 76.9% (10/13) [93] in surface water, 10.0% (1/10) [50] to 42.9% (3/7) [107] in wastewater, and 2.3% (1/44) [97] to 17.9% (5/28) in piped water. Lower *T. gondii* detection rates in drinking water and groundwater compared to surface water have been reported previously [78]. This could be due to the water treatment received or natural filtration through soil, stones, and organic matter, respectively. However, this last hypothesis will depend on the characteristics of the ground, since material of smaller diameter could retain more oocysts, as experimentally proven for *Cryptosporidium* spp. oocysts [149]. It is also possible that inhibitors that might affect molecular tests are more likely present in groundwater or wastewater, leading to an underestimation of the contamination with oocysts. Surface water may be directly in contact with definitive host feces or accumulated rainfall runoff from surfaces, leading to higher oocyst contamination.

The public health importance of different contaminated water sources is determined by their uses. In relation to this, one article reported 9.0% (9/100) recreational water samples to be oocyst positive [78], while the detection rates were between 16.7% (9/54) [95] and 50.0% (1/2) [88] in irrigation/washing water, and 2.3% (1/44) [97] and 100% (8/8) [51] in potable water. The origin of these samples was not specified in all cases; the recreational water corresponded to lakes and pools, the irrigation/washing water was from a river, and in one study, the potable water corresponded to water kept in tanks/towers from houses, in fountains, and from the water and sewage company. One study with 100% of

positive potable water samples was linked to a human toxoplasmosis outbreak in Santa Maria, Brazil, which was one of the largest studies worldwide with around 902 confirmed cases [51] and one of the few that used piglet bioassay for parasite detection; thus, oocysts infectivity was confirmed.

Previous studies have stated that untreated surface irrigation water is a relevant source of waterborne pathogens including *T. gondii* [111] and that human *T. gondii* infection seropositivity is significantly more frequent among those consuming unboiled water [104]. Herein, three out of eight articles that analyzed treated water clearly specified the treatment received, all from North and South America, with detection rates of 5.4% (4/74) in chlorinated water [103], 6.0% (2/30) in boiled water [92], and 60.0% (12/20) in water from the distribution system of a treatment plant after a coagulation, flocculation, sedimentation, filtration, and chlorination process [93]. However, oocyst viability was not assessed in these studies, and it is documented that sporulated oocysts lose their infectivity at 60 °C for 1 min [150], while treatments such as chlorination, ultraviolet (UV), and ozone are not effective [151]. One study that analyzed treated water reported 100% (8/8) of positive samples by piglet bioassay, which were linked to a human toxoplasmosis outbreak, but water treatment was not specified [51]. This finding could indicate that not all treatments used are effective or that treated water may become contaminated, which is more likely in countries with inadequate water supply systems. Thus, in addition to avoiding the contamination of stored water (tanks, cisterns, and others), the effectiveness of treatments and the post-treatment handling are both crucial factors to be considered in the prevention and control of water-related toxoplasmosis.

The number of samples collected was extremely variable: from three irrigation, municipal, and/or cistern water samples [60,88,111] to 1427 public water supply samples [109]. In addition, the sample volume ranged from 0.01 L [61] to up to 2500 L [100]. The analysis of large volumes is necessary because of the low oocyst load expected. However, water turbidity due to organic matter can also have an impact, since this can lead to membrane saturation [70] and increase the possibility of the presence of inhibitors affecting the molecular tests.

Altogether, nine articles recorded the presence of cats in the sampling area, and six of them reported positive samples [61,70,88,89,104,105] (Table 2). However, no clear association was established. One article suspected that reservoir contamination was due to a cat from the area that gave birth to kittens that lived on the top of the reservoir, but they could not be caught, and so it was not possible to confirm this hypothesis [89]. Since most of the studies did not specify whether cats were present, it was not possible to determine if reported detection rates were influenced by this variable. Moreover, the presence of cats alone is insufficient to explain the results, since infected cats can shed oocysts that could contaminate areas located far away from the sampling sites through water currents.

Detection rates by sampling season were not documented in most studies. One study in Scotland (UK) reported a higher number of positive samples in autumn compared to summer [109], and other studies in Mexico and Brazil found positive samples only during the rainy season [90,103]. In addition, a study in French Guiana, linked to a human toxoplasmosis outbreak, stated that climate changes, mainly flooding and warming, were the prelude to the event [70]. Therefore, it seems that there is a positive association between wet seasons and the presence of oocysts in water samples; however, further studies are required to confirm this. A higher probability of detecting *Cryptosporidium* spp. and *G. duodenalis* in fresh surface water during and after extreme weather events has been also documented, with mean odds ratios of 2.61 (95% CI = 1.63–4.21) and 2.87 (95% CI = 1.76–4.67), respectively [152]. Accordingly, apart from classical climatologic parameters such as temperature and humidity, extreme weather events might strongly influence oocyst presence, and this should be taken into account in risk assessments.

3.3.3. Fresh Produce

Regarding fresh produce, sampling strategies varied in terms of matrix composition, number and mass of samples, season, origin (growing location: gardens/orchards, open fields, green houses; market: local markets and fairs, supermarkets, or restaurants), production type (organic versus conventional), and product presentation (bulk, prepacked, and RTE) (Table 3, Supplementary Table S4).

Fresh produce is a heterogeneous matrix that was divided into four distinct categories in this study: leafy greens including mixed salads (n = 13), non-leafy vegetables including root crops and others (brinjal, asparagus, beet, radish, carrot, chives, chili, tomato; n = 9), herbs (basil, dill, chicory, coriander, thyme, parsley; n = 3), and fruit (apple, banana, guava, blueberry, raspberry, and strawberry; n = 3). Leafy greens included different types of lettuce, chicory, rocket (syn. Arugula), watercress, chard, spinach/baby spinach, and *Brassica* vegetables (cabbage, red cabbage, rape, pak choi). Lettuce was investigated in 12 studies, but a harmonized nomenclature was not found, since the type of lettuce was not specified in seven studies, whilst in others, the authors mentioned romaine, red and green leaf lettuce, regular lettuce, curly lettuce, butterhead lettuce, iceberg (syn. Crisp) lettuce, little gem, lollo lettuce, escarole, or simply "varieties of lettuce". In addition to this, 13 studies analyzed one type of sample, whereas three studies analyzed a mix of vegetables. The composition of fresh produce might influence the possibility of being contaminated with oocysts, since the production process and growing period differ notably between baby leaves, grown and cut leafy greens, roots, and fruit.

According to the product presentation, three studies analyzed a mixture of leafy greens, and two of them were specifically RTE products, with detection rates of 0.8% (5/648) [120] and 33.3% (2/6) [37]. Apart from these mixed salads, samples of arugula and watercress were also RTE, with 66.7% (2/3) of positive samples only in the last case [37]. The differences in detection rates may be at least partially explained by the number of samples collected and tested. It is clear that current RTE production processes do not guarantee a product free from parasites of fecal origin, as not only *T. gondii* but also *Cryptosporidium* spp., *G. duodenalis*, *Cyclospora cayetanensis*, *Blastocystis hominis*, and *Dientamoeba fragilis* have been detected in RTE products, [120]. By contrast, a recent study performed in Italy did not detect *T. gondii* in 324 locally produced RTE mixed salads [153]. However, *Echinococcus granulosus* was detected in one sample, providing evidence for the risk of being contaminated with parasites of fecal origin [153].

The number of samples analyzed varied from one study to another, from one arugula, spinach, or chard sample [37,61] to 387 baby spinach samples [52], and three pools of cabbages [92] to 100 pools of lettuces [121] for leafy greens, from five chive [61] to 18 asparagus samples [19], and one pool of carrots [37] to 109 pools of cucumbers [94] for roots and other types of fresh produce, from three coriander [37] to 16 *Chrysanthemum coronarium* samples for herbs [19], and from two pools of raspberries or blueberries [37] to 120 pools of strawberries for fruit [119]. Moreover, six [37] to 648 mixed salad samples were analyzed [120]. In general, the sample amount ranged from one to 20 units or 35 to 3600 g. Since no validation data of the detection methods used for pooled samples were reported, pooling may also have influenced the sensitivity of the detection assays.

Seasonal oocyst detection rates were reported in a few studies on fresh produce with inconclusive results. One study reported a higher detection rate in autumn compared to summer in Switzerland [121], while others reported higher detection rates in samples collected in summer [19,120] or in autumn/winter [37], but in most of these studies, there were no significant differences in detection rates. As discussed previously, extreme weather effects should be also recorded.

Altogether, four articles specified the type of production, and *T. gondii* was detected in both organic and conventional fresh produce [37,52,61,118]. One study performed statistical analysis and reported no significant differences between the two types of production [37]. Similarly, no significant differences were reported between conventional and organic samples in a recent study performed in the United States [154]. Moreover, the sampling locations were diverse, and *T. gondii* was detected in samples collected from

open fields, community fairs, storage, local markets, farmlands, school restaurants, and supermarkets [19,37,52,61,92,118]. A relevant issue that could favor or hamper oocyst contamination through cat feces may be growing the vegetables in open fields *vs*. in greenhouses. Unfortunately, although a greenhouse origin was recorded for one study [19], whether the vegetables were grown in open fields or in greenhouses was not specified in other studies.

Data on water sources, irrigation systems, and types of fertilization was limited or not provided in studies included here. Nevertheless, these variables have been identified as relevant risk factors for other foodborne pathogens and may explain some figures reported by the EFSA [155]. A study from the Czech Republic specified that vegetables were irrigated with water from rivers, lakes, or wells and washed with water from the distribution system or wells [94]. Another study from Egypt also stated that the vegetables were irrigated with water from river canals [95]. Water samples were tested in both studies, and *T. gondii* was detected in one of them [95], while oocysts were detected in fresh produce in both cases (Table 3). The presence of *T. gondii* oocysts in soil and surface water used for fresh produce production and processing (including packinghouse operations) suggests that there may be a risk of contamination of these products, as previously reported for *Cryptosporidium* spp. and *G. duodenalis* in the fresh produce industry [156]. Organic fertilizer (compost/sludge/manure) might not pose a major risk for *T. gondii* since cat feces are not usually used as fertilizer. In contrast, it is likely that access of cats to crops and weather events spreading oocysts pose a risk for the presence of *T. gondii* oocysts on fresh produce. Thus, oocyst contamination is more unlikely to occur in greenhouses *vs*. open fields, where the access of cats can be more easily restricted and fresh produce is protected from weather events. One study stated that vegetables grown close to farms are at higher risk of *T. gondii* contamination, which is probably due to the presence of felines [38]. The presence of cats was a variable recorded in four of the selected studies [38,60,61,88], but only two of them reported positive samples [38,61].

3.3.4. Bivalve Mollusks

Different genera of clams, oysters, and mussels were analyzed worldwide to determine the presence of *T. gondii*. Detection rates varied between 3.6% (1/28) [139] and 6.6% (4/61) [140] in pooled clams (*Ruditapes decussatus*) from Italy and Tunisia, respectively, 1.3% (2/160) [54] and 31.0% (446/1440) [55] in different species of pooled or individual oysters (*Crassostrea* spp.) from Brazil and the United States, 1.4% (13/959) [22] and 46.3% (19/41) [53] in individual samples of different species of mussels (*Mytilus* spp.) from the United States, and 3.1% (3/96) in pooled zebra mussels (*Dreissena polymorpha*) from France [136] and 12.5% (13/104) in individual samples of New Zealand mussels (*Perna canaliculus*) from New Zealand [23] (Table 4, Supplementary Table S5).

It has been reported that filtering activity is multifactorial and affected by bivalve genera, variations in salinity (fluctuates more in coastal marine areas), temperature, level of contamination, and kinetics of parasite diffusion, among other factors [18]. In the studies reviewed here, the same genera of bivalves were collected in different parts of the world, in different locations (coastal, bay or beach, farms and depuration plants, markets/outlets, water treatment plant discharge points), seasons, and at different depths. Moreover, there were other variations in sampling, e.g., pooled samples *vs*. individual samples, and different tissues selected for oocyst detection. This variation hampers comparisons between studies.

Regarding sampling location, detection rates varied from 1.4% (13/959) in *Mytilus californianus* samples [22] to 31.0% (446/1440) in *Crassostrea virginica* [55] samples from coastal sites in the United States, 1.3% (2/160) [54] to 16.6% (1/6) [139] in pools of *Crassostrea* spp. from farms in Brazil and Italy, respectively, 2.5% (55/2215) to 12.5% (13/104) in individual samples of *Mytilus edulis* [134] and *Perna canaliculus* [23] from markets/outlets in China and New Zealand, respectively, and 3.1% (3/96) in pools of *Dreissena polymorpha* from different discharge points of wastewater treatment plants in France [136].

The latter study was the only one that documented the depth of the sample collection, which was 20–100 cm. The results from the coastal sites may be influenced by several aspects, including seasonality, human settlements due to the presence of domestic cats, and industrial wastewater. Proximity to freshwater runoff has been associated with the presence of pathogens, including *T. gondii*, in bivalves [117].

In relation to seasonality, *T. gondii* oocyst contamination in bivalves was more frequent in spring and autumn compared to other seasons in Italy [138], in summer compared to winter in New Zealand [23], and during the wet season in the United States [22,117]. This could be explained by region-specific weather patterns in each country, so that higher contamination coincides with the rainy season. Extended dry periods may lead to a greater accumulation of oocysts on land that can be mobilized into runoff in subsequent periods of heavy rainfall.

The anatomical regions analyzed were documented in some articles that investigated the presence of *T. gondii* based on molecular assays, but few of them reported the positive samples by tissue type. Most studies analyzed the digestive glands, followed by gills and the hemolymph (Table 4). The digestive glands and hemolymph seem to be appropriate target organs according to the few spiking studies done. *Toxoplasma gondii* was most often detected in digestive glands compared with hemolymph or gills after experimental contamination under laboratory conditions carried out in mussels [157]. In a later experimental infection done in zebra mussels followed by a depuration process, the greatest concentrations of *T. gondii* DNA were observed in hemolymph and mantle tissues [158]. In field studies, there is evidence that oocysts are more frequently detected in the digestive system and/or hemolymph than in the gills [134,141]. However, other studies only found positive gills [54,139] or a higher frequency of positive gills compared to digestive samples [131]. Thus, the three tissues, or at least gills and digestive glands, should be analyzed independently or in pools to optimize parasite detection, since oocyst concentration may vary in these tissues with time post-infection [157]. Indeed, it is recommended to pool the gills and digestive tract, since this strategy optimizes parasite detection in mussels and clams based on the literature published in the presence of *T. gondii*, as well as *Cryptosporidium* spp. and *G. duodenalis*, in marine mollusks [18]. Conversely, other researchers suggest the use of hemolymph instead of all tissues due to the presumed presence of lower levels of inhibitors and less viscous material, which may improve the sensitivity of the technique [22].

Another important sampling variable is the number of samples analyzed, which varied from 41 [53] to 2215 [134] samples, and from 53 [141] to 208 [131] pools composed of three [131] to 30 [139] units per pool. On the other hand, only a handful of articles (n = 3) reported the length of the samples collected, which were longer than 5 cm in the case of *Mytilus galloprovincialis*, with similar detection rates in two studies, 10.5% (43/409) of individual samples [137] and 9.4% (5/53) of pools [141], and 18–25 mm in the case of *Dreissena polymorpha*, with 3.1% (3/96) of positive pools [136].

None of the studies determined the relationship between the presence of oocysts in bivalves and human toxoplasmosis cases caused by their consumption. There is no estimate of the number of *T. gondii* outbreaks associated with consumption of shellfish, including bivalves. According to a study performed in the United States, the consumption of raw oysters, clams, and mussels was identified as a risk for recent *T. gondii* infection (OR = 2.22, 95% CLs 1.07–4.61) [159].

Although the viability of the oocysts detected is unknown, and the only attempt to isolate *T. gondii* by mouse bioassay was not successful [130], bivalves cannot be ruled out as a potential source of infection to humans when they are consumed raw or undercooked. Moreover, it was previously suggested that the sampling strategy should focus on edible mollusk species raised under controlled conditions to better estimate the load and infectivity of filtered parasites that may pose a risk for consumers [18].

3.4. Toxoplasma gondii Oocyst Detection Methodology

A lack of harmonized methods for detecting *T. gondii* oocysts was observed in all environmental matrices (Tables 1–4), and it has been extensively discussed for fresh produce [9]. This issue is supported by the high degree of heterogeneity reported in Section 3.3, and it is also reflected in the range of analytical sensitivities reported for the detection of spiked oocysts in environmental samples in a limited number of studies. In soil samples, ranges of 10–1000 oocysts or 5–50 tachyzoites could be detected in 1 to 300 g by PCR, qPCR, and nPCR [12,66,79]. The analytical sensitivity in water was 1–1000 oocysts per L by PCR and qPCR [90,97,98]. In the case of fresh vegetable samples, the sensitivity reported was 10 oocysts per 30 g of sample [119], and the number of spiked oocysts detected in bivalve tissues was in the range of 5–1000 oocysts in hemolymph per mL or sample by nPCR [22,23] and 100 oocysts in gill tissue by real-time PCR [53].

The recovery of *T. gondii* oocysts and parasite detection are two key sequential steps. Higher variability among different matrices was observed regarding oocyst recovery compared with oocyst detection methods. In fresh produce and bivalve mollusks, a first key point for oocyst recovery was the sampling of individual *vs.* pooled samples. The analysis of pooled samples may facilitate oocyst detection [120,139], but the recovery and detection methods should be standardized in order to determine the maximum number of samples included in the pool to detect the minimum number of oocysts established by spiking assays. Accordingly, spiking experiments are highly recommended to evaluate the oocyst recovery rate as well as PCR analytical sensitivity and specificity in these complex matrices.

Second, the most extensively used recovery methods for soil and fresh produce were a combination of washing and centrifugation steps that may also include filtration or flotation. Large volumes can be a limiting factor, and filtration has been suggested for fresh produce when working with large volumes of wash buffer or samples [9]. Filtration was the preferred method for water and bivalve samples in combination with centrifugation and/or flotation, although the direct analysis of samples, without a previous oocyst recovery/concentration procedure, was also frequent in bivalves (Tables 1–4). A filtration–centrifugation method is the basis of the official USEPA method 1623 recommended by the U.S. Environmental Protection Agency (EPA) to evaluate waterborne parasites, such as *Cryptosporidium* spp. and *G. duodenalis*, in drinking water. However, this method also includes an immunomagnetic separation (IMS) step with specific commercial antibodies. A few specific polyclonal antibodies directed against *T. gondii* oocyst wall components have been generated that could be used for this purpose [160,161], but unfortunately, there is no commercially available anti-*T. gondii* oocyst antibody. Although several IMS methods have been developed [162,163], improvement of the recovery rate with IMS needs to be demonstrated for environmental matrices [18]. A recent study has achieved a proof-of-principle method for oocyst capture and separation from water using lectin magnetic separation that was later followed by qPCR, and this could be considered in future studies [164].

Recovery efficiency can be also influenced by the formation of foam that can be a challenge in handling fresh produce matrices rich in saponins [9]. It is unclear how different buffers employed for oocyst recovery could work with the different matrices and with the different mixes of vegetables analyzed. Thus, the avoidance of detergents in washing buffers (at least for fresh produce) should be considered. In order to confirm the use of appropriate buffers and efficient separation methods, spiking experiments with oocysts should be done in order to maximize the efficiency of oocyst recovery during the method standardization process [165].

Regarding detection methods, PCR was extensively employed and predominated *vs.* microscopy and bioassay methods (Tables 1–4). The sensitivity of different PCR techniques can be influenced by the different assays used to rupture the robust oocyst wall, the presence of PCR inhibitors, and the PCR protocols/procedures. Different methods to achieve efficient rupture of the oocyst wall such as bead beating, ultrasound, and freeze-and-thaw have been discussed [9], and many of the PCR-based studies reviewed here

described the use of freeze-and-thaw or bead beating prior to DNA extraction that may increase analytical sensitivity [9]. In addition, the inclusion of an internal DNA amplification control (IAC) is recommended [9], as the presence of PCR inhibitors has been reported in soil, water (e.g., organic material), fresh produce, and bivalves, and IAC is mandatory for the detection of foodborne pathogens according to ISO 22174: 2005. It was rare for studies to report the use of an IAC (soil: [12,81], water: [109], fresh produce: [19,94], bivalves: [139]). Instead, some studies used commercial kits that included an appropriate PCR inhibitor removal step [53] or bovine serum albumin (BSA) [50,63,66,69,72,97,98,109], but the inhibition problem was not always solved.

DNA amplification methods (conventional PCR, nested PCR, qPCR, LAMP) targeted either B1, SAG1, 18sRNA, ITS-1, MIC3, GRA6, and 529RE markers [9]. The most commonly employed marker was B1 (in water, fresh produce, and bivalves) followed by 529RE (in soil), and a combination of both. This finding can be easily explained by the fact that sensitivity is increased when targeting multi-copy loci (B1, 529 RE, and ITS1) compared with single-copy gene targets (e.g., SAG1 and GRA6), as shown previously [22]. These PCR methods display high sensitivity but might lack in specificity as previously evidenced [22,166]. In fact, qPCR targeting the B1 gene and 529RE without probes cross-reacted with Sarcocystidae members [167]. Thus, powerful discrimination techniques are necessary to avoid false positive results and confirm species identity [22,37,121]. In this regard, the use of TaqMan probes in qPCR guarantees high specificity [9]. Alternative methods should be also taken into consideration. Amplicon sequencing and Restriction Fragment Length Polymorphism (RFLP) analyses have been used in some of the studies to confirm positive results (Tables 1–4, Supplementary Tables S2–S5).

Toxoplasma gondii genotyping could help not only to confirm results but also to identify circulating genotypes. Genotyping tools (PCR-RFLP, microsatellite typing, PCR sequencing) were applied for this purpose in some studies, but in some instances, they were unsuccessful [19,119] or results were not reliable (e.g., based on a single marker [168]). The low oocyst burden observed in the environmental matrices (Supplementary Tables S2–S5) could limit the success of typing methods [169,170]. Currently, it is accepted that if samples are not fully characterized at the genotype level (https://toxodb.org/, accessed on 1 January 2022), the information gathered is not reliable enough for drawing robust conclusions [171,172]. Indeed, literature reviews have shown the low reliability of molecular data from environmental samples [8,172], since an unexpectedly high proportion of genotype I and non-canonical strains have been reported, which contrasts with the findings in samples from other sources such as domestic animals and humans from the same areas [172]. Therefore, additional efforts should be invested into unraveling the genotypes circulating in environmental matrices following an accurate and commonly accepted approach.

Finally, parasite quantification was not routinely carried out and was only estimated in a few studies conducted on soil, fresh produce, and bivalves. The limited data reported on parasite quantification were variable, as parasite load was referred to as the number of oocysts per g, per sample, per mL or µL, per DNA volume, or tachyzoite-equivalent copies. The quantity of *T. gondii* oocysts in soil varied from 11 to 2275 oocysts per mL [57] and 8 to 478 oocysts per 30 g of sample [81]. In fresh produce, the ranges were 1.31– 900 oocysts per g of sample [94], 62–554 oocysts per g of vegetable matter [120], 0.6–179.9 oocysts (mean of 23.5 ± 12.1 oocysts per g) [37], <10–20 oocysts per sample (mean of three oocysts per sample) [38], and 0.3–27,640 oocysts per sample [19]. On the other hand, in bivalves, it ranged from 6 to 30 oocysts per sample [141] or per 5 µL of DNA [102], 1250 to 77,500 oocysts per sample (x = 24,694, SEM = 14,254.5) [140], 0.001 to 219 copies per µL of DNA [55], and 0.14 to 1.18 copies per g [137]. Means of 40-546 tachyzoite equivalents per mL were reported [139], as were Ct means of 39.1–40.7, which were equivalent to 0.1–1.4 oocysts [136]. However, the effect of matrix, as well as the effect of the performance of the reagents used and the lack of validation of the procedures, make the quantification questionable. Despite these variable results, parasite load was occasionally very high in the

three matrices. Ideally, in this scenario, parasite viability should be estimated to define more clearly the risk that these matrices pose for humans.

Viability assays can be employed as detection methods and mouse bioassay has been suggested as a reference test for parasite detection, as mice are highly susceptible species to *T. gondii* infection [173]. In addition, bioassay methods can help to check the infectivity of the oocysts, and mouse bioassay has been widely employed for this purpose. The analysis of the literature evidenced that 15 of the studies attempted to isolate viable parasites mainly by bioassay in mice but also in pigs, chickens, and cats, and 11 obtained positive results (Tables 1–4, Supplementary Tables S2–S5). Although standardized bioassay methods are needed [173], due to ethical concerns, new alternative techniques are required to discriminate between viable and inactivated oocysts. To date, there are some new proposals to estimate oocysts viability: propidium monoazide coupled with qPCR [29,31], staining with propidium iodide [31], reverse transcription quantitative PCR (RT- qPCR) [31,174], reverse transcription PCR (RT-PCR), excystation and dyes [175], and cell culture after oocysts excystation [174]. However, further studies are necessary to standardize these processes for different matrices and guarantee their correct performance.

4. Conclusions and Considerations for Future Research

The worldwide detection rates reported for the different environmental matrices covered in this systematic review, together with the published reports of confirmed human toxoplasmosis outbreaks due to contaminated soil, water, and fresh produce, provide evidence that environmental contamination with *T. gondii* oocysts poses a risk to public health. This is supported by the oocyst load/burden detected in different studies, which should not be underestimated given the fact that a single oocyst can cause infection, and that oocysts can persist in the environment for months or years, including in the marine environment [31]. Moreover, environmental oocyst contamination is a major source of infection for animal hosts, including animal hosts raised and hunted for human consumption [176–178]. This exemplifies that *T. gondii* is a pathogen that needs to be addressed using a One Health approach.

The timeline of the studies conducted on the different matrices is noteworthy. Fresh produce has been investigated only recently, and the number of studies is still limited. The timeline appears to be in line with the increasingly understood importance of other food- and waterborne zoonotic protists, in particular *Cryptosporidium* spp. Geographical gaps were also evident; many areas of the world of the world are significantly underrepresented in the studies: for example, sub-Saharan Africa. The overall detection rates of *T. gondii* were highly variable for each matrix, which can be partially explained by the different sampling strategies and methodologies employed. Differences in *T. gondii* detection in fresh produce have been attributed to variables such as the geographical location and methods used for parasite detection [9,33], which could also apply to other environmental matrices. Thus, it is important to consider both the sampling strategy and the methodology, as they can potentially influence parasite detection success and hamper comparisons between different studies. Regarding the sampling strategy, sampling areas, sample type, number, and mass or volume must be based on previous studies and available data such as reported toxoplasmosis cases in humans and animals, reported detection rates in environmental samples, expected detection rates, variability, and others. Regarding methodologies employed for environmental matrices, the most crucial steps to be considered are the spiking assays and the inclusion of an IAC to validate the recovery and detection methods. This would enable an estimation of analytical sensitivity and specificity and avoid false negatives results so that correct interpretation of the results would be guaranteed. Well-documented and standardized bioassays and genotyping methods will also help to determine the risk of exposure and how *T. gondii* circulates in the environment. Unfortunately, consensus guidelines have not yet been proposed by the scientific community. In the meantime, it would be advisable to include as much information as possible in publications, including details of experimental design and methodology.

More studies on *T. gondii* in environmental matrices are needed, and the focus should be on the gaps identified in this review. The impact of water contamination can be high, since its consumption is not limited by eating habits, as happens for vegetarians with meat-borne toxoplasmosis cases. In addition, water can contaminate soil, seafood, or fresh produce. Significant detection rates were found in surface water, in samples collected after a long treatment process, in irrigation/washing, and potable water. Moreover, the survival of oocysts in soil and the widespread consumption of minimally processed fresh produce and bivalves support the recommendation that *T. gondii*, as well as other cyst/oocyst forming protist parasites, should be included in regular food and water quality control guidelines within the food sector. Meanwhile, basic measures should be adopted by consumers such as washing of hands after handling soil or cat feces, washing fresh produce with clean water regardless of product presentation, and proper cooking of bivalves.

Altogether, the relative contribution of different environmental matrices as *T. gondii* sources of infection to humans and animals remains unknown. Baseline data for risk assessment are limited and challenging to compare, since results may be influenced by sampling and methodological variables. Moreover, risk factors have not been adequately addressed in the context of the whole food chain including agricultural production and processing (incorporating soil, water, fresh produce including RTE products, and bivalves) given the limited and heterogeneous literature published. As an example, future work should investigate oocyst detection at the different steps of the RTE production workflow to implement mitigation strategies that might also help to avoid contamination with a wide variety of protozoa, helminths, fungi, and insects [179] and reduce infection risk for humans. Surveillance studies should ideally be accompanied by viability and genotyping assays to accurately determine the potential risk for consumers and enable tracing the sources. In general, all gaps identified evidenced the need to implement standardized procedures that could help to establish an ISO method and harmonize future studies focusing on environmental matrices. In Figure 3, we summarize the key aspects that should be considered when designing and implementing a study investigating *T. gondii* contamination of environmental matrices. As a minimum, these aspects should be explicitly addressed when reporting on the outcomes of such a study. Additional data to be considered could be extracted from systematic reviews and meta-analyses of risk factors for human infection with *T. gondii* (e.g., [180–182]). Similarly, the present review could also help further meta-analyses of risk factors in humans to identify relevant data. We appreciate the challenge of designing an adequately powered study, taking into account the multiple factors we have highlighted that can influence oocyst detection in environmental matrices. However, through the implementation of well-designed studies in the future, it will be possible to assess the contribution of different environmental matrices as sources of *T. gondii* infection to humans and animals and provide appropriate advice to policy makers, food producers, and consumers.

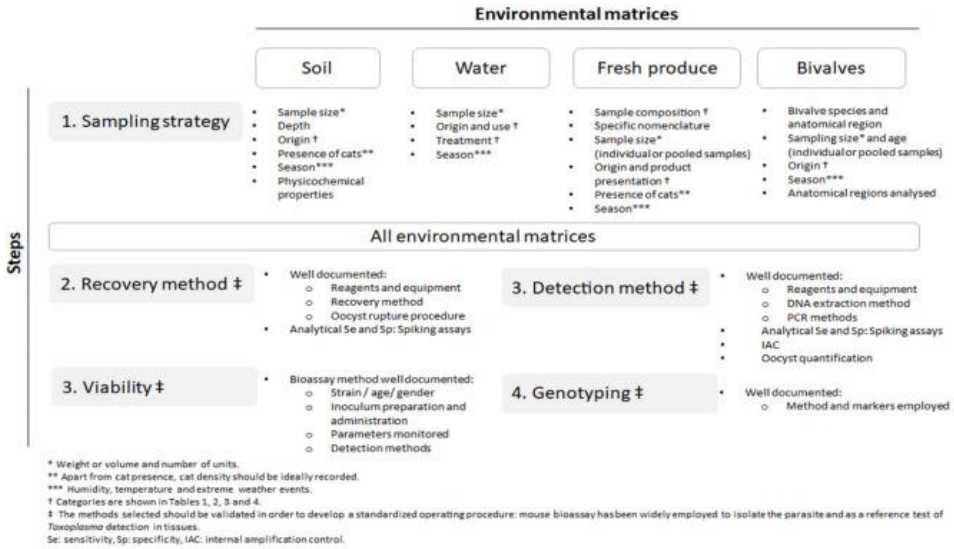

Figure 3. Workflow and key considerations for standard sampling strategies and detection methods for *Toxoplasma gondii* in environmental matrices.

Supplementary Materials: The following are available online at www.mdpi.com/article/10.3390/microorganisms10030517/s1, Supplementary Figures S1–S4 (Forest plots for each matrix), Supplementary Figure S5 (Funnel plots for each matrix) and Supplementary Tables S1–S5: Excel file with the list of papers selected from each database and combination of search terms (Supplementary Table S1) and detailed data extracted from articles on soil (Supplementary Table S2), water (Supplementary Table S3), fresh produce (Supplementary Table S4), and bivalve mollusks (Supplementary Table S5).

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Objective 2: To develop and validate a serological method that discriminate between *T. gondii* oocyst- *versus* tissue cyst-driven infections.

- Sub-objective 2.1: To develop and harmonize conventional serological tests frequently used in the diagnosis of *T. gondii* infections in three species whose meat consumption supposes a burden of infection for humans: pigs, sheep and goats, and to properly characterize the serum panels for sub-objective 2.2.

The outcomes from this sub-objective related to the tests used in **pigs** were published in the following open access article **(paper No. 2)**: López-Ureña, N.M., Calero-Bernal, R., González-Fernández, N., Blaga, R., Koudela, B., Ortega-Mora, L.M., Álvarez-García, G., 2023. **Optimization of the most widely used serological tests for a harmonized diagnosis of *Toxoplasma gondii* infection in domestic pigs**. Veterinary Parasitology 322, 110024. https://doi.org/10.1016/j.vetpar.2023.110024. This journal had an impact factor of 2.6 and was classified as Q1 (veterinary Sciences) and Q2 (Parasitology) in 2022.

Abstract: The intake of *T. gondii* tissue cysts through raw or undercooked pork meat is identified as one of the main infection sources for humans. Although serology is purposed in this species with public health commitment, there is a lack of comparative studies that offer updated diagnostic performance. Thus, herein three in-house serological tests (TgSALUVET WB, ELISA 2.0 and IFAT) were developed and preliminary validated with sera from experimentally infected pigs (n= 202) for later being included a comparative study together with three commercial ELISA tests (IDScreen, PrioCHECK and Pigtype), using an additional panel of sera from natural infections (n= 244). All tests showed good to excellent diagnostic performance and agreement with all serum panels, except for PrioCHECK that showed low specificity and agreement in all cases, and TgSALUVET WB, which presented lower sensitivity with sera from natural infections. However, the ELISA tests cutoff readjustment allowed an improvement on performance and data harmonization.

These findings were also presented as oral communication in a research dissemination day for doctoral students at the Veterinary Faculty (UCM), VI VETINDOC, in Spain (October 2020), and as an online poster communication in the 13th European Multicolloquium of Parasitology (October 2021).

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Optimization of the most widely used serological tests for a harmonized diagnosis of *Toxoplasma gondii* infection in domestic pigs

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ABSTRACT

The intake of *Toxoplasma gondii* tissue cysts through raw or undercooked pork meat is one of the main infection sources for humans. Thus, surveillance is recommended to control and prevent infection in domestic pigs. However, the lack of comparative studies hampers the updating of their performance and the comparison of seroprevalence data. Therefore, the aim of this study was to develop and validate three in-house tests and accomplish a comparative analysis of the most widely used serological tests employed in pigs. A panel of sera from pigs experimentally infected with either oocysts or tissue cysts from type II and III isolates (n = 158) was used to develop and validate a tachyzoite-based Western blot assay. Then, this technique was used as a reference to develop and preliminary validate a lyophilized tachyzoite-based enzyme-linked immunosorbent assay and an immunofluorescence antibody test. Next, a comparative study of the three in-house tests and three widely used commercial ELISAs (IDScreen®, PrioCHECK™ and Pigtype®) was accomplished with the abovementioned sera together with an additional serum panel of pigs experimentally infected with oocysts from the type II isolate (n = 44) and a panel of naturally infected pigs (n = 244). The results obtained by the majority of the tests were regarded as reference, and data analyses included TG-ROC calculations and agreement tests. Finally, the kinetics of anti-*T. gondii* IgGs from experimentally infected pigs was analyzed. Excellent sensitivity (Se) and specificity (Sp) values (≥ 93%) and moderate to near perfect agreement (k = 0.63–0.91) were observed using sera from experimental infections without requiring further readjustment, except for PrioCHECK (100% Se, 73% Sp). However, the Se of IDScreen® (87%) and TgSALUVET WB (71%) and the Sp of PrioCHECK (72%) were slightly or notably reduced when sera from naturally infected animals were analyzed, which also influenced the *kappa* values (k = 0.30–0.91). Cutoff readjustments increased the Se and Sp values to equal to or above 97% for all tests, except for TgSALUVET WB, which can be used as a reference for initial validation of tests, but it is not recommended for routine diagnosis. Seroconversion was recorded from two weeks post-infection by most of the tests, with significantly higher IgG levels in sera from pigs infected with the *T. gondii* type III *vs.* type II isolate. Again, differences regarding the test employed were observed. Differences in the diagnostic performance among tests evidenced the need to harmonize serological techniques to obtain comparable and reliable results.

1. Introduction

Toxoplasma gondii, the etiological agent of toxoplasmosis, is a worldwide distributed apicomplexan parasite that can infect any warm-blooded animal, including humans (Dubey, 2022). It has globally ranked as the third most important foodborne parasite (WHO and FAO, 2014). It is estimated that, at present, one-third of the global human population is chronically infected, with 90% of asymptomatic infections in immunocompetent individuals (McCall et al., 2022). However, clinical infections may include reproductive failures in pregnant women, especially during primary infections, such as abortion and ocular disease, as well as encephalitis, pneumonia, and death in immunocompromised patients

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(Koutsoumanis et al., 2018; Rostami et al., 2020; WHO and FAO, 2014). Thus, *T. gondii* is an opportunistic pathogen of concern for public health that should be approached through a One Health perspective.

There are three major routes of transmission for humans: the meat route through the ingestion of undercooked or raw meat containing tissue cysts; the environmental route through the consumption of sporulated oocysts that contaminate soil, water, vegetables, fruits and bivalves; and the maternal-fetal route by tachyzoites that pass from the mother to the fetus through the placenta (Almería and Dubey, 2021; Attias et al., 2020; Dubey, 2009; López-Ureña et al., 2022). Up to 47.1% (16/34) of reported toxoplasmosis outbreaks from 1960 to March 2018 were associated with the consumption of contaminated meat and derivates (Pinto-Ferreira et al., 2019). In particular, pigs have been recognized as the principal food source of *T. gondii*, responsible for 41% and 50% of the cases in studies conducted in the United States and the Netherlands, respectively (Koutsoumanis et al., 2018).

In pigs, serology has been recommended as an epidemiological indicator for *T. gondii* infection with food safety purposes (EFSA, 2011). Indeed, a literature review indicated that *T. gondii* tissue cysts could be detected by bioassay or PCR methods in the meat of 58.8% (348/529) of *T. gondii*-seropositive pigs (Opsteegh et al., 2017). A wide variety of serological techniques have been used for the detection of anti-*T. gondii* antibodies in pigs, including enzyme-linked immunosorbent assay (ELISA), immunofluorescence antibody test (IFAT), Western blot (WB), indirect hemagglutination assay (IHA), modified agglutination test (MAT), Sabin-Fieldman dye test (DT), latex agglutination test (LAT) (Dubey et al., 2020) and protein microarray-based assay (Loreck et al., 2020). ELISA methods are the most widely used tests (Huertas-López et al., 2023; Liyanage et al., 2021), especially commercial ready-to-use kits, followed by MAT (Dubey et al., 2020) and IFAT (Huertas-López et al., 2023). In contrast, WB has been poorly used (Al-Adhami and Gajadhar, 2014; Basso et al., 2013; García et al., 2008; Huertas-López et al., 2023) and has been recognized as a highly specific test. However, to date, there is no commonly adopted IFAT cutoff or WB positive pattern criterion. Moreover, the usefulness of WB for daily diagnosis purposes is unclear. In addition, despite the wide variety of serological tests used worldwide, the lack of comparative studies hampers the updating of their performance and the comparison of seroprevalence data. Only a restricted number of serological tests have been compared, and the main limitations addressed are as follows: i) commercial tests have been scarcely evaluated; ii) in the absence of a gold standard test, either in-house or commercial tests have been employed as a reference technique with unknown diagnostic performance data (Al-Adhami and Gajadhar, 2014; Garcia et al., 2006; Hill et al., 2006; Steinparzer et al., 2015); iii) sera from experimentally and naturally infected pigs were only used by Hill et al. (2006) *vs.* most studies that employed either sera from experimentally infected animals (Al-Adhami and Gajadhar, 2014; Garcia et al., 2006) or sera from natural infections (Basso et al., 2013; Cubas-Atienzar et al., 2019; Damriyasa et al., 2004; Kunic et al., 2022; Limon et al., 2017; Pablos-Tanarro et al., 2018; Papatsiros et al., 2016; Pardini et al., 2012; Steinparzer et al., 2015); and iv) the influence of *T. gondii* doses and isolates/strains remains unknown. In this context, the European Food Safety Authority (EFSA) pointed out the need for ring trials of the most widely used serological techniques to unify criteria and obtain comparable results since some of the tests and antigens currently accessible are not validated or standardized, and no standard sera samples are available (EFSA, 2011).

Accordingly, herein, a comparative study of the most widely used serological techniques for the diagnosis of *T. gondii* infections in domestic pigs following World Organization for Animal Health (WOAH) guidance (WOAH, 2023; 2021; 2018) was performed. First, the criterion of seropositivity was defined for a tachyzoite-based Western blot assay (TgSALUVET WB), which was later used as a reference test to develop and preliminary validate a lyophilized tachyzoite-based ELISA (TgSALUVET ELISA 2.0) and an immunofluorescence antibody test (TgSALUVET IFAT). Next, three commercial ELISA kits (IDScreen®, PrioCHECK™ and Pigtype®) and three in-house serological tests (TgSALUVET WB, TgSALUVET ELISA 2.0 and TgSALUVET IFAT) were compared and validated by analyzing a wide panel of sera from experimentally and naturally infected pigs. Finally, the influence of parasite stage and isolate on the kinetics of anti-*T. gondii* IgGs was studied for each test with sera from experimental infections.

2. Materials and methods

2.1. Panels of sera

Different serum panels obtained from pigs either experimentally or naturally infected with *T. gondii* were used in the different steps of this study (Fig. 1). Serum samples were obtained from the jugular vein and kept at − 80 °C until use.

2.1.1. Sera from pigs experimentally infected with Toxoplasma gondii

Two panels of sera from pigs experimentally infected with *T. gondii* were used. These experiments were performed considering the European regulations, as well as the animal welfare and the good practices guidance for animals used for experimental research and other scientific practices, European Directive 2010/63/EU. The procedures were approved by the Ministry of Education, Youth and Sports from the Czech Republic (PP 55/2016) (Dámek, 2023a) and the Agency for Food, Environmental and Occupational Health & Safety (ANSES) from France (APAFIS N◦ 14,363–2018032908554996v3) (Dámek et al., 2023a, 2023b).

Panel 1 included sera from prepubertal Dan Hybrid-LY sows orally infected with 400 oocysts or 10 tissue cysts from either type II (CZ-Tiger isolate, ToxoDB#3, n = 6 and 7, respectively) or type III (CZ-Šimková isolate, ToxoDB#2; n = 6/group) isolates. Samplings were carried out prior to the infection and weekly from one to six weeks post-infection (wpi), with a few exceptions in which the samples were collected only in some weeks (n = 158) (Dámek, 2023a). These sera were used in step 1 to define the pattern of *T. gondii* tachyzoite antigen recognition by TgSALUVET WB, as well as to validate the TgSALUVET ELISA 2.0 and TgSALUVET IFAT, and in step 2 related to the comparative study (Fig. 1).

Panel 2 was composed of sera obtained from three 97-day-old Large White female pigs orally infected with 1000 oocysts of the clonal type II reference strain ME49 (ToxoDB #1). Sampling was carried out at 0, 2, 5, 9, 12, 14, 19, 26, 33, 40, 47, 54, and 58 days post-infection (dpi). One noninfected pig was used as a negative control, and blood samples were collected at 0, 26, 40, 47 and 54 dpi (n = 44) (Dámek et al., 2023a, 2023b). This panel of sera was used in step 1 for the initial validation of TgSALUVET ELISA 2.0 and TgSALUVET IFAT and in step 2 in the comparative study (Fig. 1).

2.1.2. Sera from pigs naturally exposed to Toxoplasma gondii

Panel 3 consisted of sera collected from slaughtered Black Iberian pigs (50% cross with Duroc-Jersey breed) during 2019–2020. These pigs had been reared in different systems ranging from extensive to semi-extensive regimes in farms allocated within the Dehesa ecosystem, composed mostly of acorn Mediterranean forest in the Extremadura region (southwestern Spain) (n = 244). Ten positive and 10 negative samples from this panel, based on TgSALUVET WB results, were used to test the precision of TgSALUVET ELISA 2.0 (inter- and intraplate variability) (step 1). The whole panel was included in the comparative study (step 2) (Fig. 1).

2.2. Parasites and antigen preparation

Parasite culture and antigen preparation were performed as previously described by García-Lunar et al. (2017) with slight modifications. Briefly, *T. gondii* tachyzoites from the type II ME49 strain were cultured in a monolayer of the VERO-81 cell line using DMEM (Dulbecco's

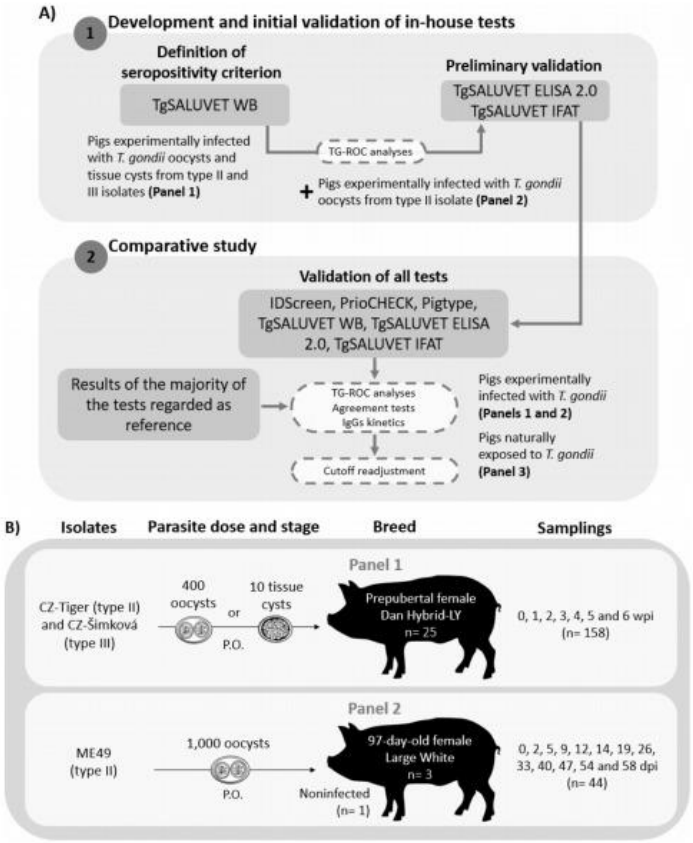

Fig. 1. A) Workflow followed in this study, including the serum panels used in each step. B) Experimental design of *Toxoplasma gondii* infections in pigs. P.O.: *Per os*. Wpi: weeks post-infection. Dpi: days post-infection. A checklist of the items included in the standards for reporting diagnostic accuracy studies (Kostoulas et al., 2017) has been provided as Supplementary Table 1.

Modified Eagle's Medium – high glucose, Sigma®, Ref. 6429) supplemented with 10% fetal bovine sera (FBS) and 1% antibiotics (Lonza, Ref. H317–745E). Fetal bovine serum was seronegative for *T. gondii* and other closely related parasites (*Besnoitia besnoiti* and *Neospora caninum*) to avoid cross-reactivity (García-Lunar et al., 2015). At 72 h post-infection (hpi), the culture was scraped off, and the parasites were purified using 3-µm Millipore filters (Millipore, Ref. TSTP02500) and counted in a Neubauer chamber. Parasites were pelleted by centrifugation at 1350 x *g* for 15 min (min) at 4 °C. For TgSALUVET WB, pellets of 1×10^8 tachyzoites were kept at − 80 °C until use. For TgSALUVET ELISA 2.0, glass vials, each with 1×10^8 tachyzoites in 4 mL of PBS, were stored at − 80 °C until being lyophilized (García-Lunar et al., 2017). For the TgSALUVET IFAT, pellets of 1×10^7 tachyzoites were resuspended in 1 mL of cold PBS, 5 µL of 37–38% formaldehyde (PanReac AppliChem, Ref. 131328.1211) was added, and the vials were stored at 4 °C until use.

2.3. Serological techniques

Three commercial ELISA kits (IDScreen, PrioCHECK and Pigtype) and three in-house tests (TgSALUVET WB, TgSALUVET ELISA 2.0, and TgSALUVET IFAT) were included in this comparative study (Fig. 1A, Table 1). Commercial tests were used as specified by the manufacturers' instructions.

2.3.1. Western blot (TgSALUVET WB)

Aliquots of 2×10^7 tachyzoites were resuspended in loading buffer (final concentration: 10% glycerol, 50 mM TRIS at pH 6.8, 2% SDS, 0.05% bromophenol blue and 100 mM DTT). The samples were subjected to a cold wet ultrasonic bath for 15 min, followed by a wet bath at 100 °C for 5 min. Then, the samples were loaded in a one-well comb in a 15% polyacrylamide gel for electrophoresis at 150 V (constant) for approximately 1.5 h in a Mini Protean® Tetra System (Bio-Rad Laboratories). Prestained protein standards (Precision Plus Protein™ Kaleidoscope™, Bio-Rad Laboratories, Ref. 1310375) were added to one side of each gel to estimate the molecular weight of the antigens. After that,

Table 1
Serological techniques used for the detection of anti-*Toxoplasma gondii* IgGs in domestic pigs included in this comparative study.

Technique	Antigen	Target species	Sample type	Sample dilution: type	Cutoff	Diagnostic performance*
ID Screen® Toxoplasmosis Indirect Multi species (IDScreen)	P30	Ruminants, swine, dogs, cats	Serum, plasma and meat juice	1/10: serum and plasma 1/2: meat juice	S/P% ≤ 40, ≥ 50	Se= 100%, Se= 100%[a]
PrioCHECK® Porcine Toxoplasma Ab Kit (PrioCHECK)	Tachyzoite**	Pigs	Serum, plasma and meat juice	1/50: serum and plasma 1/10: meat juice	PP ≥ 20	Not specified[b]
Pigtype® Toxoplasma Ab Indical Bioscience/ Qiagen (Pigtype)	Tachyzoite**	Cattle, sheep, goats, cats, dogs, foxes, pigs and wild boars	Serum, plasma (meat juice only from pigs and wild boars)	1/100: serum and plasma 1/10: meat juice	S/P ≥ 0.3 ***	Not specified[c]
TgSALUVET WB (reduced conditions)	Tachyzoites	Pigs	Serum	1/20	To be determined in this study	To be determined in this study
TgSALUVET ELISA 2.0	Lyophilized tachyzoites	Pigs	Serum	1/100	To be determined in this study	To be determined in this study
TgSALUVET IFAT	Tachyzoites	Pigs	Serum	1/100 and 1/200	To be determined in this study	To be determined in this study

Se: sensitivity, Sp: specificity. S/P%, PP and RIPC= ([sample OD- negative control OD]/[positive control OD- negative control OD]) x 100, S/P = ([sample OD- negative control OD]/[positive control OD- negative control OD]). *According to the information provided in the user's manual within the kit. **No more details were provided. *** Short protocol suggested in the manual was followed. [a]Based on 33 positive samples from France and 300 negative samples tested (animal species not specified). Intra-plate repeatability= 3–4%, inter-plate reproducibility= 4–6%. [b]No data available in the kit manual, only available in an online flyer from ThermoFisher (https://www.thermofisher.com/order/catalog/product/es/es/7610230); sera: Se= 98% and Sp= 100% (based on 50 positive and 270 negative porcine samples), meat juice: Se= 97%, Sp= 100% (based on 33 positive and 116 negative samples), using as reference IFAT, WB ad ELISA. [c]Microtiter plate coefficient of variation= 6.8%.

the antigens were transferred to a 0.2-μm nitrocellulose membrane (Bio-Rad, Ref. 1620112) in a Mini Protean II™ (Bio-Rad Laboratories) at 400 mA (constant) for 1 h. The membranes were stained with 0.1% Red Ponceau (Sigma Ref. P3504) to visualize the electrotransferred protein bands. Next, the membranes were washed with 0.05% TBS-Tween 20 (TBS-T) and blocked with 5% powdered skim milk TBS-T for 2 h at room temperature. Then, they were washed three times for 5 min each with TBS-T, carefully dried with filter paper, and stored in dry filter paper and plastic bags (Ziploc) at − 20 °C until further use.

For immunoblots, the membranes were soaked in TBS-T and cut into strips of approximately 1–2 mm. The strips were placed on stands with individual rails. Serum samples were diluted at 1/20 in blocking solution, added to separate rails with a strip and incubated in a rocker for 1.5 h at room temperature. After that, three washes with TBS-T for 5 min each were performed. Then, protein G conjugated with peroxidase (Sigma Ref. P8170) was added at a dilution of 1/600 in TBS-T and incubated under the same conditions and protected from light. Finally, two washes of 5 min each were performed with TBS-T followed by an additional wash with TBS. The reaction was revealed using 4-chloro-1-naphthol solution (Thermo Scientific, Ref. 34010) and stopped with Milli-Q water using the colorimetric reaction of the positive and negative controls as a reference, which were initially selected from pigs naturally exposed to *T. gondii* (Panel 3) based on this technique; these results were later confirmed by all included serological tests in the comparative study. The strips were placed in a template and scanned with the GS-800 Calibrated Densitometer (Bio-Rad) for analysis.

2.3.2. Lyophilized tachyzoite-based ELISA (TgSALUVET ELISA 2.0)

A lyophilized *T. gondii* tachyzoite-based ELISA was developed and validated for diagnostic purposes and previously used for analyzing the kinetics of anti-*T. gondii* IgGs in experimentally infected piglets (Largo-de la Torre et al., 2022). The detailed protocol is as follows. Initially, 1×10^5 *T. gondii* tachyzoites diluted in 100 μL of 0.1 M carbonate-bicarbonate, pH 9.6, were added per well in 96-well plates (Thermo Scientific MaxiSorp, Ref. 442404) and kept at 4 °C overnight. After three washes with 0.05% PBS-Tween 20 (PBS-T), the plates were blocked with 300 μL per well of 5% powdered skim milk PBS-T and incubated at room temperature for 2 h. Then, they were washed, and 100 μL of diluted sera at 1/100 in blocking solution was added per well. After 1 h of incubation at 37 °C and three washes, 100 μL of protein G with peroxidase (Sigma—Aldrich, Ref. P8170), diluted at 1/6000 in PBS-T, was added per well and incubated for 1 h under the same conditions. Next, the plates were washed, and 100 μL of TMB Ultra was added per well (Thermo Fisher, Ref. 34028). The reaction was stopped by adding 100 μL/well of 2 N sulfuric acid after 10 min. The final optical density (OD) was read at 450 nm using a microplate reader (Multiscan RC 6.0, Labsystems). The results were interpreted as the relative index percent (RIPC): ([sample OD- negative control OD]/[positive control OD- negative control OD]) x 100. The same sera samples used as positive and negative controls in TgSALUVET WB were used for the TgSALUVET ELISA 2.0 test.

2.3.3. Immunofluorescence antibody test (TgSALUVET IFAT)

A total of 8×10^4 formalin-fixed tachyzoites were added per 4-mm well (Thermo Scientific Diagnostic Microscope Slides, Ref. ER-311B-CE24), dried at room temperature and then postfixed with acetone (PanReac AppliChem, Ref. 211007.1211) at − 20 °C for 10 min. Next, the slides were washed with Milli-Q water for 10 min under movement (100 rpm) and dried at room temperature. Positive and negative controls were diluted at 1/100, samples were diluted at 1/100 and 1/200 in PBS, and 8 μL of each was placed in the wells. The slides were incubated in wet, dark chambers for 30 min at 37 °C. After that, a quick wash with PBS was performed, followed by two additional washes of 10 min each in a rocker. Rabbit anti-pig IgG (whole molecule) FITC antibody (Sigma, Ref. F1638) was diluted at 1/64 in 0.2% Evans Blue Dye PBS, and 8 μL was added per well. The samples were incubated and washed under the same conditions, with an additional final wash of 10 min with Milli-Q water. The slides were dried at room temperature in the dark, and the cover slides were fixed with Fluoroshield™ (Sigma, Ref. F6182). The same serum controls used in the TgSALUVET WB were included in each slide for TgSALUVET IFAT. Samples were classified as positive if more than 50% of the tachyzoites showed complete peripheral intense fluorescence, and the results were based on the consensus of two experienced operators, with the samples whose results were discrepant being classified as doubtful.

2.4. Data analysis

The molecular weight of the detected proteins by TgSALUVET WB was estimated using Quantity One 4.5.1 software (Bio-Rad). Immunodominant antigens or fractions (IDAs) were identified by their frequency and intensity of recognition, and this was achieved by two operators to avoid bias. A chi-square test was performed to identify differences between experimental groups in the frequency of recognition of each identified antigen. Sera collected at 6 wpi (Panel 1) were used for this purpose. The intensity was scaled as weak (+), medium (++) or high (+++). A two-way analysis of variance (ANOVA) with repeated measures, followed by a *post hoc* Tukey or Dunnett test if applicable (GaphPad Prism), was performed to confirm significant differences in the total antigens and IDAs recognized after the infection with respect to prior to the infection, as well as between experimental groups (Panel 1). Differences were considered significant when the *P* values were lower than 0.05. When the criterion of positivity was defined, if a mismatch result was observed between both operators, the sample was regarded as doubtful by TgSALUVET WB.

TgSALUVET ELISA 2.0 precision was determined by analyzing each sample in triplicate in three different ELISA plates. The intraplate coefficient of variation (CV) was determined as follows: mean ([standard deviation of the three replicate ODs/mean of the three replicate ODs] x 100). The interplate CV was determined as follows: mean ([standard deviation of the OD mean of each sample from each plate/mean of the OD mean of each sample from each plate] x 100). Intra- and interplate CVs below 20% were assumed to have acceptable repeatability (Jacobson, 1998). A preliminary TgSALUVET ELISA 2.0 cutoff selection was performed with a nonparametric two-graph receiver operating characteristic (TG-ROC) analysis with SigmaPlot 12.0 software. Serum panels from experimental infections and TgSALUVET WB as a reference test in the absence of a gold standard method were used to obtain a proof-of-concept Se and Sp results.

The TgSALUVET IFAT cutoff was defined by performing an agreement test between the IFAT results with respect to the results obtained with TgSALUVET WB with sera from experimental infections. Cohen's Kappa coefficients were analyzed by the WinEpi platform (Thrusfield et al., 2001) (http://www.winepi.net/) using a confidence level of 95%. The results were expressed as *kappa* (*k*) values, and the serum dilution that presented the highest agreement was selected for the comparative study. Then, the initial diagnostic performance of TgSALUVET IFAT was defined using TgSALUVET WB as reference test in the WinEpi platform, with a confidence level of 95%.

For the comparative study, TG-ROC analyses were performed using SigmaPlot 12.0 Software, with a confidence level of 95%, independently for samples from experimentally (Panels 1 and 2) and naturally (Panel 3) infected pigs. For this, each sample was classified as positive, negative, or doubtful based on the result of most of the tests (at least by four out of six techniques). Samples with positive results by three techniques and negative results by the other three techniques were considered doubtful and were excluded from the analysis. For qualitative assays such as TgSALUVET WB and TgSALUVET IFAT, the diagnostic performance was determined using WinEpi as described before but using the results from the majority of the tests as a reference test. The agreement among tests was assessed before and after the TG-ROC analysis by analyzing Cohen's Kappa coefficients as specified above.

Anti-*T. gondii* IgG levels for each experimental group and each week/day of sampling were compared (Panels 1 and 2). For Panel 1, a mixed-effects analysis with repeated measures and multiple comparisons was performed to confirm differences within and between experimental groups based on parasite stage and isolate, followed by a Tukey test if applicable. For the results obtained with Panel 2, a one-way ANOVA was used to compare the differences in infected pigs among sampling days, followed by a Dunnett test. These tests were performed using GraphPad Prism, version 8.0.1, not assuming sphericity and applying a Geisser-Greenhouse correction if recommended. Differences were considered significant when the *P* values were lower than 0.05.

3. Results

3.1. Pattern of antigen recognition and establishment of the seropositive criterion for TgSALUVET WB

A serum sample from an experimentally infected pig (Panel 1) at 6 wpi with the highest number of antigens recognized was regarded as a reference to determine the mean molecular weight (MW) value of the detected antigens (Fig. 2A). Up to 17 antigens were recognized by seropositive animals with molecular weights that ranged between 9 and 82 kDa (Fig. 2A). The frequency and intensity of antigen recognition are shown in Table 2. Eight IDAs were identified (9–10, 19, 25, 28, 30, 33–35, 43–45, and 69 kDa). These IDAs were detected by all infected pigs from at least one of the experimental groups at 6 wpi with a medium or high intensity (Fig. 2A). Nevertheless, 64% of the pig sera recognized the 19 kDa antigen with medium intensity prior to the infection, as well as the 25, 28, 30, 33–35, 39, and 43–45 kDa antigens, but with lower frequency and/or intensity rates. Only two IDAs, the 9–10 and 69 kDa antigens, were not recognized prior to the infection, and the 9–10 kDa antigen was recognized with a higher frequency and intensity (Table 2). A given sample was classified as positive if at least three IDAs were recognized with medium or high intensity since the recognition of three IDAs with medium or high intensity was observed only in seropositive samples (Fig. 2B, Table 2). Furthermore, the presence of these IDAs was not dependent on the experimental group (stage and isolate) ($P \geq 0.05$), except for the 28 kDa antigen, which was recognized by up to 100% of the pigs infected with the type III isolate *vs.* 17–50% of the pigs infected with the type II isolate (Table 2).

Moreover, statistically significant differences in the total number of recognized antigens and IDAs were recorded from 2 wpi onward compared with samples prior to the infection (Fig. 3). After 2 wpi, the number of recognized antigens increased weekly, with significant differences in all cases at any sampling week *vs.* 6 wpi. When considering *T. gondii* stages and isolates, significant differences between experimental groups were detected from 3 or 4 wpi in the number of IDAs and total recognized antigens, respectively. In detail, a higher number of recognized antigens was observed in pigs infected with the type III isolate than in those infected with the type II isolate as follows: any stage of type III isolate *vs.* oocysts type II isolate at 4–5 wpi, tissue cysts type III isolate *vs.* oocysts type II isolate at 6 wpi, and tissue cysts type III isolate *vs.* tissue cysts type II isolate at 5–6 wpi ($P < 0.05$) (Fig. 3A). Similarly, a higher number of recognized IDAs was recorded in pigs infected with the type III isolate as follows: tissue cysts from the type III isolate *vs.* oocysts from the type II isolate at 3 wpi, any stage from the type III isolate *vs.* oocysts from the type II isolate at 4–5 wpi, and tissue cysts from the type III isolate *vs.* tissue cysts from the type II isolate at 3 and 5 wpi ($P < 0.05$) (Fig. 3B).

Finally, based on the established criterion of positivity, seropositive pigs were recorded from 2 wpi onward in Panels 1 and 2.

3.2. Optimization and diagnostic performance of TgSALUVET ELISA 2.0

The coefficients of variation for the intra- and interplate repeatability were 3.6% and 4.8%, respectively. Six out of 202 samples belonging to serum Panels 1 and 2 were excluded from the TG-ROC analysis due to doubtful results obtained with the TgSALUVET WB reference test (No. of sera samples included in the TG-ROC analysis based on TgSALUVET WB: negative= 81, positive= 115). The area under the curve (AUC) was 0.97 ($CI_{95\%}$: 0.95–0.99), and 95% Se ($CI_{95\%}$: 89–98) and 95% Sp ($CI_{95\%}$: 88–99) were obtained for the selected cutoff, RIPC $\leq$ 15.27- $\geq$ 31.09. Using this cutoff, seropositivity was recorded from 2 wpi or 19 dpi onward in Panels 1 and 2, respectively, with 7.4% doubtful results.

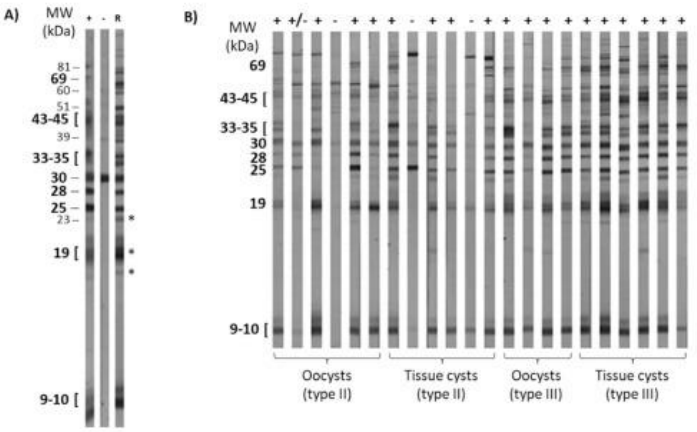

Fig. 2. *Toxoplasma gondii* tachyzoite antigens recognized by experimentally infected pigs at six weeks post-infection. A) + : positive control serum, -: negative control serum, R: positive control serum used as reference where "* " shows a high recognition (+++) of 19 kDa antigen, medium recognition (++) of 23 kDa antigen and a weak recognition (+) of 19 kDa antigen. B) Positive (+), negative (-) and doubtful (+/-) sera from experimentally infected pigs with either oocysts or tissue cysts from type II or III isolates at six weeks post-infection based on the defined positivity criteria: recognition of at least three immunodominant antigens (IDAs) with medium or high intensity. MW: molecular weight. IDAs are shown in bold letters.

Table 2
Frequency and intensity of recognition of *Toxoplasma gondii* tachyzoite antigen by sera from pigs experimentally infected with either oocysts or tissue cysts from type II and III isolates at six weeks post-infection.

Antigens (kDa)[a]	Oocysts type II isolate	Tissue cysts type II isolate	Oocysts type III isolate	Tissue cysts type III isolate	X^2	Prior to infection
81	50/++	50/+++	25/+	50/++	*	36/+
69	17/++	33/++	75/++	100/+++	Ns	0
60	0	17/++	0	17/++	Ns	0
51	83/++	17/++	50/++	67/++	****	41/++
43–45	33/++	33/+	100/++	100/+++	Ns	50/+
39	17/+	33/+	0	33/++	***	23/++
33–35	50/+	67/++	75/+++	100/+++	Ns	23/+
30	83/++	83/++	100/+++	100/+++	Ns	23/++
28	17/+++	50/++	75/++	100/+++	*	14/+
25	50/++	83/++	75/+++	100/+++	Ns	4/+
23	0	33/+	0	33/+	Ns	0
19	67/++	67/++	75/++	100/++	Ns	64/++
9–10	67/++	83/++	100/+++	100/+++	Ns	0

[a] An antigenic fraction comprised more than one antigenic band. X^2: Chi-square test. Immunodominant antigens are represented in bold letters. Frequency: percentage. Intensity: +++ (high), ++ (medium), + (weak). The intensity was the mean within each experimental group. Ns: non-significant. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

3.3. Validation of TgSALUVET IFAT

A better agreement between TgSALUVET IFAT and TgSALUVET WB was obtained for 1/200 ($k = 0.80$) *vs.* 1/100 ($k = 0.74$) cutoff. Accordingly, a 1/200 cutoff was selected for the comparative study (Supplementary Table 2), with an initial diagnostic performance of 99% Se ($CI_{95\%}$: 97–101) and 78% Sp ($CI_{95\%}$: 69–87) when using TgSALUVET WB as a reference test. Based on this cutoff, seropositivity was also recorded from 2 wpi onward in pigs from Panels 1 and 2.

3.4. Comparative study

All ELISA tests showed high Se and Sp values (97–100%) for the cutoffs suggested by the manufacturers with sera from experimentally infected pigs, except for PrioCHECK, which showed 73% Sp (Fig. 4A). Moreover, TgSALUVET WB and TgSALUVET IFAT also showed good diagnostic performance (Se= 95%, Sp= 100% and Se= 99%, Sp= 93%, respectively). Since doubtful results were obtained with IDScreen (0.99%; 2/202) and TgSALUVET ELISA 2.0 (7.43%; 15/202), the cutoffs were readjusted to avoid doubtful results and maintain good diagnostic performance (Se= 99%, Sp= 100% and Se= 97%, Sp= 100%, respectively). On the other hand, the PrioCHECK cutoff was also readjusted to increase Se and Sp up to 97% (Fig. 4A). After cutoff value readjustments, the diagnostic performance of TgSALUVET WB and TgSALUVET IFAT was hardly modified (Se= 91%, Sp= 100% and Se= 100%, Sp= 91%, respectively). The cutoffs, Se and Sp, as well as the AUC values for each ELISA, are shown in Fig. 4A. Summarized information regarding confidence intervals for each parameter is available in Supplementary Table 3. All tests also showed good to near perfect agreement before the TG-ROC analysis ($k = 0.80$–0.91) with sera from experimental infections, except for PrioCHECK with moderate agreement in all pairwise comparisons ($k = 0.63$–0.75) that improved after the cutoff readjustment ($k = 0.79$–0.92) (Table 3).

When TG-ROC analyses were carried out with sera from naturally infected pigs, several tests showed lower Se and/or Sp values (TgSALUVET WB: Se= 71%, Sp= 99%; PrioCHECK: Se= 100%, Sp= 72%; IDScreen: Se= 87%, Sp= 100%; TgSALUVET ELISA 2.0: Se= 100%, Sp= 92%). Pigtype and TgSALUVET IFAT maintained good diagnostic performance (97% Se and Sp; and 97% Se and 100% Sp, respectively). Cutoff readjustments improved the diagnostic performance of all techniques, with Se and Sp values equal to or higher than 97%, except for TgSALUVET WB, whose Se value increased up to 79%. The cutoffs, Se and Sp, as well as the AUC values for each ELISA, are shown in Fig. 4B. Summarized information regarding confidence intervals is for each parameter available as Supplementary Table 3. Low to moderate agreement between techniques was observed, and PrioCHECK showed the lowest agreement ($k = 0.30$–0.46), while TgSALUVET IFAT showed the highest *kappa* values ($k = 0.70$–0.91). After the cutoff readjustment, only IDScreen and TgSALUVET IFAT reached moderate to good agreement in some pairwise comparisons (Table 3).

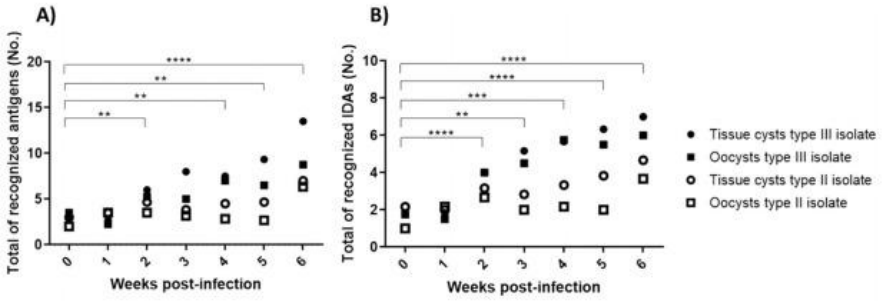

Fig. 3. Kinetics of total (A) or immunodominant (B) antigen recognition by sera from experimentally infected pigs by TgSALUVET WB. IDAs: immunodominant antigens. Significant differences between each sampling week with respect to prior to the infection are identified as follows (Dunnett's multiple comparison test): * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$ and **** = $P < 0.0001$.

3.5. Kinetics of antibody levels in experimentally infected pigs by ELISA tests

In general terms, a significant increase in anti-*T. gondii* IgGs was detected in both panels of sera from 2 to 3 wpi with respect to samples prior to the infection, except in Panel 2 for PrioCHECK, which showed an earlier increase in IgG levels (from 9 dpi), and Pigtype and TgSALUVET ELISA 2.0, which showed a delayed increase in IgG levels (from 26 dpi). Similar seroconversion results at approximately 2 wpi were obtained regardless of the cutoffs employed (initial *vs.* readjusted cutoffs). However, when the cutoff suggested by PrioCHECK was used, three animals from Panel 1 were seropositive prior to infection, and seroconversion was recorded earlier, from 1 wpi.

When the kinetics of IgGs were studied between experimental groups (sera from Panel 1), a significantly higher level of antibodies was detected in pigs infected with the type III isolate by all ELISA tests as follows: pigs infected with any stage of the type III isolate *vs.* oocysts from the type II isolate at 2–4 and 6 wpi for IDScreen, 2–6 wpi for Pigtype, and 3–6 wpi for TgSALUVET ELISA 2.0; tissue cysts from the type III isolate *vs.* oocysts from the type II isolate at 5 wpi for IDscreen, 5–6 wpi for PrioCHECK and 2 wpi for TgSALUVET ELISA 2.0; and tissue cysts from the type III isolate *vs.* tissue cysts from the type II isolate at 4 wpi for IDScreen and 3–5 wpi for Pigtype (Fig. 5A). Nevertheless, no significant differences were observed between groups infected with oocysts and tissue cysts from the same genotype.

4. Discussion

Herein, a comparative study of a wide set of serological techniques used for the detection of anti-*T. gondii* IgGs in domestic pigs has been performed, including routinely used commercial ELISA kits (IDScreen, PrioCHECK and Pigtype), as well as three in-house methods (WB, ELISA and IFAT) that were previously validated. Updated diagnostic performance data are offered, and new cutoffs are suggested to obtain harmonized results. For this study, sera from pigs experimentally infected with different stages and isolates of *T. gondii*, as well as from pigs naturally exposed to *T. gondii*, were analyzed by all the above-mentioned serological tests. Moreover, differences in the levels of anti-*T. gondii* IgGs regarding the *T. gondii* stage and isolate were investigated for all tests.

The developed in-house WB and ELISA tests showed good to excellent proof-of-concept diagnostic performance in their initial validation. Moreover, TgSALUVET IFAT was a highly specific test. *Toxoplasma gondii*-based WBs have been previously developed for pig sera. However, they have been performed under different conditions, and to date, there is no consensus on the criterion of positivity. Some authors considered the recognition of the 30 kDa antigen in addition to the 22 kDa or 42 kDa antigens under nonreducing conditions to be positive (Basso et al., 2013; Pardini et al., 2012). In contrast, Al-Adhami and Gajadhar (2014) classified a sample as positive if the pattern of antigen recognition was similar to that shown by the positive control, where the 150, 100, 79, 45, 39, 35, 30 and 24 kDa antigens were recognized. Recently, a commercial WB kit validated for humans was adapted to pig sera, and the recognition of the 30 kDa antigen together with either the 31 kDa or 33 kDa antigen was considered a positive result (Olsen et al., 2022). In this scenario, herein, an exhaustive analysis with a wide panel of sera from experimentally infected pigs using different parasite stages and isolates has been used to define a criterion of positivity based on the frequency and intensity of antigen recognition. The 9–10, 19, 25, 28, 30, 33–35, 43–45 and 69 kDa bands were identified as IDAs, and only the 9–10 and 69 kDa IDAs were not recognized prior to infection. Therefore, we established a restrictive criterion of positivity that consisted of the recognition of at least three of the IDAs (9–10, 19, 25, 28, 30, 33–35, 43–45 and 69 kDa) with medium or high intensity to avoid false-positive results. The recognition of antigens prior to infection can be due to cross-reactivity with closely related parasites since some proteins are conserved among Apicomplexan parasites (Lorenzi et al., 2016). For example, anti-*N. caninum* antibodies present in small ruminant sera cross-reacted with the 30 kDa *T. gondii* surface antigen (known as TgSAG1 or SRS29B) (Huertas-López et al., 2021; Sánchez-Sánchez et al., 2021). In fact, TgSAG1 is highly immunogenic (Velmurugan et al., 2008) and is used in serological tests (*e.g.*, IDScreen). Thus, WB results based solely on recognition of the 30 kDa antigen should be interpreted with caution.

The establishment of a restrictive criterion of positivity for TgSALUVET WB allowed the definition of a cutoff value for two additional in-house tests, namely TgSALUVET ELISA 2.0 and TgSALUVET IFAT. TgSALUVET ELISA 2.0 was previously used to analyze the kinetics of anti-*T gondii* IgGs in experimentally infected piglets with oocysts from type II and type III isolates (Largo-de la Torre et al., 2022), but there was a lack of validation for positive and negative discrimination. To date, this is the first ELISA that employs *T. gondii* lyophilized tachyzoites for diagnostic purposes in swine. Traditionally, whole or soluble tachyzoite extracts have been used as antigens in ELISA tests, and uniquely in the case of the closely related parasite *B. besnoiti*, a lyophilized tachyzoite-based ELISA was developed (García-Lunar et al., 2017). In that case, an increased Sp was reported compared to a soluble extract-based ELISA, likely due to an enrichment of membrane *vs.* cytosolic antigens (García-Lunar et al., 2017). The TgSALUVET IFAT initially showed excellent Se, with lower Sp. Since a limitation claimed for IFAT is the subjective results, consensus from two or more experienced operators is strongly recommended to avoid subjectivity and

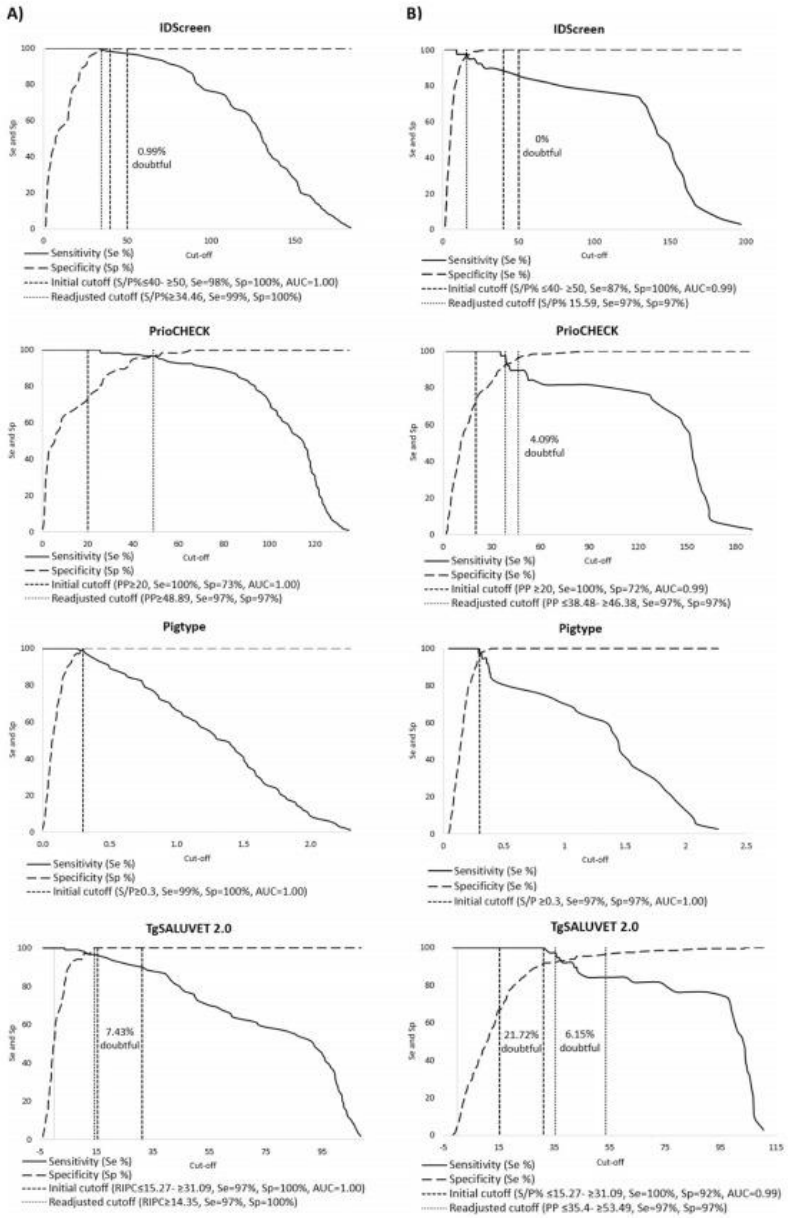

Fig. 4. Two-graph receiver operating characteristic (TG-ROC) analyses based on the reference criterion. A) Sera from experimentally infected pigs; B) sera from naturally infected pigs. Cutoff, sensitivity (Se), specificity (Sp), and area under the curve (AUC) values are shown for each ELISA.

Table 3
Agreement (*kappa* value) among techniques using sera from *Toxoplasma gondii* experimentally or naturally infected pigs before (b) and after (a) the TG-ROC analysis.

Experimental infections	IDScreen		PrioCHECK		Pigtype		TgSALUVET ELISA 2.0		TgSALUVET WB		TgSALUVET IFAT	
	b	a	b	a	b	a	b	a	b	a	b	a
IDScreen	1.00	1.00	**0.71**	0.90	0.90	0.90	0.91	0.90	0.83	0.84	0.85	0.90
PrioCHECK	**0.71**	0.90	1.00	1.00	**0.72**	0.86	**0.67**	0.92	**0.63**	**0.79**	**0.75**	0.90
Pigtype	0.90	0.90	**0.72**	0.86	1.00	1.00	0.91	0.91	0.82	0.82	0.86	0.86
TgSALUVET ELISA 2.0	0.91	0.90	**0.67**	0.92	0.91	0.91	1.00	1.00	0.89	0.81	0.85	0.86
TgSALUVET WB	0.83	0.84	**0.63**	**0.79**	0.82	0.82	0.89	0.81	1.00	1.00	0.80	0.80
TgSALUVET IFAT	0.85	0.90	**0.75**	0.90	0.86	0.86	0.85	0.86	0.80	0.80	1.00	1.00
Natural infections	**IDScreen**		**PrioCHECK**		**Pigtype**		**TgSALUVET ELISA 2.0**		**TgSALUVET WB**		**TgSALUVET IFAT**	
	b	a	b	a	b	a	b	a	b	a	b	a
IDScreen	1.00	1.00	0.36	**0.82**	0.74	0.77	0.65	0.79	0.73	0.63	**0.91**	**0.80**
PrioCHECK	0.36	**0.82**	1.00	1.00	0.43	0.71	0.46	0.78	0.30	0.71	0.39	**0.82**
Pigtype	0.74	0.77	0.43	0.71	1.00	1.00	0.72	0.75	0.64	0.64	0.77	0.77
TgSALUVET ELISA 2.0	0.65	0.79	0.46	0.78	0.72	0.75	1.00	1.00	0.55	0.73	0.70	**0.86**
TgSALUVET WB	0.73	0.63	0.30	0.71	0.64	0.64	0.55	0.73	1.00	1.00	0.73	0.73
TgSALUVET IFAT	**0.91**	**0.80**	0.39	**0.82**	0.77	0.77	0.70	**0.86**	0.73	0.73	1.00	1.00

The lowest kappa values ($k \leq 0.80$) are marked in bold letters.
The highest kappa values ($k \geq 0.80$) are marked in bold letters.

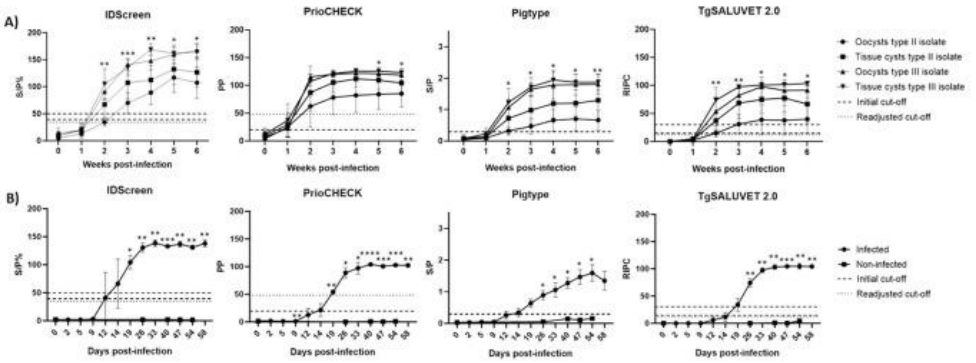

Fig. 5. Anti-*Toxoplasma gondii* IgG kinetics as assessed by different ELISAs in pigs experimentally infected with *T. gondii*. Data are presented as the mean in S/P%, PP, S/P or RIPC (as specified by manufacturers) by experimental group ± standard deviation (SD). Placed horizontally: A) Panel 1: significant differences between experimental groups within sampling weeks (Tukey's multiple comparisons test, representing the highest *P* value within sampling week; *= $P < 0.05$, **= $P < 0.01$ and ***= $P < 0.001$). B) Panel 2: significant differences between the IgG mean levels at 0 dpi *vs.* 2–58 dpi (Dunnett's multiple comparisons test; *= $P < 0.05$, **= $P < 0.01$, ***= $P < 0.001$ and ****= $P < 0.0001$). No comparisons were carried out between infected and noninfected pigs since there were missing samples on some sampling days.

obtain reliable and comparable results by IFAT tests, as done herein. Indeed, a previous interlaboratory study of serological tests employed in the diagnosis of bovine neosporosis evidenced high discrepancies among IFAT tests (Campero et al., 2018).

These results, together with the results obtained with commercial ELISAs in the comparative study, showed the usefulness of sera from experimentally infected animals for the validation of serological tests. In general terms, all tests showed excellent diagnostic performance when analyzing sera from experimental infections; thus, the cutoffs recommended by the manufacturers can be employed, except for PrioCHECK (Se= 100%, Sp= 73%, $k = 0.63$–0.75). Good diagnostic performance with sera from experimental infections is expected since these sera are from controlled infections and homogeneous experimental groups. Accordingly, the diagnostic performance of these serological tests could be overestimated. A previous comparative study of serological tests used for the diagnosis of bovine neosporosis, which also included sera samples from experimental infections and used the results obtained by the majority of the tests as a reference, also showed good to excellent test performance (Álvarez-García et al., 2013; von Blumröder et al., 2004). However, regarding the serodiagnosis of *T. gondii* infection, in contrast with these outcomes, lower Se values have been reported in previous comparative studies of different tests when using sera from experimental infections, probably due to the different techniques used as references, for which initial validation and performance were not clearly stated in all cases. Among them, a MAT and a rhoptry ELISA showed Se values of 87% and 94%, respectively, but 100% Sp in both cases when an IFAT was used as a reference (Garcia et al., 2006). Al-Adhami and Gajadhar (2014) also reported 85–89% Se and 94–97% Sp for an in-house ELISA when SafePath ELISA, MAT and WB were used as references.

Nevertheless, as expected, the scenario changed when sera from naturally infected pigs were employed following the WOAH recommendations (WOAH, 2023), which better mimics what may occurs in the field (unknown stage, doses and route, presence of co-infections, among other variables). The results evidenced the need for cutoff readjustments, especially for PrioCHECK and IDScreen, since a decrease in both the diagnostic performance and agreement was recorded. There are a

few comparative studies of commercial tests that analyzed sera from naturally infected pigs. IDScreen was used more frequently, but with variable results, probably due to the different tests employed as references. For example, in contrast to our results, IDScreen showed lower Se when compared to a commercial MAT (Toxo-Screen DA Biomérieux) (Se= 57%, Sp= 99%) (Steinparzer et al., 2015), but high agreement ($k = 0.96$) when compared to an IFAT kit (Fuller Laboratories, Fullerton CA) (Papatsiros et al., 2016), or low to moderate agreement ($k = 0.39–0.68$) when compared to the same commercial MAT kit (Cubas-Atienzar et al., 2019; Pablos-Tanarro et al., 2018; Steinparzer et al., 2015). Other commercial tests previously evaluated were PrioCHECK and Pigtype. In contrast to our results, PrioCHECK obtained lower Se and higher Sp values compared to a commercial MAT (Se= 65%, Sp= 97%, $k = 0.62$), while Pigtype showed the worst performance compared to the same test (Sp= 57%, Sp= 99%, $k = 0.64$) (Steinparzer et al., 2015). However, better performance was reported for PrioCHECK in a comparative study with an in-house TgSAG-1-ELISA, WB and IFAT that used a Bayesian latent class approach, with 99% Se and 93% Sp (Basso et al., 2013). In the present study, PrioCHECK was the test that showed the lowest Sp (72–73%), as well as the lowest agreement ($k = 0.30–0.75$) regardless of the serum panel employed. Based on the comparative study, Pigtype and TgSALUVET IFAT were initially well validated since they maintained high Se and Sp values and did not require cutoff readjustment with any serum panel. Finally, despite the low Se value obtained with TgSALUVET WB in natural infections even after the ELISA cutoff readjustments (Se= 79%), the criterion of positivity could not be changed since a lower intensity and frequency of IDAs recognition was observed with this serum panel. For example, it was found that the 9–10 kDa antigen, which was frequently detected with medium-high intensity in experimentally infected pigs (Table 2), was present in less than 50% of seropositive pigs from Panel 3 based on the defined criterion of positivity. Thus, our results demonstrate that TgSALUVET WB could be used as a reference test in an initial validation of serological techniques, but it is not recommended for routine serodiagnosis.

All in-house and commercial tests detected seroconversion at 2–3 wpi on average, except for PrioCHECK, which was earlier and could be directly related to its high Se but low Sp. Comparable anti-*T. gondii* IgG kinetics have been reported in other studies based on some of the ELISAs tested here and performed with pigs experimentally infected with *T. gondii* oocysts. One of them documented seroconversion from 1.5 to 2 wpi until the end of the experiment (8–30 wpi) based on PrioCHECK or IDScreen (Kauter et al., 2022), whereas the other reported a significant IgG increase from 14 dpi based on TgSALUVET ELISA 2.0 prior to its validation for diagnostic purposes (Largo-de la Torre et al., 2022). Interestingly, all ELISA tests, as well as TgSALUVET WB, showed significantly higher levels of anti-*T. gondii* IgGs in pigs infected with the type III isolate with respect to the type II isolate, with an earlier IgG level increase detected with IDScreen, Pigtype and TgSALUVET ELISA 2.0, from 2 wpi onward, but a more delayed increase with TgSALUVET WB and PrioCHECK, from 3 and 5 wpi, respectively. The higher antibody levels associated with the type III isolate could be explained by the higher virulence and earlier dissemination of this isolate. This was demonstrated previously in a mouse model where higher rates of mortality and morbidity of type III were shown compared to those with a set of type II isolates (Fernández-Escobar et al., 2021, 2020). Higher virulence and earlier dissemination have also been documented in type III *vs.* type II isolates in a piglet model of toxoplasmosis, with higher temperatures for a longer period in pigs infected with the type III isolate, as well as a higher frequency of detection and parasite burden quantified by qPCR in mice inoculated with tissue originating from pigs infected with the type III isolate (Largo-de la Torre et al., 2022). In this experiment, pigs infected with the type III isolate also showed higher IgG levels with respect to the other group based on TgSALUVET ELISA 2.0, while no significant differences were recorded between groups when using PrioCHECK (Largo-de la Torre et al., 2022), which agrees with the present results.

Multiple variables, such as the reference sera, the methodology and the antigen used, influence the performance of a test and therefore are key points to be considered when validating a serological technique (WOAH, 2021). Unfortunately, there is no sera panel that can represent all target infected populations and environmental conditions (WOAH, 2009) or commonly accepted reference tests. Consequently, in this comparative study, we used different panels of sera and employed the results obtained by most of the included tests as reference criteria, an approach that seems to be appropriate for further studies. The usefulness of the different serum panels has been discussed above. However, other more sophisticated statistical approaches, such as the Bayesian Latent Class Models, are highly recommended in the absence of a gold standard test (Johnson et al., 2019; Kostoulas et al., 2017). On the other hand, limited conclusions could be drawn with respect to the influence of the antigen employed since the different tests that showed good diagnostic performance employed different antigens (Table 1). Nevertheless, it was reported for small ruminants that false-positive reactions can be found with anti-*N. caninum* antibodies in TgSAG1-based tests (WB and IDScreen) due to cross reactions (Huertas-López et al., 2021; Sánchez-Sánchez et al., 2021). To date, there are only a few studies about *N. caninum* seroprevalence in domestic pigs (Damriyasa et al., 2004; Lopes et al., 2021; Villa et al., 2022), probably because it has low relevance in this species since its contribution to inducing transplacental infections and reproductive failures in natural infections is still unclear (Snak et al., 2019). However, the presence of anti-*N. caninum* antibodies in naturally exposed domestic pigs has been documented (Damriyasa et al., 2004; Lopes et al., 2021; Villa et al., 2022), so cross-reactivity with this parasite, as well as with other closely related ones, should be considered in future studies.

It must be taken into consideration that serum, plasma, or meat juice can be used in some of the commercial ELISA kits, and different performances have been observed in the same test based on the type of sample (García et al., 2008). Thus, similar approaches should be followed with any other sample types (*e.g.*, meat juice, whole blood) and with sera from other host species considering the multispecies specifications of some of the commercial ELISA kits evaluated here.

5. Summary and conclusions

We offer updated performance information of widely employed serological tests, including commercially available methods that are accessible for research and diagnostic laboratories. In this comparative study, all tests proved to be well validated when analyzing sera from experimental infections, except for PrioCHECK, but several ELISAs required further readjustments for sera originating from natural infections to obtain harmonized data. TgSALUVET WB, which turned out to be useful as a reference test for an initial validation of serological techniques, is not recommended for routine diagnosis. These results must be taken into consideration for epidemiological surveys and studies that aim to evaluate IgG kinetics based on different stages or isolates since some tests were more sensitive than others in detecting variations among experimental groups. The establishment of an international network for creating a common well-characterized serum biobank would favor future test validations.

CRediT authorship contribution statement

Blaga Radu: Writing – review & editing, Investigation. **López-Ureña Nadia María:** Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Calero-Bernal Rafael:** Writing – review & editing, Visualization, Supervision, Resources, Methodology. **González-Fernández Nuria:** Methodology, Investigation, Formal analysis, Data curation. **Álvarez-García Gema:** Writing – review & editing, Project administration, Methodology, Funding acquisition, Formal analysis,

Conceptualization. **Koudela Bretislav:** Writing – review & editing, Investigation. **Ortega-Mora Luis Miguel:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Nadia Maria Lopez-Urena, Rafael Calero-Bernal, Radu Blaga, Bretislav Koudela, Luis Miguel Ortega-Mora, Gema Alvarez-Garcia reports financial support was provided by Horizon Europe.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.vetpar.2023.110024.

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Objective 2: To develop and validate a serological method that discriminate between *T. gondii* oocyst- *versus* tissue cyst-driven infections.

- Sub-objective 2.1: To develop and harmonize conventional serological tests frequently used in the diagnosis of *T. gondii* infections in three species whose meat consumption supposes a burden of infection for humans: pigs, sheep and goats, and to properly characterize the serum panels for sub-objective 2.2.

The outcomes from this sub-objective related to the tests used in **small ruminants (sheep and goats)** were published in the following open access article **(paper No. 3)**: López-Ureña, N.M., Calero-Bernal, R., Vázquez-Calvo, Á., Sánchez-Sánchez, R., Ortega-Mora, L.M., Álvarez-García, G., 2023. **A comparative study of serological tests used in the diagnosis of *Toxoplasma gondii* infection in small ruminants evidenced the importance of cross-reactions for harmonizing diagnostic performance**. Research in Veterinary Science 165, 105052. https://doi.org/10.1016/j.rvsc.2023.105052. This journal had an impact factor of 2.4 and was classified as Q1 (Veterinary Sciences) in 2022.

Abstract: *Toxoplasma gondii* is a significant zoonotic foodborne parasite transmitted through undercooked meat from small ruminants. While serology is proposed as an epidemiological indicator, the diagnostic performance of available tests is currently uncertain. Hence, herein was defined a criterion of positivity for in-house Western blot test (TgSALUVET WB) considering cross-reactivity with anti-*N. caninum* IgGs. Then, this test was used to initially validate an in-house ELISA test based on lyophilized tachyzoites (TgSALUVET ELISA 2.0) for goats and sheep. After that, both in-house tests were subjected to a comparative study together with four commercial ELISA tests (IDScreen, PrioCHECK, Pigtype and IDEXX) using sera from sheep experimentally and naturally infected with *T. gondii*, and cross-reactivity with anti-*N. caninum* was also analyzed. All tests showed good to excellent performance with sera from experimental or natural infections, although this parameter could be improved with a cutoff readjustment. However, cross-reactivity with anti-*N. caninum* IgGs was observed in all ELISA tests, and so an additional cutoff readjustment was required to avoid false positive results.

These outcomes were also presented as an oral communication in an Apicowplexa virtual meeting (February 2021).

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A comparative study of serological tests used in the diagnosis of *Toxoplasma gondii* infection in small ruminants evidenced the importance of cross-reactions for harmonizing diagnostic performance

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ABSTRACT

Toxoplasma gondii is a major foodborne zoonotic pathogen that can be transmitted through the consumption of raw or undercooked meat of small ruminants, among others. Serology has been suggested as an epidemiological indicator and several tests are available nowadays. However, there is no comparative study with the most used ones. Therefore, the objective of this study was to develop and validate two in-house tests (Western blot -TgSALUVET WB- and ELISA -TgSALUVET ELISA 2.0-) and perform a comparative study including such tests and four commercial ELISA kits (IDScreen®, PrioCHECK®, Pigtype® and IDEXX). First, a specific pattern of recognition of immunodominant antigens by TgSALUVET WB was determined with serum panels of noninfected sheep and sheep infected with *T. gondii* or *Neospora caninum*. Next, TgSALUVET WB was used as a reference to preliminary validate TgSALUVET ELISA 2.0 using sera from sheep and goats naturally infected with *T. gondii*. Then, the abovementioned sheep serum panels were analyzed by all tests and subjected to TG-ROC analyses and agreement tests, and cross-reactivity with the anti-*N. caninum* IgGs was studied.

All the techniques were accurate enough for the cutoff values initially suggested with all serum panels (Se and Sp $\geq$ 94%), except for PrioCHECK®, which showed 83% Sp. However, a cutoff readjustment improved their diagnostic performance. Additionally, cross-reactions between anti-*N. caninum* antibodies and *T. gondii* antigens were detected with all tests. Thus, a second cutoff readjustment was carried out and the use of both readjusted cutoff values is recommended to obtain comparable data and avoid false-positive results.

1. Introduction

Toxoplasma gondii, the causative agent of toxoplasmosis, is a widespread apicomplexan parasite able to infect any warm-blooded animal species, including humans. Toxoplasmosis is usually asymptomatic in immunocompetent hosts, with an estimation of one-third of the global human population chronically infected or having had past episodes of contact with the organism. However, this zoonosis is of concern since it can induce severe respiratory, neurological and ocular diseases in immunocompromised people, as well as reproductive failures in pregnant women, mainly via primary infection (WHO, and FAO, 2014). In humans, horizontal transmission of *T. gondii* mainly occurs through the consumption of sporulated oocysts that contaminate soil, water, vegetables, fruits and bivalves (environmental route) or tissue cysts contained in raw or undercooked meat (meat route) (Dubey, 2021; Pinto-Ferreira et al., 2019). Primary infections in humans and small ruminants during gestation may also lead to vertical transmission by tachyzoites that reach the fetus/es through the placenta, causing severe harm such as congenital malformation, ocular disease, hydrocephaly, abortion, reabsorption, and neonatal death, among others (Innes et al., 2009; Koutsoumanis et al., 2018; Lindsay and Dubey, 2020; Stelzer et al., 2019). In this context, *T. gondii* is a perfect paradigm of a pathogen

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whose control should be achieved through a One Health approach (Djurković-Djaković et al., 2019).

Small domestic ruminants are highly susceptible to *T. gondii* infections. Indeed, *T. gondii* has been identified as one of the most common and important reproductive transmissible agents in ewes and goats (Stelzer et al., 2019), which translates to great economic losses for producers, with approximately 1.5 million lambs lost per year in Europe (Innes et al., 2009). In addition, *T. gondii* infections in small ruminants are of public health concern since the consumption of raw or undercooked sheep/lamb meat has been identified as a primary food source of infection (WHO, and FAO, 2014) and a risk factor associated with acute toxoplasmosis in humans (Odds Ratio = 3.6–3.9 (95% CI 1.3–9.8, 1.9–8.0)) (Belluco et al., 2017; Friesema et al., 2023). Furthermore, *T. gondii* has been identified as a high priority in meat inspections based on ranking biological hazards in small ruminants (EFSA, 2013a), with global pooled seroprevalence of 33.86% and 31.78% in sheep and goats, respectively (Ahaduzzaman and Hasan, 2022).

Serology is a valuable strategy for monitoring the infection with a public health commitment (EFSA, 2013b). Accordingly, serological techniques are the most commonly used tools for the diagnosis of *T. gondii* infections in small ruminants (Dubey et al., 2020a, 2020b). However, there are several issues that hamper the interpretation of serological results and that could lead to misdiagnosis. First, diagnostic performance and analytical specificity data are not always available, and the scarce number of comparative studies hinders access to updated information. Furthermore, cross-reactivity with closely related parasites is of major concern in small ruminants. For example, there is increased evidence of the relevance of *Neospora caninum* infection in sheep and the coexistence of *T. gondii* and *N. caninum* infections in flocks (Gharekhani et al., 2018; Gondim et al., 2017; González-Warleta et al., 2014; Sun et al., 2020; Villagra-Blanco et al., 2019). Second, a few limitations have been identified concerning the comparative studies carried out to date: a) the initial diagnostic performance of the evaluated techniques was not always specified, b) the majority of the studies arbitrarily defined an in-house technique as a reference test, c) the serum panel consisted of a low number of sera, initially characterized with a limited set of serological tests (Glor et al., 2013; Mainar-Jaime and Barberán, 2007; Mangili et al., 2009; Opsteegh et al., 2010), and d) commercial ELISA tests have barely been evaluated (within comparative studies) despite being very frequently used since not all laboratories are specialized in the development and validation of serological tests (Glor et al., 2013; Opsteegh et al., 2010).

Therefore, the objective of this study was to compare the performance of a wide panel of serological techniques routinely used for the detection of anti-*T. gondii* IgGs in small domestic ruminants. For this, a total of 840 well-characterized serum samples from experimentally and naturally infected animals were employed. Initially, two in-house tests, a Western blot (WB) and a lyophilized tachyzoite-based ELISA, were standardized. Second, the abovementioned in-house tests and the most frequently used and/or known commercial ELISA kits (IDScreen,

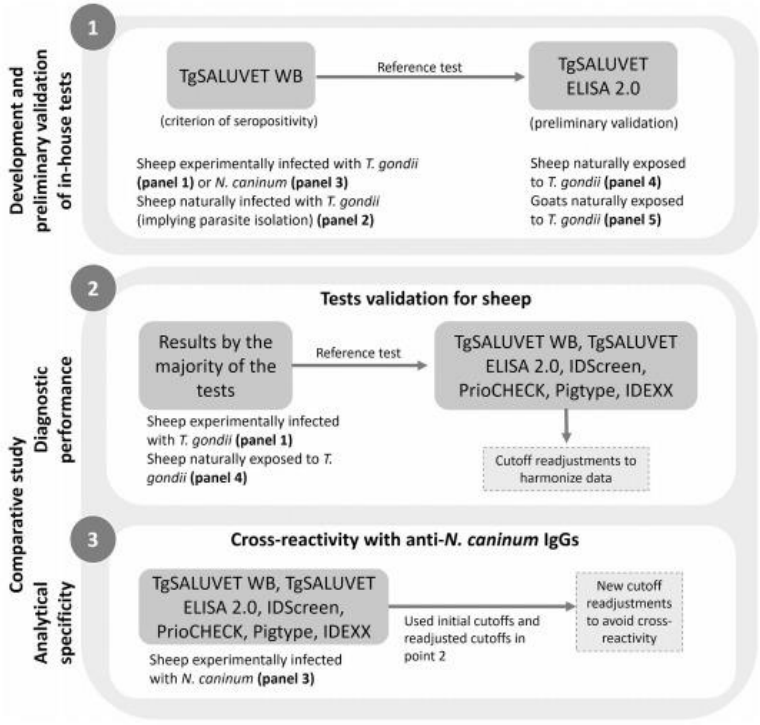

Fig. 1. Experimental design followed in this comparative study. A checklist of the items included in the standards for reporting diagnostic accuracy studies (Kostoulas et al., 2017) has been provided as Supplementary Table 1.

Table 1
Serological techniques used for the detection of anti-*Toxoplasma gondii* IgG in small domestic ruminants included in this comparative study.

Technique	Antigen	Host species	Sample type	Sample dilution	Cutoff	Diagnostic performance[a]	Reference
TgSALUVET WB	Tachyzoites	Sheep and goats	Serum	1/20	To be determined in this study	To be determined in this study	This study
TgSALUVET ELISA 2.0	Lyophilized tachyzoites	Sheep and goats	Serum	1/100	To be determined in this study	To be determined in this study	This study
IDScreen	P30	Ruminants, swine, dogs, cats	Serum, plasma and meat juice	Serum and plasma: 1/10, meat juice1/2	S/P% ≤ 40, ≥ 50	Se = 100%, Sp = 100%[b]	Basso et al., 2022; Gazzonis et al., 2020; Mangili et al., 2009; Villagra-Blanco et al., 2018, 2019
PrioCHECK	Tachyzoite[**]	Small ruminants	Serum, plasma and meat juice	Serum and plasma: 1/100, meat juice: 1/10	PP ≥ 20	Not specified	Glor et al., 2013; Martínez-Rodríguez et al., 2020
Pigtype	Tachyzoite[**]	Cattle, sheep, goats, cats, dogs, foxes, pigs and wild boars	Serum, plasma (meat juice for pigs and wild boars)	Serum and plasma: 1/100, meat juice: 1/10	S/P ≥ 0.3	Not specified[b]	None
IDEXX	Not specified[***]	Small ruminants	Serum and plasma	1/400	S/P%<20, ≥ 30 (weak positive), ≥ 100 (positive)	Not specified	Maitar-Jaime and Barberán, 2007; Opsteegh et al., 2010

Se: sensitivity, Sp: specificity. S/P%, PP and RIPC = ((sample OD- negative control OD)/(positive control OD- negative control OD)) x 100, S/P = ((sample OD- negative control OD)/(positive control OD- negative control OD)).
[*] According to the information provided within the kit.
[**] No additional details provided.
[***] Only specified that the microtiter plates are coated with inactivated antigen. The short protocol described by the manufacturer was followed for Pigtype.
[a] Based on 33 positive samples from France and 300 negative samples tested (the origin species was not specified). Intra-plate repeatability= 3–4%, inter-plate reproducibility= 4–6%.
[b] Microtiter plate coefficient of variation = 6.8%.

PrioCHECH, Pigtype and IDEXX) were compared. Finally, cross-reactivity with the anti-*N. caninum* IgGs was investigated in all tests.

2. Materials and methods

2.1. Experimental design and panels of sera

The experimental design that was followed and the tests that were evaluated in this study are shown in Fig. 1 and Table 1, respectively. The sera panels employed in each step of the workflow are detailed below:

2.1.1. Pattern of recognition of immunodominant antigens and criterion of seropositivity for TgSALUVET WB

Panel 1 ($n = 124$) consisted of samples from 14 Rasa Aragonesa breed sheep that had been orally inoculated with 10 *T. gondii* oocysts (TgSpSh1, genotype II) at 90 days of gestation, as well as sera from 6 noninoculated pregnant sheep. Sampling was carried out at −2, 3, 8, 12, and 14 days post-infection (dpi). In addition, serum samples from 9 of these animals (six infected and three noninfected sheep) were also collected at 21 and 27 dpi (Vallejo-Blanco et al., 2023). Samples collected prior to the infection tested negative for *N. caninum* by a specific soluble antigen-based ELISA (Sánchez-Sánchez et al., 2021a; Vallejo-Blanco et al., 2023) and were kept at −80 °C until analysis. Negative ($n = 20$, prior to the infection) and positive ($n = 9$, from 27 dpi) serum samples from this panel were used to define the pattern of recognition of *T. gondii* tachyzoite immunodominant antigens (IDAs) by TgSALUVET WB and the criterion of seropositivity. All the serum samples were also included in the comparative study of all the serological tests (see Section 2.3).

Panel 2 consisted of 17 sheep naturally infected that tested positive for *T. gondii* by a soluble antigen-based ELISA. In all these animals, *T. gondii* was isolated by mouse bioassay from myocardial tissue (Fernández-Escobar et al., 2020). These samples tested negative for *N. caninum* by a specific soluble antigen-based ELISA (Sánchez-Sánchez et al., 2021a) and were kept at −80 °C until use. This panel of sera was employed to define the pattern of recognition of *T. gondii* tachyzoite IDAs by TgSALUVET WB and the criterion of seropositivity.

Panel 3 was composed of 26 serum samples from sheep inoculated intravenously with *N. caninum* tachyzoites (Sánchez-Sánchez et al., 2018, 2021b). All of them tested positive for *N. caninum* by a specific soluble antigen-based ELISA (Sánchez-Sánchez et al., 2021a), with low (RIPC = 35–49, $n = 8$), medium (RIPC = 50–79, $n = 7$) and high (RIPC≥80, $n = 11$) IgG levels. This sera panel was used to test cross-reactivity between anti-*N. caninum* IgGs and *T. gondii* tachyzoite IDAs in TgSALUVET WB to define the criterion of seropositivity for TgSALUVET WB. It was also used in the comparative study to evaluate cross-reactivity in all the ELISA tests.

2.1.2. Development and initial validation of TgSALUVET ELISA 2.0

Panel 4 ($n = 239$) was composed of serum samples from sheep naturally exposed to *T. gondii*. These samples were collected at two slaughterhouses in Spain (Fernández-Escobar et al., 2020). All sera tested negative for *N. caninum* by a specific soluble antigen-based ELISA (Sánchez-Sánchez et al., 2021a) and were kept at −80 °C. This panel was used to develop and validate TgSALUVET ELISA 2.0. This panel was also included in the comparative study. The precision of TgSALUVET ELISA 2.0 was tested using TgSALUVET WB-positive (n = 11) and -negative (n = 11) serum samples from Panel 1.

Panel 5 ($n = 434$) was composed of serum samples from goats naturally exposed to *T. gondii*. These samples were collected from different flocks with a previous history of *T. gondii*-associated abortions. These samples tested negative for *N. caninum* by a specific soluble antigen-based ELISA (Sánchez-Sánchez et al., 2021a) and were kept at −80 °C until use. This panel was used to test the suitability of TgSALUVET ELISA 2.0 for goats, using TgSALUVET WB as a reference.

2.1.3. Comparative study of all serological tests

The sera from sheep experimentally and naturally infected with *T. gondii* were analyzed separately in the comparative study (Panels 1 and 4, respectively). In addition, the sera from sheep experimentally infected with *N. caninum* were included in this step to test cross-reactivity in all the ELISA tests (Panel 3).

2.2. Parasite culture and antigen production

Toxoplasma gondii tachyzoites of the ME49 strain were cultured in the VERO-81 cell line with a multiplicity of infection (MOI) of 4:1, parasite:cell, with DMEM (Dulbecco's Modified Eagle Medium – high glucose, Sigma®, Ref.6429) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (Lonza, Ref. H317-745E). The FBS used tested negative for *T. gondii*, *Besnoitia besnoiti* and *N. caninum* to avoid cross-reactivity (García-Lunar et al., 2015). After 72 h, the culture was syringed 3 times through a 25G (0.5 × 16 mm) needle, and tachyzoites were purified using 3-μm Whatman® filters (Millipore, Ref. TSTP02500), quantified and centrifuged at 1350 xg for 15 min at 4 °C. Pellets of 1×10^8 tachyzoites were kept at −80 °C until use for TgSALUVET WB. For TgSALUVET ELISA 2.0, glass vials with 1×10^8 tachyzoites in 4 mL of phosphate-buffered saline (PBS) were stored at −80 °C until being lyophilized as specified in a previous study (García-Lunar et al., 2017; López-Ureña et al., 2023).

2.3. Serological techniques

The main characteristics of the serological tests evaluated in this comparative study are shown in Table 1.

2.3.1. TgSALUVET WB

Antigen preparation, as well as the electrophoresis and electro-transfer of proteins were carried out following the procedure described by Sánchez-Sánchez et al. (2019b), with a few changes mentioned below. Aliquots of 2×10^7 tachyzoites were subjected to a cold wet ultrasonic bath for 15 min, followed by a wet bath at 100 °C for 5 min, in loading buffer under reducing conditions (10% glycerol, 6.8 pH 50 mM TRIS, 2% SDS, 0.05% bromophenol blue and 100 mM DTT final concentration). Then, the content was transferred to a 15% polyacrylamide gel and then to a 0.2-μm nitrocellulose membrane (Bio Rad laboratories). Membranes were cut into strips (1–2 mm each) and placed on stands with individual rails. Samples diluted at 1/20 in blocking solutions (5% powdered skim milk 0.05% tris-buffered saline-Tween 20 (TBS-T)) were added in separate rails and incubated for 1.5 h at room temperature. After that, three washes with TBS-T, each for 5 min, were performed, and the secondary antibody was added diluted at 1/1000 in TBS-T (monoclonal anti-goat/sheep IgG antibody conjugated with peroxidase, Sigma, Ref. A9452). It was incubated and washed under the same conditions, including one additional wash with TBS. The bounded antibodies were revealed using 4-chloro-1-naphthol solution (Thermo Scientific, Ref. 34010) and stopped with ultra-pure water based on the reaction developed in the positive controls. The strips were scanned with the GS-800 Calibrated Densitometer (Bio-Rad) for further analysis. The positive and negative controls used were obtained from Panel 1.

The analysis of the pattern of recognition of immunodominant antigens (IDAs) was performed by two experienced operators to avoid bias. Herein, the antigens of 9–10, 18–20, 24–26, 30 and 37–40 kDa were considered IDAs based on previous studies performed with small ruminant serum samples and *T. gondii*-based Western blot tests (Wastling et al., 1994; Conde et al., 2001). The criterion of seropositivity was established based on the IDAs frequency and intensity of recognition (see Section 2.4).

2.3.2. TgSALUVET ELISA 2.0

The assay was carried out following a previous described procedure with a few modifications (López-Ureña et al., 2023). Ninety-six-well microtiter plates (Thermo Scientific, Fisher Brand Maxisorp®, MA, USA, Ref. 10554831) were coated with 1×10^5 lyophilized tachyzoites per well in 0.1 M carbonate buffer (pH 9.6) (100 μL/well) overnight at 4 °C. The plates were then washed three times with 0.05% PBS-Tween 20 (PBS-T) and blocked with 5% powdered skim milk PBS-T for 2 h at room temperature (300 μL/well). After that, 100 μL per well of sera controls and samples diluted at 1/100 in blocking solution was placed per well and incubated at 37 °C for 1 h. Three additional washes were performed under the same conditions, and 100 μL of diluted secondary antibody at 1/10000 in PBS-T was dispensed per well (monoclonal anti-goat/sheep IgG antibody conjugated with peroxidase, Sigma Ref. A9452) and incubated at 37 °C for 1 h. For the detection of bound antibodies, the plates were washed again, and 100 μL of ABTS® (Roche, 11684302001) was added per well. The reaction was stopped with 100 μL per well of 0.3 M oxalic acid, when the OD of the positive control was between 1 and 1.1. Data were normalized as relative index percentage (RIPC) with the following formula: ([sample OD- negative control OD]/[positive control OD- negative control OD]) x 100. Here, the same positive and negative controls employed for TgSALUVET WB were used.

2.3.3. Commercial ELISA tests

Four commercially available ELISA tests used to detect anti-*T. gondii* IgGs in small ruminants were included in this comparative study: ID Screen® Toxoplasmosis Indirect Multispecies (Innovative Diagnostics, France); PrioCHECK® Toxoplasma Ab SR (Prionics, Switzerland); Pigtype® Toxoplasma Ab (Indical Bioscience, Germany) and IDEXX Toxotest Ab (IDEXX Laboratories, Inc., the United States), named IDScreen, PrioCHECK, Pigtype and IDEXX, respectively. The manufacturers' protocols were followed, and the characteristics of the ELISA tests are summarized in Table 1.

2.4. Data analysis

The frequency and intensity of IDAs recognition were used to define the criterion of seropositivity for TgSALUVET WB. The intensity of recognition was classified as high (+++), medium (++) or low (+). The frequency of the recognition of each IDA corresponding to the different panels of sera employed was compared by a contingency test (Fisher's exact test). This analysis was performed with GraphPad Prism, version 8.0.1.

To determine the precision of TgSALUVET ELISA 2.0, four replicates of each serum sample were run within a plate, and three different plates were analyzed in parallel. The following formula was used to determine the intraplate coefficient of variation (CV): mean ([standard deviation of the three replicate ODs/mean of the three replicate ODs] x 100). The interplate CV was determined as follows: mean ([standard deviation of the OD mean of each sample from each plate/mean of the OD mean of each sample from each plate] x 100). Coefficients of variation below 20% were interpreted as acceptable (Jacobson, 1998). Diagnostic performance and preliminary cutoff were estimated by a nonparametric two-graph receiver operating characteristic (TG-ROC) analysis with SigmaPlot 12.0 Software, and the WinEpi platform (Thrusfield et al., 2001) (http://www.winepi.net/), using TgSALUVET WB as reference test.

For TG-ROC analyses, the result obtained by the majority of the tests (four out of six techniques) was regarded as a reference in the comparative study. If a serum sample was positive by three tests and negative by the other three tests, it was considered doubtful and was excluded from the analysis. After that, an agreement test, expressed as *kappa* values, was performed using the WinEpi platform with a confidence level of 95%.

The kinetics of anti-*T. gondii* IgGs was also studied for all tests using serum Panel 1. Significant differences between noninfected and infected sheep within sampling days were analyzed with a mixed-effects analysis, followed by Sidak's multiple comparison test if applicable, using GraphPad Prism, version 8.0.1. Sphericity was not assumed, and the

Geisser-Greenhouse correction was automatically applied when necessary.

Fisher's exact test was performed for each ELISA to determine if the presence or absence of anti-*N. caninum* IgGs had a significant influence on the number of false-positive results in *T. gondii*-based ELISA tests.

Significant differences were considered when *P* values were lower than 0.05.

3. Results

3.1. Pattern of recognition of immunodominant antigens and criterion of positivity for TgSALUVET WB

The 18–20, 24–26, 30 and 37–40 kDa IDAs were recognized with high intensity for 100% of sheep experimentally infected with *T. gondii* at 27 dpi (Panel 1), as well as for all the sheep naturally infected with *T. gondii* (Panel 2), while the 9–10 kDa IDA was recognized with high and medium intensity for 100% and 82.4% of the sheep from Panel 1 (at 27 dpi) and Panel 2, respectively (Table 2, Fig. 2). Remarkably, the 30 and 37–40 kDa IDAs were recognized with medium and low intensity for 90% and 40% of the noninfected sheep, respectively. Furthermore, for all sheep experimentally infected with *N. caninum* (Panel 3) the 30 and 37–40 kDa IDAs were recognized with high intensity, and, respectively, for 30.8% and 11.5% of sheep experimentally infected with *N. caninum* the 18–20 and 24–26 kDa IDAs were recognized with low intensity, whereas the 9–10 kDa IDA were recognized for none of the sheep experimentally infected with *N. caninum*. Nonsignificant differences were observed in the frequency of recognition of the 30 kDa antigen between the noninfected sheep and the sheep infected with either *T. gondii* or *N. caninum*. Moreover, the 9–10, 18–20 and 24–26 kDa IDAs showed significant differences in the frequency of recognition between sheep naturally infected with *T. gondii* and sheep experimentally infected with *N. caninum* (Table 2). Thus, based on these results, the recognition of the 9–10 and/or 24–26 kDa antigens together with two other IDAs (18–20, 30 and 37–40 kDa) with medium-high intensity was established as a criterion of seropositivity. Accordingly, none of the sheep experimentally infected with *N. caninum* tested positive by TgSALUVET WB (Table 2, Fig. 2). Seroconversion was recorded from 14 dpi onward in Panel 1 based on TgSALUVET WB.

3.2. TgSALUVET ELISA 2.0 initial validation

The mean CV values for the intra and interplate repeatability for TgSALUVET ELISA 2.0 were 4.59 (standard deviation (SD) = 0.02) and 9.48 (SD = 0.05), respectively. This ELISA test showed a high performance with an AUC of 0.99 for the preliminary cutoff defined, RIPC≥19.18, with 93% Se and 96% Sp based on the TG-ROC analysis from sheep naturally infected with *T. gondii* (Panel 4) and using TgSALUVET WB as a reference test. Seroconversion was recorded from 21 dpi onward in Panel 1 when applying the defined cutoff. Furthermore, TgSALUVET ELISA 2.0 was suitable for goats (Panel 5), showing 100% Se and 98% Sp when using the abovementioned cutoff value and TgSALUVET WB as a reference test.

3.3. Comparative study

All the ELISA tests showed high Se and Sp values when sera from experimentally infected sheep were analyzed (Panel 1) (Se and Sp values equal to or higher than 94%, AUC = 1; Fig. 3A, no doubtful results were observed by the majority of the tests). TgSALUVET WB showed 100% Se and 94% Sp. The highest diagnostic performance corresponded to IDScreen and TgSALUVET ELISA 2.0, followed by PrioCHECK. In general terms, moderate to perfect agreements were also recorded (k = 0.78–1.00), and after cutoff readjustments, Se, Sp and *kappa* values reached almost perfect values (Fig. 3A; Table 3).

Seroconversion was recorded from 21 dpi when the initial ELISA test cutoff values were considered (Fig. 4). Seroconversion was detected earlier when the mean levels of anti-*T. gondii* IgGs were compared between noninfected and infected sheep during the experimental assay, with a significant increase in IgG levels starting at 12 dpi with PrioCHECK and TgSALUVET ELISA 2.0, 14 dpi with IDScreen and Pigtype, and 21 dpi with IDEXX (Fig. 4).

All the ELISA tests also showed excellent diagnostic performance when analyzing sera from sheep naturally infected (Panel 4) (Se and Sp values equal to or higher than 95%, AUC = 1; Fig. 3B, and only 4 out of 239 samples that resulted doubtful based on the results obtained by the majority of the tests), except for PrioCHECK, which showed 83% Sp. Pigtype was the test that showed the best diagnostic performance, followed by IDScreen and TgSALUVET ELISA 2.0. TgSALUVET WB presented 94% Se and 97% Sp. The *kappa* values are shown in Table 3 (k = 0.73–0.98), with PrioCHECK presenting the lowest values (k = 0.73–0.80). Both the performance and agreement of the tests improved when their cutoff values were readjusted based on the TG-ROC analyses, with 98–100% Se and Sp and 0.84–1.00 *kappa* values (Fig. 3B, Table 3), except for TgSALUVET WB with 93% Se. The cutoff, Se, Sp and AUC values are specified for each ELISA in Fig. 3B.

All the ELISA tests showed false-positive results with the anti-*N. caninum* IgGs (Panel 3) when using the cutoff values suggested by the manufacturers, as follows: IDScreen (14/26), PrioCHECK (13/26), Pigtype (11/26), IDEXX (6/26) and TgSALUVET ELISA 2.0 (6/26) (Table 4). False-positive results remained when the readjusted cutoff values were applied (Table 4). There was a significant association between the number of false-positive results and the presence of anti-*N. caninum* IgGs when using both the initial and readjusted cutoff values (Table 4). Consequently, new cutoff readjustments were required for all the ELISA tests to avoid false-positive results, as detailed in Table 4.

When both readjusted cutoff values were applied to TgSALUVET ELISA 2.0 using the goat serum panel (Panel 5), whether it was the one meant to obtain comparable data among tests or the one targeted to avoid cross-reactivity with the anti-*N. caninum* IgGs, TgSALUVET ELISA 2.0 maintained excellent performance, with 100% Se and 94% Sp and 99% Se and 99% Sp, respectively.

Table 2
Frequency and intensity of recognition of *Toxoplasma gondii* tachyzoite immunodominant antigens (IDAs) by TgSALUVET WB.

IDAs	Noninfected sheep	*T. gondii* infected sheep		*N. caninum* infected sheep	C vs. D Significance
	(A) n = 20 Frequency/intensity	(B) n = 9 Frequency/intensity/significance	(C) n = 17 Frequency/intensity/significance	(D) n = 26 Frequency/intensity/significance	
37–40	40/ +	100/ +++/ **	100/ +++/ ****	100/ +++/ ****	ns
30	90/ ++	100/ +++/ ns	100/ +++/ ns	100/ +++/ ns	ns
24–26	0	100/ +++/ ****	100/ +++/ ****	11.5/ +/ ns	****
18–20	0	100/ +++/ ****	100/ +++/ ****	30.8/ +/ **	****
9–10	0	100/ +++/ ****	82.4/ ++/ ****	0/ ns	****

A: sera collected prior to the infection (Panel 1). B: sera collected at 27 days post-infection (Panel 1). C: sera from Panel 2. D: sera from Panel 3. Frequency: percentage (%). Intensity: +++ (high), ++ (medium), + (low), mean within each group. For statistical analyses, columns B, C, D were compared to column A or column C to column D. ns: no significant differences. Significant differences are represented as follows: * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$, and **** = $P < 0.0001$.

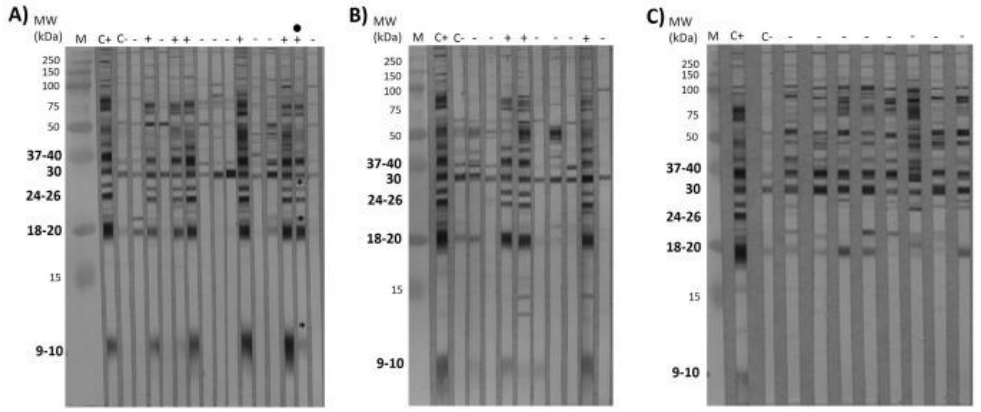

Fig. 2. Recognition of *Toxoplasma gondii* tachyzoite antigens by TgSALUVET WB. **(A)** Sera from *T. gondii* experimentally infected sheep; **(B)** Sera from *T. gondii* naturally infected sheep. *Toxoplasma gondii* was isolated by mouse bioassay from myocardial tissue from all positive samples; **(C)** Sera from *Neospora caninum* experimentally infected sheep; MW: molecular weight. IDAs are marked in bold letters. M: molecular weight marker, C+: *T. gondii* positive control serum. C-: *T. gondii* negative control serum. Strips with a "+" or "-" were classified as positive or negative for *T. gondii*, respectively. In the strip identified with "●", antigens with a "*" above show low intensity for the 10 kDa band, medium intensity for the 28 kDa band and high intensity for the 18–20 kDa band.

4. Discussion

A comprehensive comparative study was carried out in terms of the number of serological tests included and the range of reference serum panels employed. We have developed and validated two in-house serological techniques that were later included in the comparative study together with four commercially available ELISA tests. All the tests showed good diagnostic performance and agreement when using sera from both experimental and natural infections, although further readjustments improved Se and Sp values. However, all the ELISA tests showed a high number of false-positive results when *N. caninum*-positive sera were tested. Thus, additional cutoff value readjustment was suggested based on the epidemiological scenario.

The two in-house tests developed herein showed good diagnostic performance and can be indistinctly employed with ovine or caprine sera. For TgSALUVET WB, we established an exhaustive and restrictive criterion of seropositivity, and the remarkable cross-reactions found between *T. gondii* antigens and anti-*N. caninum* antibodies were considered. López-Ureña et al. (2023) recently defined a criterion of positivity for pig sera that consisted of the recognition of three out of eight IDAs (9–10, 19, 25, 28, 30, 33–35, 43–45, and 69 kDa). Despite the similarities found between both studies with commonly reported IDAs, the criterion established with pig sera was less restrictive, including additional IDAs, which could be explained by the fact that cross-reactions were not studied. However, considerations of cross-reactivity should not be discarded since most of such IDAs were also recognized prior to infection in pigs, except for the 9–10 and 69 kDa IDAs, and cross-reactions with other apicomplexan parasites relevant in this species were not investigated (e.g., *Cystoisospora suis*, *Sarcocystis* spp.). Hebbar et al. (2022) also studied cross-reactivity between anti-*N. caninum* IgGs and *T. gondii* antigens in goats by WB and reported minimal cross reactivity at 1/200 serum dilution. However, whether sensitivity could be compromised was not studied. Herein, the most problematic IDAs in terms of cross-reactivity were those corresponding to 30 and 37–40 kDa; thus, the unique recognition of these IDAs cannot be considered a criterion of seropositivity. Cross-reactivity between anti-*N. caninum* IgGs and *T. gondii* protein SAG1, which has a predictable molecular weight of 30 kDa, was previously described for other *T. gondii*-based serological tests, such as IDScreen (Sánchez-Sánchez et al., 2021b) and a SAG1-GRA8 chimeric antigen-based time-resolved fluorescence immunoassay (Huertas-López et al., 2021). The 30 kDa antigen has been described as an IDA together with others: 11–13, 18, 24, 34 and 42 kDa antigens in sheep sera (Wastling et al., 1994) and 28 and 34 kDa (Conde et al., 2001) or 12, 17, 23, 32, 55 and 75 kDa antigens in goats sera (Hebbar et al., 2022). Thus, it should be considered together with more specific IDAs in the criterion of seropositivity. In this regard, the 9–10 kDa IDA was the most specific antigen, as similarly reported by López-Ureña et al. (2023)).

TgSALUVET ELISA 2.0 also showed high initial diagnostic performance for both sheep and goats, as corroborated later in the comparative study. These results were expected, as lyophilized tachyzoites have been previously employed in ELISA tests that were highly sensitive and specific (García-Lunar et al., 2017; López-Ureña et al., 2023). This preliminary validation was carried out considering TgSALUVET WB as reference since this test mitigate the issue of false-positive reactors based on the specific criterium of seropositivity established herein. Although IFAT has been regarded as reference on several occasions, this assay may yield false-positive results and is further hampered by subjectivity in result interpretation as it is operator-dependent (Campero et al., 2018). Similarly, MAT was not deemed suitable as reference test due to its susceptibility to cross-reactivity issues with closely related Apicomplexan parasites (Mazuz et al., 2018).

We have compared a wide set of serological techniques, including in-house assays and commercial ELISA tests. This is the first time that the performance of Pigtype has been studied, whereas IDScreen, PrioCHECK and IDEXX had already been included in previous comparative studies. Herein, all tests showed good to excellent diagnostic performance and agreement regardless of the serum panel tested, with the exception of PrioCHECK, which presented a slight decrease in Sp and *K* values with respect to the other tests when analyzing serum samples from sheep naturally infected with *T. gondii*. However, a further improvement of all the ELISA tests was possible by readjusting their cutoff values, obtaining better performance and more harmonized results. Slight differences in terms of time of seroconversion were observed in the ELISA tests with sera from experimental infections, with time of seroconversion being earlier when considering a significant increase of IgG levels between

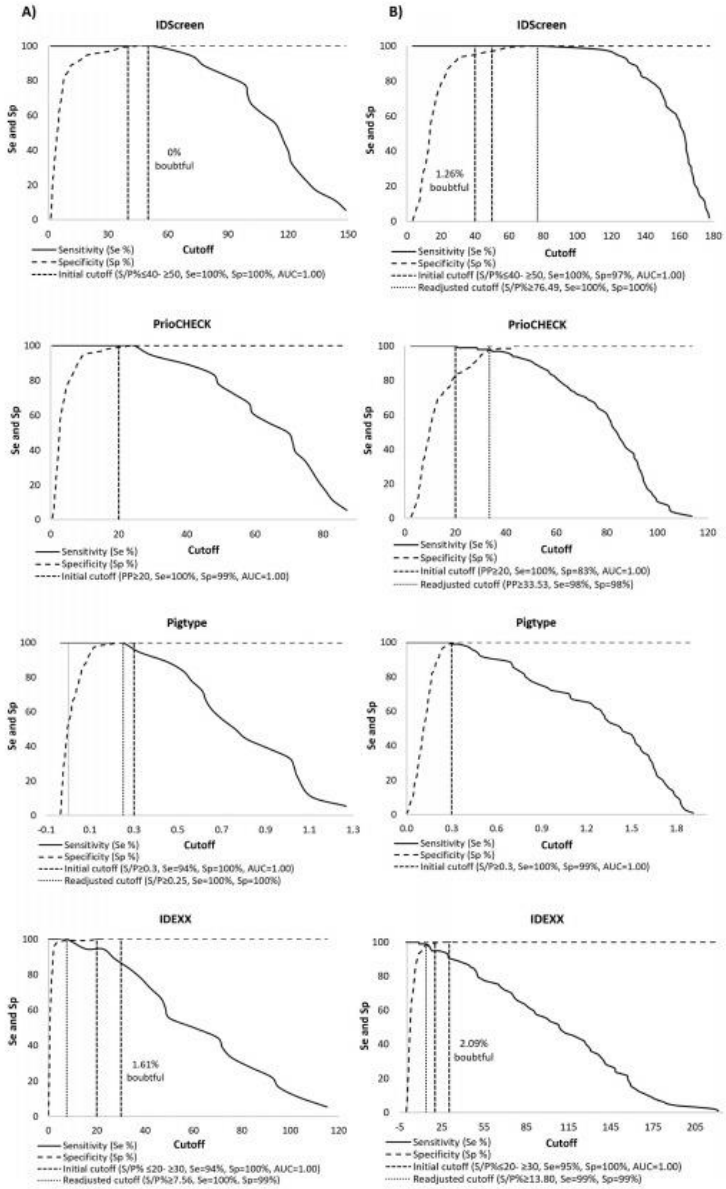

Fig. 3. TG-ROC graphs of four commercial and one in-house ELISA tests based on the reference criterion, using serum samples from sheep experimentally (Panel 1) **(A)** or naturally (Panel 4) **(B)** infected with *Toxoplasma gondii*. Cutoff, sensitivity (Se), specificity (Sp), and the area under the curve (AUC) values are shown for each ELISA test.

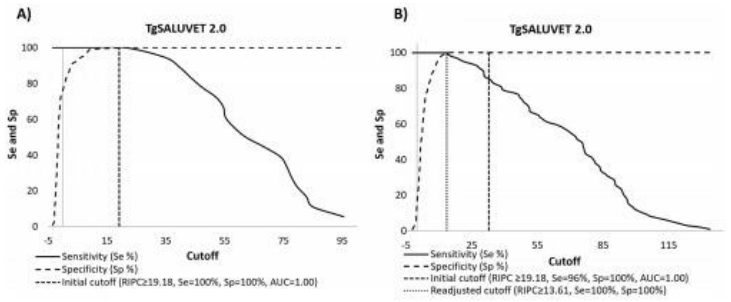

Fig. 3. (*continued*).

Table 3
Agreement between serological techniques using sera from *Toxoplasma gondii*-infected sheep before **(b)** and after **(a)** the TG-ROC analyses and cutoff value readjustment.

Experimental infections (Panel 1)	IDScreen		PrioCHECK		Pigtype		IDEXX		TgSALUVET ELISA 2.0		TgSALUVET WB	
	b	a	b	a	b	a	b	a	b	a	b	a
IDScreen	1.00	1.00	0.97	0.97	0.97	1.00	0.96	0.97	1.00	1.00	0.83	0.83
PrioCHECK	0.97	0.97	1.00	1.00	0.94	0.97	0.93	1.00	0.97	0.97	0.86	0.86
Pigtype	0.97	1.00	0.94	0.97	1.00	1.00	0.96	0.97	0.97	1.00	0.80	0.83
IDEXX	0.96	0.97	0.93	1.00	0.96	0.97	1.00	1.00	0.96	0.97	**0.78**	0.86
TgSALUVET ELISA 2.0	1.00	1.00	0.97	0.97	0.97	1.00	0.96	0.97	1.00	1.00	0.83	0.83
TgSALUVET WB	0.83	0.83	0.86	0.86	0.80	0.83	**0.78**	0.86	0.83	0.83	1.00	1.00

Natural Infections (Panel 4)	IDScreen		PrioCHECK		Pigtype		IDEXX		TgSALUVET ELISA 2.0		TgSALUVET WB	
	b	a	b	a	b	a	b	a	b	a	b	a
IDScreen	1.00	1.00	**0.79**	0.95	0.96	0.99	0.89	0.97	0.91	1.00	0.84	0.88
PrioCHECK	**0.79**	0.95	1.00	1.00	0.80	0.94	**0.74**	0.95	**0.75**	0.95	**0.73**	0.84
Pigtype	0.96	0.99	0.80	0.94	1.00	1.00	0.92	0.96	0.93	0.99	0.87	0.87
IDEXX	0.89	0.97	**0.74**	0.95	0.92	0.96	1.00	1.00	0.98	0.97	0.88	0.89
TgSALUVET ELISA 2.0	0.91	1.00	**0.75**	0.95	0.93	0.99	0.98	0.97	1.00	1.00	0.89	0.88
TgSALUVET WB	0.84	0.88	**0.73**	0.84	0.87	0.87	0.88	0.89	0.89	0.88	1.00	1.00

The lowest *kappa* values (below 0.80) based on the cutoff values suggested by the manufacturers are marked in bold letters.

noninfected and infected sheep, from 12 to 21 dpi depending on the ELISA test, compared to seropositivity based on their initial cutoff values, from 21 dpi in all the ELISA tests, as similarly observed with PrioCHECK by Glor et al. (2013). Differences can be also explained by the different experimental designs followed (e.g., dose, strain) since seroconversion can vary between 12 dpi and 21 dpi even if the same test/antigen is employed as reported by other authors when using a *T. gondii* soluble antigen-based ELISA test (Castaño et al., 2014, 2019; Sánchez-Sánchez et al., 2019a).

Regarding the results obtained with sera from naturally infected sheep, IDScreen showed higher performance vs. PrioCHECK, which showed lower Sp compared with previous studies. Mangili et al. (2009) reported 83% Se for IDScreen when compared to an IFAT. In contrast, PrioCHECK showed 93–100% Se and Sp values when compared to an in-house IFAT and a commercial indirect hemagglutination test (ELI.H.A Toxo, ELITech Group, Switzerland) (Glor et al., 2013). These differences could be explained by the different sera and tests used as references. Furthermore, IDEXX showed similar results, as reported by other researchers, with 85–91% Se and 97–98% Sp (Mainar-Jaime and Barberán, 2007) or 91–92% Se and 97–99% Sp (Opsteegh et al., 2010), with both studies based on a Bayesian approach.

Finally, the cross-reactivity observed between anti-*N. caninum* antibodies and a high number of *T. gondii* antigens by TgSALUVET WB was reflected in the results obtained with all the ELISA tests that showed an elevated proportion of false-positive results (15–65%) regardless of the cutoff value employed. Accordingly, additional cutoff values were proposed to avoid false-positive results. The study of cross-reactions between anti-*N. caninum* antibodies and *T. gondii* antigens is highly recommended to obtain accurate results since *N. caninum* is an Apicomplexan parasite closely related to *T. gondii*, both with an orthologous cluster of proteins (Lorenzi et al., 2016). Cross-reactivity between *T. gondii* and *N. caninum* was observed as early as 1994 (Bjerkas et al., 1994) and has been continuously documented in different studies (Gondim et al., 2017; Huertas-López et al., 2021; Nishikawa et al., 2002; Sánchez-Sánchez et al., 2021b). Furthermore, *N. caninum* has also been identified as an important cause of reproductive failure in small ruminants (Moreno et al., 2012; Sánchez-Sánchez et al., 2018, 2021a), and *N. caninum* and *T. gondii* coinfections in small ruminant flocks have been noted (Moreno et al., 2012; Rossi et al., 2011; Sun et al., 2020; Villagra-Blanco et al., 2019). However, cross-reactivity with other closely related parasites, such as *Hammondia* spp. and *Sarcocystis* spp., cannot be ruled out since antibodies against such parasites were not tested prior to the infection in the sera panel employed. The existence of false-positive results with IDScreen has already been reported (Sánchez-Sánchez et al., 2021b), but this troubleshooting seems to be common to all the ELISA tests evaluated. Whether the nature or type of antigen might influence the results should be clarified since the antigens used in the tests evaluated were either a recombinant SAG1 protein, whole-tachyzoite

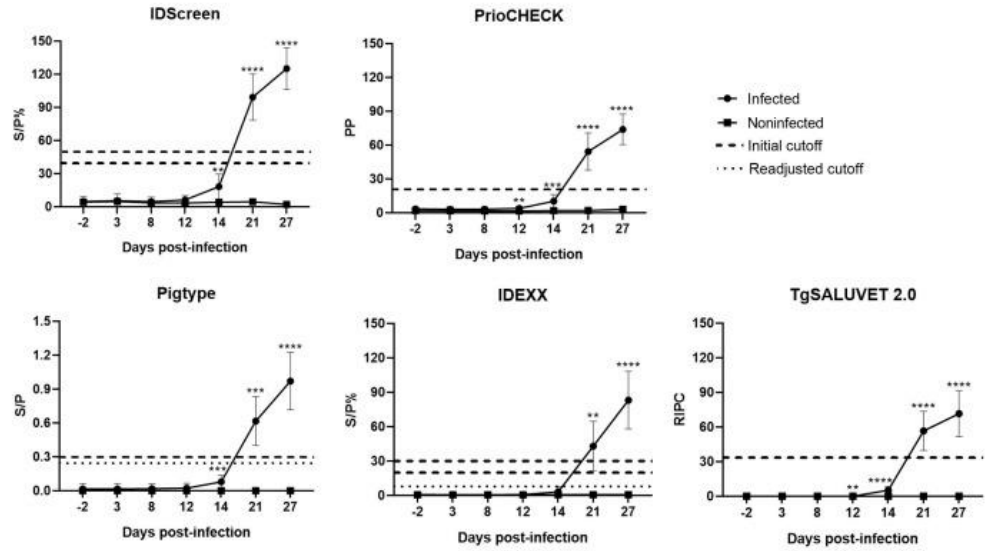

Fig. 4. Kinetics of anti-*Toxoplasma gondii* IgGs by all the ELISA tests using serum samples from sheep experimentally infected with *Toxoplasma gondii* oocysts (Panel 1). Significant differences were analyzed within sampling days between noninfected and infected groups for each ELISA test and were identified as follows (Sidak's multiple comparisons tests): * = $P < 0.05$, **: $P < 0.01$, *** = $P < 0.001$, and **** = $P < 0.0001$.

Table 4
Cross-reactivity between anti-*Neospora caninum* IgG and *Toxoplasma gondii* antigens in *Toxoplasma gondii*-based ELISA tests according to the different cutoff values employed.

ELISA tests	Positive-*N. caninum* serum samples (Panel 3, n = 26) that tested positive by *T. gondii*-based ELISA tests							
	Initial cutoff values			First readjusted cutoff values			Second readjusted cutoff values	
	Cutoff value[*]	n	*P*	Cutoff value[*]	n	*P*	Cutoff[**]	n
IDScreen (S/P%)	≤ 40, ≥ 50	14	<0.0001	≥ 76.49	4	0.1213	≥ 99.23	0
PrioCHECK (PP)	≥ 20	13	0.0001	≥ 33.53	5	0.0593	≥ 53.91	0
Pigtype (S/P)	≥ 0.3	11	0.0010	N/A	11	0.0010	≥ 0.48	0
IDEXX (S/P%)	<20, ≥ 30	6	0.0287	≥ 13.80	17	<0.0001	≥ 69.08	0
TgSALUVET 2.0 (RIPC)	19.18	6	0.0287	≥ 13.61	10	0.0024	≥ 32.21	0

N/A: do not apply. S/P%, PP and RIPC = ([sample OD- negative control OD]/[positive control OD- negative control OD]) x 100, S/P = ([sample OD- negative control OD]/[positive control OD- negative control OD]).

[*] The corresponding diagnostic performance are specified in Fig. 3.

[**] The diagnostic performance parameters were as follows: IDScreen= 99% Se, 100% Sp; PrioCHECK= 88% Se, 100% Sp; Pigtype= 94% Se, 100% Sp; IDEXX= 72% Se, 100% Sp; TgSALUVET ELISA 2.0= 86% Se, 100% Sp. The negative serum samples used as control in this analysis (Panel 1 prior to the infection, n= 20) tested negative in all ELISA tests when applying the initial or both readjusted cutoff values.

extract or lyophilized tachyzoites. It has been claimed that recombinant or chimeric antigens might be more specific (Liyanage et al., 2021). For example, Holec-Gąsior et al. (2014) reported the absence of false-positive results with an ELISA based on *T. gondii* GRA1, P22 and ROP1 recombinant proteins or on four tetravalent chimeric proteins (AMA1N-SAG2-GRA1-ROP1, AMA1C-SAG2-GRA1-ROP1, AMA1-SAG2-GRA1-ROP1, and SAG2-GRA1-ROP1-GRA2) when testing *N. caninum*-positive sheep sera. However, the characteristics of these sera were not mentioned (e.g., experimental or natural infection origin and IgG levels). These results contrast with the results presented herein since IDScreen, which initially showed the highest number of false-positive results, was the only ELISA based on a recombinant protein, P30 (SAG1).

In summary, all the serological tests compared herein are accurate enough for serological diagnosis of *T. gondii* infection in small ruminants. However, ideally, readjusted cutoff values are recommended for a higher diagnostic performance. Moreover, cross-reactions between anti-*N. caninum* antibodies and *T. gondii* antigens harm the analytical specificity more than initially thought and should be considered when defining a criterion of seropositivity by WB and when using such serological tests for diagnostic purposes. To discard false-positive reactors, a practical recommendation for diagnostic laboratories could be the use of both readjusted cutoff values estimated with sera from naturally infected sheep, and those samples with results in between (doubtful results), should be further analyzed by a specific and confirmatory WB test. The employment of well-characterized sera, including false-positive reactors, should be an essential requirement for future method developments or validation studies carried out in small ruminants.

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Declaration of Competing Interest

Authors declare no conflict of interest.

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Objective 2: To develop and validate a serological method that discriminate between *T. gondii* oocyst- *versus* tissue cyst-driven infections.

- Sub-objective 2.2: To develop and validate an enzyme-linked immunosorbent assay (ELISA) based on *T. gondii* sporozoite- or sporocyst/oocyst wall-specific proteins to differentiate between oocyst- *versus* tissue cyst-driven infections.

The outcomes from this sub-objective related to **the identification of oocyst-specific proteins and the development of a oocyst-attributing serological test** were published in the following open access article **(paper No. 4)**: López-Ureña, N.M., Calero-Bernal, R., Koudela, B., Cherchi, S., Possenti, A., Tosini, F., Klein, S., San-Juan-Casero, C., Jara-Herrera, S., Jokelainen, P., Regidor-Cerrillo, J., Ortega-Mora, L.M., Spano, F., Seeber, F., Álvarez-García, G., 2023. **Limited value of current and new *in silico* predicted oocyst-specific proteins of *Toxoplasma gondii* for source-attributing serology**. Frontiers in Parasitology, Section of Parasite Diagnostics, 2. DOI: 10.3389/fpara.2023.1292322. This is a new journal within the prestigious Frontiers editorial, thus data on impact factor and quartile is not available yet.

Abstract: The One Health approach seeks to identify postnatal toxoplasmosis resulting from the consumption of tissue cysts or sporulated oocysts through the application of serological tools. This study was the first to assess the source-attribution potential of a wide range of identified *in silico* predicted sporozoite- or oocyst/sporocyst wall-specific proteins using *T. gondii* omics data and strict validation guidelines, which included the employment of a wide well-characterized serum panels of pigs and sheep experimentally infected with oocysts and tissue cysts (38 pigs and 20 sheep sampled from 0 to 6 weeks 50 post-infection [wpi], n= 385). A total of 32 proteins, including those previously described, were screened by Western blot using selected samples prior and after the infection from both abovementioned species. Only two proteins, TgCCp5A and TgSR1, were nominated for an ELISA development for pigs since they induced seroconversion and showed to be stage specific. However, when the complete pig serum panels were tested by both techniques, both proteins resulted low antigenic and lacked stage-specificity. Thus, no useful oocyst-attributing antigen has been identified for serological tools.

These outcomes were also presented as an oral communication in 2nd Environmental Toxoplasmosis Workshop and the 16th Biennial International Congress on Toxoplasmosis in California (May 2022), as well as in the VIII VETINDOC in Spain (June 2022), winning in this last event the second-best oral communication. They were also presented as a poster communication at the 15th International Congress of Parasitology in Copenhagen (August 2022) and at the Apicowplexa meeting in Bern (October 2022).

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Limited value of current and new *in silico* predicted oocyst-specific proteins of *Toxoplasma gondii* for source-attributing serology

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Toxoplasma gondii is a zoonotic parasite infecting all warm-blooded animals, including humans. The contribution of environmental contamination by *T. gondii* oocysts to infections is understudied. The aim of the current work was to explore *T. gondii* serology as a means of attributing the source of infection using a robust stepwise approach. We identified *in silico* thirty-two promising oocyst-specific antigens from *T. gondii* ˙omics data, recombinantly expressed and purified them and validated whether serology based on these proteins could discriminate oocyst- from tissue cyst-driven experimental infections. For this, three well-characterized serum panels, sampled from 0 to 6 weeks post-infection, from pigs and sheep experimentally infected with *T. gondii* oocysts or tissue cysts, were used. Candidate proteins were initially screened by Western blot with sera from pigs or sheep, infected for different times, either with oocysts or tissue cysts, as well as non-infected animals. Only the recombinant proteins TgCCp5A and TgSR1 provoked seroconversion upon infection and appeared to discriminate between oocyst- and tissue cyst-driven infections with pig sera. They were subsequently used to develop an enzyme-linked immunosorbent assay test for pigs. Based on this assay and Western blot analyses, a lack of stage specificity and low antigenicity was observed with all pig sera. The same was true for proteins TgERP, TgSporoSAG, TgOWP1 and TgOWP8, previously described as source-attributing antigens, when analyzed using the whole panels of sera. We

conclude that there is currently no antigen that allows the discrimination of *T. gondii* infections acquired from either oocysts or tissue cysts by serological tests. This work provides robust new knowledge that can inform further research and development toward source-attributing *T. gondii* serology.

KEYWORDS

Toxoplasma gondii, oocyst-specific proteins, diagnosis, serology, antigen prediction

1 Introduction

Toxoplasma gondii (Apicomplexa) is a cosmopolitan zoonotic intracellular protist responsible for toxoplasmosis, which is considered to cause the third highest disease burden associated with food-borne infections in humans (EFSA Panel on Biological Hazards et al., 2018). Clinical toxoplasmosis usually occurs during the acute phase caused by tachyzoites (fast-replicating stage). The infection is usually mild for healthy and immunocompetent individuals, but may cause e.g. ocular disease (Gomez-Marin and de-la-Torre, 2020). However, especially immunosuppressed individuals are at risk of severe, even fatal, toxoplasmosis. When the infection occurs in pregnant women, it can lead to abortion, stillbirth, and fetal malformations. Human congenital toxoplasmosis accounts for 5.8 cases per 100,000 live births, and it is ranked among the top causes of disease burden in EU/EEA when disability-adjusted life years are considered (Cassini et al., 2018).

T. gondii has a complex life cycle, with a wide host range and various routes of transmission. Therefore, its control requires a One Health approach. Members of the Felidae family act as definitive hosts (DH), while almost all homeothermic animals may serve as intermediate hosts (IH) (Dubey, 2021a). All three stages of the parasite (oocyst, tachyzoite and bradyzoite) are infective to the hosts, but with differences in infectivity efficiency to IH and DH (reviewed by Dubey, 2021a). When DH ingest raw or undercooked tissues derived from IH and harboring bradyzoites-containing tissue cysts, the parasite can start both asexual and sexual multiplication in the small intestine, leading to the formation of oocysts. DH shed oocysts that are resistant to environmental stresses and that, upon sporulation, become infectious. Oocysts can contaminate soil, water bodies, vegetables, fruits and shellfish, constituting an environmental reservoir and a source of infection. While tachyzoites are usually associated with the vertical (congenital) route of transmission, bradyzoites and oocysts are responsible for most horizontal (postnatal) transmissions. Although the importance of both routes has been acknowledged (Pinto-Ferreira et al., 2019; Dubey, 2021b; López-Ureña et al., 2022), the relative importance of meat-borne *vs.* oocyst-driven transmission of *T. gondii* is still unknown. Available literature indicates that 30–60% of infections could be attributed to the meat route *vs.* 6–17% to the environmental one (Cook et al., 2000; Hald et al., 2016). A recent case-control study carried out in the Netherlands indicated a significant risk of acute toxoplasmosis through the consumption of raw or undercooked meat or meat products (Friesema et al., 2023). In contrast, in a highly endemic country such as Brazil, a compilation of outbreaks data indicated that the suspected meat-source accounted for 21.4%, whereas the environmental route was suspected in 45.2% of toxoplasmosis cases (Balbino et al., 2022). Furthermore, available data on global human toxoplasmosis outbreaks showed that 47.1% were associated with tissue cyst and 44.1% with oocyst ingestion (Pinto-Ferreira et al., 2019).

A major objective of the One Health approach is the prevention of *T. gondii* infections by designing multidisciplinary intervention strategies able to tackle the main transmission routes. Stage-specific serology could be highly useful to inform efficient interventions, and not only for humans but also for animals along the food chain. Several attempts have been made to discriminate *T. gondii* infections caused by oocysts *vs.* tissue cysts through serology. The antigens evaluated and serological assays used have been extensively reviewed (Álvarez García et al., 2021). However, validated methods are still missing. Several oocyst wall-specific proteins (TgOWP1-12; (Possenti et al., 2010; Salman et al., 2017)) and sporozoite-specific proteins (TgERP, TgSporoSAG, TgCCp5A; Radke et al., 2004; Hill et al., 2011; Santana et al., 2015) have been proposed as antigens that could serve as indicators for an oocyst-derived infection. However, contradictory results have been obtained when evaluating their potential diagnostic value. In humans, TgERP was identified as an early infection marker (Hill et al., 2011) and an indicator of environmental contamination with oocysts (Vieira et al., 2015; Mangiavacchi et al., 2016), whereas TgCCp5A was recognized by human sera in a toxoplasmosis outbreak (Santana et al., 2015). TgSporoSAG was later described as non-immunogenic in humans (Crawford et al., 2010). The assumed low level of immune stimulation by oocysts in the small intestine (Fabian et al., 2021) and the different experimental designs and procedures used by various authors indicate that it is difficult to identify antigens capable of differentiating infection routes. Accordingly, the search for such proteins should be pursued using a more standardized validation workflow (Álvarez García et al., 2021).

The present study was a comprehensive investigation of stage-specific serology aimed at identifying sporozoite- or oocyst/sporocyst wall-specific antigens. It was based on a genome-wide *in silico* prediction approach for proteins likely to be exposed to the immune system, followed by the evaluation of the derived recombinant proteins for their diagnostic potential in terms of source attribution. To this end, reference pig and sheep serum panels were selected and used in a rigorous validation workflow that consisted of screening of proteins-of-interest (POIs), development

of POI-based enzyme-linked immunosorbent assay tests (ELISA) and evaluation of their analytical specificity.

2 Materials and methods

2.1 Bioinformatic analyses

2.1.1 Selection of proteins-of-interest

T. gondii protein candidates were identified *in silico* for oocysts-specific diagnostic purposes based on findings obtained from large-scale protein microarray approaches to identify antigens of diagnostic value from pathogens (Liang and Felgner, 2015) and taking into account the possibilities and challenges discussed previously (Álvarez García et al., 2021). Accordingly, such proteins, besides being sporozoite- or oocyst/sporocyst wall-specific, should be either secreted or surface-exposed and of relatively high abundance. Additionally, antigenicity prediction was further considered. The starting set of 8,284 annotated proteins and the algorithms and additional sources used for filtering have been previously described by us and are available as Supplementary Table 2 in Álvarez García et al. (2021) (Figure 1). The final 90 candidates were then manually curated for consistency and further checked based on literature data. Their designation as being Coccidia- or *T. gondii*-specific was based on Ortholog Group assignments from OrthoMCL DB (https://orthomcl.org), which can be accessed for each gene from Supplementary Table 1. They were verified by BLAST searches within ToxoDB (https://toxodb.org).

2.1.2 Epitope predictions

We used the following algorithms for prediction of either linear or 3D epitopes, respectively, based on whole primary protein sequences: BepiPred 3 (Clifford et al., 2022); (https://services.healthtech.dtu.dk/services/BepiPred-3.0); EpiDope (Collatz et al., 2021) and DiscoTope 3 using the AlphaFold 2 mode (Høie et al., 2023); (https://services.healthtech.dtu.dk/services/DiscoTope-3.0). For seven proteins (TGME49_205658, TGME49_223700, TGME49_228240, TGME49_235200, TGME49_235315, TGME49_235390, TGME49_264070) no DiscoTope 3 predictions were performed due to the size restriction of <1,280 amino acids (aa) of AlphaFold 2/UniProt models and thus available template structures for these proteins.

As another criterion we used a representation of protein disorder either as a graphical representation of consecutive six or more disordered residues along the aa sequence of a given protein (described in Arranz-Solís et al., 2023), based on the consensus disorder predictions from MobiDB (Piovesan et al., 2020), or the pLDDT score provided by DiscoTope 3, a per-residue measure of AlphaFold2's model accuracy. A score of <0.5 can be regarded as a strong predictor of disorder (Jumper et al., 2021). Disordered regions of proteins can be fairly antigenic (MacRaild et al., 2016; Uversky and Van Regenmortel, 2021), also reflected e.g. in some TgGRA proteins in Supplementary Figure 1. The algorithm-specific values for each individual aa were combined into a single graph for each protein and represented the individual scores along the protein sequence. For BepiPred 3 and EpiDope, the default thresholds of 0.15 and 0.82, respectively, are indicated. No threshold exists for DiscoTope 3 (but we used 0.25 for calculation of the combined epidope prediction score (cEPS)), instead, higher values are better, but should be considered together with the quality of the AlphaFold2 model. For calculation of cEPS see Supplementary Figure 1B.

2.2 Cloning of selected protein candidates

T. gondii nucleotide sequences encoding selected full-length proteins or portions thereof were amplified by PCR using as

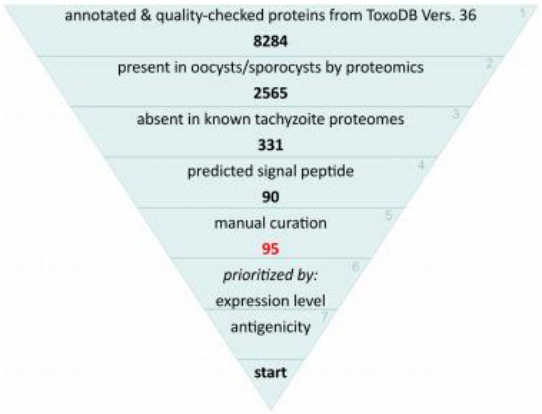

FIGURE 1
Selection and prioritizing scheme for the *in silico* prediction of *T. gondii* oocyst-derived proteins.

template either the genomic DNA of strain ME49 (type II) or a lambdaTriplex cDNA library from partially sporulated oocysts (strain VEG, type III), kindly provided by Dr. M. White (Montana State University, MT, USA). Leader peptides, as predicted by SignalP 5.0, (https://services.healthtech.dtu.dk/services/SignalP-5.0/) were always excluded from the amplified DNA regions. Amplicons were either cloned via Gibson assembly (NEB) in frame with the N-terminal six histidine tag (6His tag) encoded by the pQE80L (Qiagen) expression vector, linearized with restriction enzymes *Bam*HI and *Hin*dIII, or ligated at the appropriate restriction sites of plasmid pQE30 in frame with the N-terminal 6His tag. Gene-of-interest (GOI)-specific primers (Supplementary Table 2) consisted of a 22-28 nt sequence complementary to the open reading frame of interest preceded by the respective sequences complementary to the ends of the linearized plasmid. Alternatively, for cloning into pAviTag-C-Kan (Lucigen) as fusion with a C-terminal 6His tag, we first generated pAviTag-ccdB. It contains the counterselectable ccdB toxin amplified from pDONR221 (Invitrogen), which was inserted into pAviTag-C-Kan according to the manufacturer's protocol, using primers rki1 and rki2. For cloning of the GOIs, pAviTag-ccdB was linearized with *Sap*I, thereby releasing the ccdB insert, and the amplified GOI open reading frames were inserted via Gibson assembly using the respective primers given in Supplementary Table 2. A similar approach was followed to clone GOIs as N-terminal fusions with MBP and C-terminal 6His tag. pAviTag-MBP-SAG1 (Klein et al., 2020), cut with *Bam*HI and *Pst*I to release the SAG1 insert, was used as template for the insertion of ccdB via homologous recombination using primers rki3 and rki4. This resulted in pAvi-MBP-ccdB. For in-frame cloning of GOIs into this plasmid, we amplified it using primers rki5 and rki6 (thereby releasing ccdB) and combined the amplicon with the genes amplified with the respective GOI-specific primers (Supplementary Table 2) using Gibson assembly. The correct insertion of the amplicons in all assembled expression vectors was verified by sequencing.

2.3 Expression and purification of proteins-of-interest

Consistent with the prediction rate of several algorithms (fDETECT; Meng et al., 2017, or Protein-Sol; Hebditch et al., 2017), that indicated low solubility for more than half of the 95 selected proteins (data not shown), only 32 proteins could be sufficiently purified. The pQE80L encoded proteins were expressed in the *Escherichia coli* strain DH5alpha for 3 hrs at 37 °C upon addition of 1 mM isopropyl-β-D-1-galactopyranoside. The histidine-tagged polypeptides were purified from total bacterial lysates by nickel affinity chromatography under denaturing conditions (8 M urea, 0.1 M sodium phosphate, 0.01 M Tris, pH 8.0) and successively dialyzed against multiple changes of PBS, pH 7.2. The concentration of the recombinant proteins was determined by the Bradford method (Bio-Rad). For pAviTag-based proteins, *E. coli* T7 Shuffle (NEB) was used as an expression host. Their induction and subsequent purification were done as described previously (Klein et al., 2020). For all purified proteins, their integrity and identity were subsequently determined by SDS-PAGE and Western blot (WB) analysis using anti-6His antibodies (Qiagen). They usually reached a purity of 90-95% as judged by SDS-PAGE (data not shown).

2.4 Experimental design for proteins-of interest screening and ELISA development

We followed a recommended workflow for the screening of *T. gondii* oocyst stage-specific antigens aiming at the development of a serological tool with source-attribution usefulness (Álvarez García et al., 2021). The experimental design is detailed in Figure 2. Three panels of sera from either experimentally infected pigs (panels 1 and 2) or sheep (panel 3), previously well-characterized by a battery of conventional serological tests (Largo-de la Torre et al., 2022; López-Ureña et al., 2023a; López-Ureña et al., 2023b; Supplementary Figure 2, and Supplementary Table 3), were used. First, a selection of pig and sheep sera were used as reference for the screening of the POIs by WB: three pigs infected with oocysts and two pigs infected with tissue cysts, up to 3 weeks post-infection (wpi) (panel 1), ii) one non-infected pig and two pigs infected with oocysts, up to 42 days post-infection (dpi) (panel 2) and iii) one non-infected sheep and two sheep infected with oocysts, up to 21 dpi (panel 3). For both pig and sheep reference sera, POIs were considered as putative candidates for source-attribution based on two main criteria: i) the detection of seroconversion (reactivity exclusively after the infection) and ii) reactivity to a protein band compatible with the molecular weight predicted for the recombinant polypeptide. Further criteria for antigen selection using pig sera were the ability to differentiate between oocyst- *vs.* tissue cysts-driven infection and the concordance between the two pig serum panels. Selected POIs were used to develop and optimize an ELISA test. Then, the whole set of samples from serum panels were analyzed by POI-based WBs and the newly developed POI-based ELISAs, and POI-based WBs were regarded as reference for a TG-ROC analyses and further POI-based ELISAs standardization. After analyzing the pattern of recognition by both POI-based techniques using all serum samples from selected panels, a POI was regarded as environmental source-attributing if it continued to be recognized only by animals infected with oocysts after the infection (up to 6 wpi for pig sera from panel 1, 42 dpi for pig sera from panel 2 and 21 dpi for sheep sera from panel 3). The kinetics of anti-POIs IgGs was later studied. In parallel, cross-reactivity of anti-*Neospora caninum* antibodies (IgGs) was tested by the POI-based WBs (Figure 2).

2.4.1 Serum panels

The experimental infections were performed for unrelated previous studies under the corresponding regulations (panel 1: approved by the Ministry of Education, Youth and Sports from Czech Republic, PP 55/2016; panel 2: approved by the Animal Welfare Committee of the Community of Madrid from Spain, PROEX 293.7/20, PROEX 290.4/20 and PROEX 062/19; panel 3: approved by the Animal Ethic Committee from the Spanish National Research Council (CSIC), 1063/2021). In the absence of a gold standard for the detection of anti-*T. gondii* IgGs in pigs and

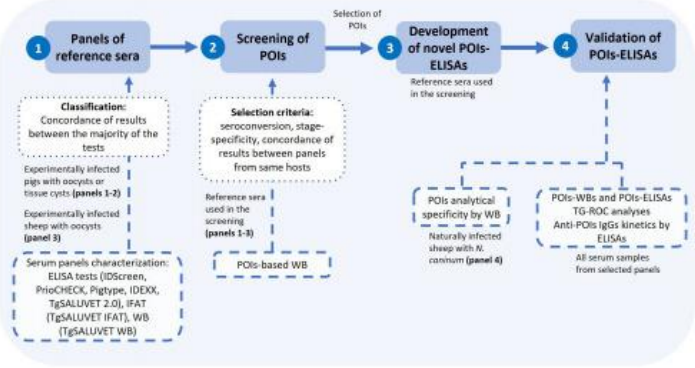

FIGURE 2
Workflow followed in the screening of proteins-of-interest from *T. gondii* oocysts and the enzyme-linked immunosorbent assay (ELISA) development. IDScreen: ID Screen® Toxoplasmosis Indirect Multi species; PrioCHECK: PrioCHECK® Porcine Toxoplasma Ab Kit or PrioCHECK® Toxoplasma Ab SR; Pigtype: Pigtype® Toxoplasma Ab from Indical Bioscience/Qiagen; IDEXX: IDEXX Toxotest Ab; IFAT: immunofluorescence antibody test; WB: Western blot test; POIs: proteins-of-interest.

sheep, multiple commercial and in-house conventional serological tests were used for the characterization of the different available panels of sera (Largo-de la Torre et al., 2022; López-Ureña et al., 2023a; López-Ureña et al., 2023b; Supplementary Figure 2, and Supplementary Table 3). Commercial and in-house serological tests were performed as specified by the manufacturers and López-Ureña et al. (2023a; 2023b), respectively. Different host- and parasite-dependent factors were considered as they can greatly influence the immune response elicited by parasite antigens: two parasite infective stages (sporulated oocysts and tissue cysts), different inoculation doses, parasite strains, animal breeds and ages. Samples were classified as positive or negative based on the majority criteria (result obtained by most of the tests), seroconversion was recorded at 2-3 wpi and higher antibody levels corresponded to groups inoculated with type III isolates (Largo-de la Torre et al., 2022; López-Ureña et al., 2023a; López-Ureña et al., 2023b; Vallejo et al., 2023).

Panel 1 consisted of orally inoculated prepubertal sows with 400 *T. gondii* oocysts (n= 6) or 10 tissue cysts (n= 7) from type II isolate (CZ-Tiger strain, ToxoDB RFLP genotype #3), and 400 *T. gondii* oocysts (n= 6) or 10 tissue cysts (n= 6) from type III isolate (Šimková strain, ToxoDB#2, isolated from feces of a domestic cat (*Felis catus*), Dámek (2023)), respectively. Blood samples were collected weekly (from 0 to 6 weeks), with a few exceptions when the samples were not collected in all sampling weeks (n= 158). This panel was previously characterized by López-Ureña et al. (2023a), and based on the results from most of the tests from that study, seroconversion was recorded from 2 wpi (positive: n= 97, negative: n= 48, doubtful; n= 13). In addition, all serum samples were analyzed by a WB based on soluble antigen from sporulated oocysts, and seroconversion was also recorded from 2 wpi in all experimental groups (unpublished data). This panel was used for the screening of POIs and the development of the novel POI-based ELISAs. For the screening of POIs and the development of the POI-based ELISAs, sera from three and two pigs infected with oocysts (type II and III) or tissue cysts (type III), respectively, from 0, 1, 2 and 3 wpi, were selected.

Panel 2 was composed of non-infected (n= 3) and orally inoculated three-months-old female pigs with 1,000 oocysts from the isolates TgShSp1 (type II, ToxoDB#3, n= 5) and TgShSp24 (type III, ToxoDB#2, n= 5), bled at -4, 0, 4, 7, 14, 21, 28 and 42 dpi (n= 103) (Largo-de la Torre et al., 2022). This panel of sera was characterized within this study (Supplementary Figure 2) and pigs seroconverted from 14 dpi by most of the serological tests (positive: n= 40, negative: n= 63, and no doubtful results). This panel was used for the screening of POIs, the development of POI-based ELISAs and further analyses. For the screening of POIs and the development of the POI-based ELISAs, sera from one non-infected and two infected pigs (with both isolates), from all sampling days, were used.

Panel 3 consisted of sera from non-infected (n= 6) and experimentally infected pregnant sheep (n= 14) orally inoculated with 10 *T. gondii* oocysts from the isolate TgShSp1 (type II, ToxoDB #3, n= 124) bled at -2, 3, 8, 12, and 14 dpi, with 6 of these sheep also sampled at 21 and 27 dpi (Vallejo et al., 2023). This panel of sera was characterized by López-Ureña et al. (2023b) and seroconversion was recorded from 21 dpi based on most of the tests (positive: n= 18, negative: n= 106, and no doubtful results). It was used for the screening of POIs, the development of POI-based ELISAs and further analysis. Sera from one non-infected and two infected sheep from -2, 8, 14 and 21 dpi were used for the screening of the POIs and the development of POI-based ELISAs.

Panel 4 consisted of twenty-three serum samples from sheep naturally infected with *N. caninum* from semi-intensive flocks. All these samples tested positive by a *N. caninum* tachyzoite-based WB under reducing conditions and a *N. caninum* tachyzoite soluble

antigen-based ELISA, but were found negative by a *T. gondii* lyophilized tachyzoite-based ELISA and a *T. gondii* tachyzoite based-WB under reducing conditions (Sánchez-Sánchez et al., 2021). This panel of sera was used to test for cross-reactivity of selected POIs with anti-*N. caninum* IgGs by POIs based-WBs. The sera employed were grouped based on the level of anti-*N. caninum* IgGs as follows: low (RIPC= 37-60, n= 5), medium (RIPC= 61-89, n= 10), and high (RIPC≥ 90, n= 8).

2.4.2 Protein-of-interest-based Western blot tests

All POIs were quantified using the Bradford method (Bio-Rad), following manufacturer's instructions. Appropriate amounts (5-15 µg) of each POI were resuspended in loading buffer (10% of glycerol, 50 mM of TRIS pH 6.8, 2% of SDS, 0.05% of bromophenol blue and 100 mM of DDT), and incubated at 100°C for 5 min. Following resolution by SDS-PAGE on one-comb 15% polyacrylamide gels along with a pre-stained protein standard (Precision Plus Protein™ Kaleidoscope™, Bio-Rad), the POIs were transferred to a 0.2 µm nitrocellulose membrane and visualized by staining with 0.1% Ponceau Red (Sigma). Then, the membranes were blocked for 2 h at room temperature (RT) with 5% of skimmed milk in 0.05% TBS-Tween 20 (TBS-T), washed three times, each for 5 min, and kept at -20°C until use. For the screening of serum panels, samples were diluted 1/20 in blocking solution and incubated for 1.5 h at RT (1-2 mm membrane strips were used). After three washes with TBS-T, each for 5 min, peroxidase-conjugated secondary reagents were added (pigs: peroxidase-conjugated protein G at 1/600 (Sigma); sheep: peroxidase-conjugated monoclonal anti-goat/sheep IgG at 1/1,000 (Sigma)) and incubated for 1.5 h. Then, the strips were washed as described above, and finally with TBS. Bound primary antibodies were visualized colorimetrically using a 4-chloro-1-naphthol solution (Thermo Scientific). The reaction was stopped after 30 min of incubation at RT by adding ultra-pure water. The nitrocellulose strips were scanned using a GS-800 Calibrated Densitometer (Bio-Rad) for further analysis. In the case of TgOWP8, one important limitation was the recognition of multiple bands, which hampered the interpretation of the results. Thus, we only considered the recognition of a 70 kDa band corresponding to TgOWP8 predicted molecular weight.

2.4.3 Protein-of-interest-based ELISAs

After testing several experimental variables, the ELISA conditions yielding the better discrimination between reference serum samples before and after infection were selected. Briefly, 100 µL/well of up to 4 µg/mL of individual POIs (TgCCp5A and TgSR1) diluted in cold PBS were used to coat 96 wells plates (MaxiSorp™, Thermo Scientific) overnight at 4°C. Then, the plates were washed three times with 0.05% PBS-Tween 20 (PBS-T) and blocked with 300 µL/well of 5% w/v powdered skim milk in PBS-T for 2 h at RT. The plates were subsequently washed and 100 µL/well of 1/100 diluted serum samples in blocking solutions were incubated for 1 h at 37 °C. Following three washes, 100 µL/well of 1/3,000 diluted peroxidase-conjugated Protein G in PBS-T were added. The plates were incubated and washed as described above and then 100 µL/well of TMB Ultra were added (Thermo Scientific). The reaction was stopped after 10 min of RT incubation by adding 100 µL of 2 N sulfuric acid, and optical densities were immediately read at 450 nm using a microplate reader (Multiscan RC 6.0, Labsystems). Positive and negative controls were selected based on the results from the screening of WBs from each selected POI. Results were interpreted as relative index percent (RIPC): ([sample OD-negative control OD]/[positive control OD-negative control OD]) x 100.

The average coefficient values (CV) of the intra- and inter-plate repeatability of the CCp5A-ELISA were below 5 (standard deviation (SD)= 0.01). TgCCp5A-ELISA showed an area under the curve (AUC) of 0.82 with 80% sensitivity (Se) and 74% specificity (Sp) for the selected cut-off, RIPC≥ 23.69, using as reference TgCCp5A-WB (TgCCp5A-WB results, positive: n= 55, negative: n= 206). For the TgSR1-ELISA, the mean CV values of intra- and inter-plate repeatability were also below 5 (SD= 0.001). TgSR1-ELISA showed an area under the curve (AUC) of 0.97 with 100% Se and 85% Sp for the cut-off selected, RIPC≥ 24.78, using as reference TgSR1-WB (TgSR1-WB results, positive: n= 17, negative: n= 243).

2.5 Data analysis

For the precision (intra- and inter-plate variability) of POI-based ELISAs, positive and negative controls were tested in triplicate, with three different plates for each selected POI. The coefficient of variation (CV) was determined as follows: mean ([standard deviation of the three replicate ODs/mean of the three replicate ODs] x 100), or mean ([standard deviation of the OD mean of each sample from each plate/mean of the OD mean of each sample from each plate] x 100). The POI-ELISAs cut-off values were defined with a non-parametric two-graph receiver operating characteristic (TG-ROC) analysis, performed with SigmaPlot 12.0, using as reference test the POI WB.

Anti-POIs IgG kinetics were studied with serum panels from experimental infections based on the defined cut-off values, as well as based on statistically significant differences. For the statistical analysis, all animals that tested positive for the POIs prior to infection, based on the established cut-off values for each ELISA, were discarded. A two-way ANOVA or a mixed-effects analysis with multiple comparisons and repeated measures was performed with two different approaches: i) comparing the mean RIPC of each sampling day/week after the infection with respect to the RIPC prior to infection within each experimental group to confirm seroconversion; ii) comparing the mean RIPC between experimental groups within each sampling day to confirm if there were differences based on the stage and isolate of *T. gondii*. When applied, the analysis was followed by a Tukey test. These analyses were done using GraphPad Prism, version 8.0.1, and differences were considered statistically significant when *P* values were lower than 0.05. The same approach was followed for the characterization of sera from panel 2, which included the kinetics of anti-*T. gondii* IgGs by different conventional ELISA tests (Supplementary Figure 2).

3 Results

3.1 *In silico* selection of antigen candidates

We filtered our previously curated set of 8,284 annotated proteins (Álvarez García et al., 2021) taken from ToxoDB (www.toxodb.org) (Harb and Roos, 2020) for oocyst-specific proteins which were absent in available tachyzoite and bradyzoite proteome datasets and which should be either secreted or surface-exposed and of relatively high abundance (Figure 1). This resulted in 331 proteins, of which 90 had a predicted signal peptide (determined with SignalP 5.0). After manual curation, five more sequences were added, resulting in 95 candidate proteins (Supplementary Table 1). They contained three antigens that have previously been reported to distinguish oocyst- from tissue cyst-derived infection, e.g. TgERP (TGME49_276850) (Hill et al., 2011); TgCCp5A (TGME49_258400) (Santana et al., 2015) and TgOWP8 (TGME49_271590) (Liu et al., 2019). In a final step we prioritized their cloning and expression according to their putative abundance in oocysts, using respective data from proteomic and transcriptomic studies when available (Fritz et al., 2012a; Fritz et al., 2012b). Finally, we evaluated the presence of predicted linear B cell epitopes using two algorithms, BepiPred2 (Jespersen et al., 2017) and EpiDope (Collatz et al., 2021). However, this was not followed further (see Discussion section) and did not influence our prioritization.

Using as templates *T. gondii* genomic DNA or a cDNA library from partially sporulated oocysts, we attempted PCR amplification of the full-length coding sequences, or parts thereof, of 83 out of the 95 selected genes. Of these 83 candidates, 50 (60%) did not reach the serological screening phase, either because of PCR failure or due to no/very low bacterial expression level. The purification of bacterially expressed proteins in sufficient amounts for serological analysis was successfully achieved for 32 antigens (see section 3.2. and Table 1).

3.2 Selection of two putative oocyst-specific serological markers by applying our criteria

A total of 32 POIs were initially screened by WB with the subset of experimentally infected animals. Most of the POIs were discarded for the following reasons: i) they were not recognized at any sampling day by WB, ii) they were recognized prior to infection, iii) they did not discriminate between oocysts- and tissue cysts-driven infections, and/or iv) they showed discrepant reactivity with pig sera from panels 1 and 2 (see section 2.4.1 for panel description) (Table 1). Noteworthy, applying these criteria we excluded four proteins from subsequent experimental steps, which had been previously reported as antigens with source-attributing potential, i.e., TgERP (LEA850) (Hill et al., 2011; Vieira et al., 2015; Burrells et al., 2016; Mangiavacchi et al., 2016), TgSporoSAG (Crawford et al., 2010; Döşkaya et al., 2014), TgOWP1 (Santana et al., 2015) and TgOWP8 (Liu et al., 2019). The recombinant proteins TgCCp5A and TgSR1 reacted stage-specifically with pig sera from both panels (2/3 pigs from panel 1 and 1/2 pigs from panel 2) only after infection, indicating seroconversion of the animals (Table 1). In contrast, none of the POIs passed the initial screening when using sera from sheep (panel 3) (Table 1). Based on these results, TgCCp5A and TgSR1 were selected for the development of a source-attributing ELISA for pigs.

3.3 TgCCp5A and TgSR1 lack source-attribution properties

To quantify serological responses, we established a specific TgCCp5A-ELISA (see section 2.4.3). The analysis of all serum samples from both pig panels (n= 261) by TgCCp5A-ELISA and TgCCp5A-WB revealed a lack of stage specificity and low antigenicity of this POI, reflected by 28% (7/25) of pigs from panel 1 and 10% (1/10) from panel 2 giving positive results prior to infection. Thus, these samples were excluded from the seroconversion analysis. Seroconversion was recorded in 50% (5/10) and 67% (4/6) of pigs infected with oocysts and tissue cysts from panel 1, respectively, and in 44% (4/9) of infected pigs from panel 2. In addition, a high variability within experimental groups was observed: i) a few animals seroconverted to TgCCp5A; ii) the RIPC values remarkably varied within the groups (Figure 3). A similar scenario was observed with TgCCp5A-WB (see Figure 3 description).

Notably, compared to prior infection, none of the experimental groups from panels 1 and 2 showed a significant increase of anti-TgCCp5A IgGs by ELISA throughout the sampling period, except for panel 1 at 4 wpi in pigs infected with type II tissue cysts. Considering differences between experimental groups within a sampling week, there was a significant higher level of anti-TgCCp5A IgGs in pigs infected with type II tissue cysts with respect to pigs infected with oocysts from the same isolate at 0, 3 and 4 wpi ($P< 0.05$) (Figure 3).

We also developed a TgSR1-ELISA (see section 2.4.3). When its cut-off was applied, 36% (9/25) of infected pigs from panel 1 and 10% (1/10) of infected pigs from panel 2 tested positive prior to infection. When these animals were excluded, 14% (1/7) of pigs from panel 1 infected with oocysts and 57% (4/7) of those infected with tissue cysts seroconverted, in contrast to 11% (1/9) of infected pigs from panel 2. A high variability among experimental groups was also observed, and a similar scenario was recorded with TgSR1-WB (see Figure 3 description).

A non-significant increase of anti-TgSR1 IgGs was observed by SR1-ELISA in the different experimental groups after the infection (Figure 3).

3.4 Failure of previously described proteins with source-attribution properties to meet our criteria

In our study, TgERP consistently showed low immunogenicity and lacked stage-specific recognition. Only three pigs from panel 1 tested positive by TgERP-WB at 3 wpi, all of them had been infected with tissue cysts. Although two pigs from panel 2 reacted with TgERP at 4 dpi and 7 dpi, another five oocyst-infected pigs from the

TABLE 1 Serological screening of proteins-of-interest from *T. gondii* oocysts using serum samples from pigs and sheep experimentally infected with *T. gondii* oocysts and tissue cysts.

Gene ID	ToxoDB annotation	Localization	Screening by antigen-based WB in pigs and sheep The number of reactive animals is indicated in parenthesis for each group (*see legend for color interpretation*†)								# of amino acids of full length protein	position of the 6-histidine tag	Reference
			Pigs (Panel 1)				Pigs (Panel 2)		Sheep (Panel 3)				
			Oocysts (n= 3)		Tissue cysts (n= 2)		Oocysts (n= 2)		Oocysts (n= 2)				
			No recognition before Inf.?	Recognition after Inf.?	No recognition before Inf.?	Recognition after Inf.?	No recognition before Inf.?	Recognition after Inf.?	No recognition before Inf.?	Recognition after Inf.?			
TGME49_204420	TgOWP1	Oocyst wall	†	(1)		†					499 (25-252)	N	Possenti et al., 2010
TGME49_209610	TgOWP2	Oocyst wall					(1)	(2)		(1)**	462 (26-291)	N	Possenti et al., 2010
TGME49_271590	TgOWP8	Oocyst wall		(1)	(2)						229 (24-229)	N	Salman et al., 2017
TGME49_223700	F5/8 type C domain-containing protein	Sporozoite cytoplasm	(2)	(2)		(2)	(1)	(1)		(2)**	1571 (21-335)	N	Spano F., unpublished
TGME49_256840	PA14 domain-containing protein	Sporozoite cytoplasm			(1)	(1)					998 (501-998)	N	Spano F., unpublished
TGME49_258400	TgCCp5A	Sporozoite cytoplasm		(2)				(1)	(1)	(1)	996 (555-996)	N	Spano F., unpublished
TGME49_258850	TgSAG-related protein TgSRS28	Sporozoite surface	(1)	(1)		(2)		(2)	(1)	(1)	291 (25-291)	N	Radke et al., 2004
TGME49_259670	von Willebrand factor type A domain-containing protein	NA									1080 (45-424)	N	NA
TGME49_267410	Scavenger receptor protein TgSR1 precursor (TgSR1 C-term)	Sporozoite cytoplasm		(2)				(1)		(1)**	1245 (761-1245)	N	Spano F., unpublished
TGME49_268310	TgOWP3	Oocyst wall				(1)	(1)	(1)		(1)**	640 (71-477)	N	Possenti et al., 2010
TGME49_276830	Late embryogenesis abundant protein (TgERP)	NA						(1)			104 (1-104)	C	Hill et al., 2011
TGME49_276860	Late embryogenesis abundant protein	NA	(2)	(2)	(1)	(2)			(2)	(2)	517 (27-517)	C	Fritz et al., 2012a
TGME49_276870	Late embryogenesis abundant protein	NA									171 (1-171)	C	Fritz et al., 2012a

(*Continued*)

TABLE 1 Continued

Gene ID	ToxoDB annotation	Localization	Screening by antigen-based WB in pigs and sheep The number of reactive animals is indicated in parenthesis for each group (see legend for color interpretation!)								# of amino acids of full length protein	position of the 6-histidine tag	Reference
			Pigs (Panel 1)				Pigs (Panel 2)		Sheep (Panel 3)				
			Oocysts (n= 3)		Tissue cysts (n= 2)		Oocysts (n= 2)		Oocysts (n= 2)				
			No recognition before Inf.?	Recognition after Inf.?	No recognition before Inf.?	Recognition after Inf.?	No recognition before Inf.?	Recognition after Inf.?	No recognition before Inf.?	Recognition after Inf.?			
TGME49_276880	Late embryogenesis abundant protein	NA									130 (1-130)	C	Fritz et al., 2012a
TGME49_315730	Apical membrane antigen 3 (TgAMA3)	Sporozoite micronemes				(2)					651 (393-566)	N	Fritz et al., 2012b
TGME49_205090	Toxoplasma family D protein (TgOWP11)	Oocyst wall*									575 (20-575)	N	Salman et al., 2017
TGME49_232940	Hypothetical protein	Oocyst wall*	(1)	(2)	(1)	(1)	(1)	(1)			489 (48-489)	N	NA
TGME49_236975	Toxoplasma family D protein	Oocyst wall*	(2)	(3)	(1)	(1)		(2)	(1)	(1)	582 (21-582)	N	NA
TGME49_272240	Toxoplasma family D protein (OWP12)	Oocyst wall*									634 (28-634)	N	Salman et al., 2017
TGME49_316560	Toxoplasma family D protein	Oocyst wall*									569 (22-569)	N	NA
TGME49_316670	Toxoplasma family D protein	Oocyst wall*	(1)	(1)	(1)	(1)					644 (26-644)	N	NA
TGME49_209920	PAN-domain containing protein	NA							(1)	(2)	991 (17-991)	N	NA
TGME49_296640	Peptidase family M13 protein	NA					(1)	(2)			1038 (32-1038)	N	NA
TGME49_262470	C protein immunoglobulin-A-binding beta antigen	NA							(2)	(2)	303 (24-303)	C	NA
TGME49_204520	Hypothetical protein	NA									369 (20-369)	N	NA
TGME49_270950	Hypothetical protein	NA						(1)			357 (22-357)	C	NA
TGME49_292350	Hypothetical protein	NA			(1)	(2)		(1)	(2)	(2)	169 (21-169)	C	NA

(Continued)

TABLE 1 Continued

Gene ID	ToxoDB annotation	Localization	Screening by antigen-based WB in pigs and sheep The number of reactive animals is indicated in parenthesis for each group (see *legend for color interpretation*†)								# of amino acids of full length protein	position of the 6-histidine tag	Reference
			Pigs (Panel 1)				Pigs (Panel 2)		Sheep (Panel 3)				
			Oocysts (n= 3)		Tissue cysts (n= 2)		Oocysts (n= 2)		Oocysts (n= 2)				
			No recognition before Inf.?	Recognition after Inf.?	No recognition before Inf.?	Recognition after Inf.?	No recognition before Inf.?	Recognition after Inf.?	No recognition before Inf.?	Recognition after Inf.?			
TGME49_287250	Hypothetical protein	NA									222 (25-222)	N	NA
TGME49_254780	Hypothetical protein	NA									112 (23-112)	N	NA
TGME49_244260	Hypothetical protein	NA									167 (18-167)	N	NA
TGME49_273705	Hypothetical protein	NA									424 (27-424)	N	NA
TGME49_316890	Hypothetical protein	NA									407 (24-407)	N	NA

†Cells in green indicate affirmative answer to the column question and white cells indicate a negative answer, thus two consecutive cells in oocysts-infected groups is an expected result for an oocyst-specific marker as long as there is a white cell in the "recognition after the infection" column for the tissue cyst-infected group. *Putative localization; ** Serum samples from the non-infected sheep (negative control) also showed reactivity to these antigens, reason why they were not selected; NA: not available; WB: Western blot test.

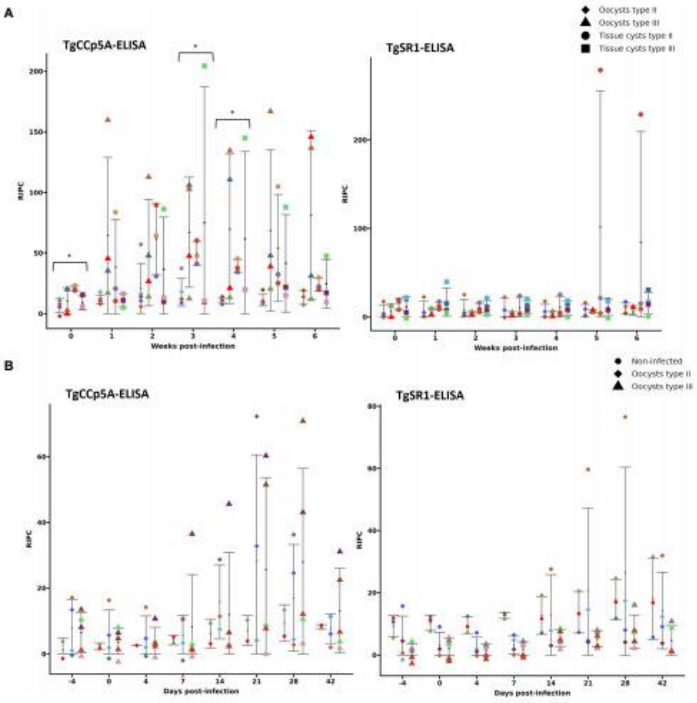

FIGURE 3
Kinetics of anti-TgCCp5A and anti-TgSR1 IgGs in serum panels 1 **(A)** and 2 **(B)** based on the novel developed enzyme-linked immunosorbent assays (ELISAs). Data presented as the mean of the relative index percentage (RIPC) by experimental group ± the standard deviation (SD). Each color represents one pig through the experiment within each graph. A pig color could vary between graphs, but not within graphs. Significant differences between groups infected with oocysts *vs.* tissue cysts within sampling weeks/days from each ELISA are identified as follow: *= $P< 0.05$. When these panels were analyzed by TgCCp5A-WB, 36.5% (4/11) and 58.3% (7/12) of animals infected with oocysts and tissue cysts from panel 1 showed positive reactivity, respectively, *vs.* 28.6% (2/7) of pigs infected with oocysts from panel 2 (pigs with TgCCp5A-WB-positive reactivity prior to infection were excluded). Based on TgSR1-WB, 18.2% (2/11) and 25% (3/12) of pigs infected with oocysts and tissue cysts from panel 1 tested positive, respectively, *vs.* 10% (1/10) of infected pigs from panel 2 (TgSR1-WB-positive animals prior to infection were also excluded).

same panel reacted with TgERP prior to infection, as well as two non-infected pigs included in the negative control group.

Despite its higher antigenicity, TgSporoSAG was not considered suitable as a source-attributing indicator. In panel 1, 77.8% (7/9) and 72.7% (8/11) of pigs infected with oocysts or tissue cysts tested positive, respectively, from 1 wpi to 6 wpi. In panel 2, 100% (10/10) of infected pigs seroconverted from 4 dpi to 42 dpi. However, also one non-infected pig serum reacted with TgSporoSAG, starting at 21 dpi until the end of the experiment.

On the other hand, only 18.2% (2/11) and 16.7% (2/12) of pigs infected with oocysts or tissue cysts from panel 1 developed antibodies against TgOWP1 by WB, respectively, from 2 to 6 wpi, and 10% (1/10) of infected pigs from panel 2 seroconverted at 14 dpi.

TgOWP8 was recognized by 14% (1/7) of pigs from panel 1 infected with tissue cysts, while none of the pigs infected with oocysts tested positive. In addition, some pigs showed reactivity prior to infection. In panel 2, 37.5% (3/8) of infected pigs recognized TgOWP8, starting 4 dpi, and two infected and three non-infected pigs recognized TgOWP8 prior to infection.

4 Discussion

The difficulty to discriminate between infections from the meat-borne route *vs.* the environmental route is one of the most relevant gaps for the prevention and control of *T. gondii* infections from a One Health perspective. Stage-specific serology could be a game-changer to fill this knowledge gap. It could be applied to humans as well as animals along the food-chain, to inform interventions targeting the relevant transmission routes. Indeed, a few sporozoite- or oocyst/sporocyst wall-specific proteins have been

suggested as putative diagnostic markers for this purpose (reviewed by Álvarez García et al., 2021). This study represents the first attempt, using strict guidelines for the validation of diagnostic tests (Newberry and Colling, 2021), to evaluate the source-attribution potential of previously described and novel predicted sporozoite- or oocyst/sporocyst wall-specific proteins.

At the beginning of this study we used the two most recent algorithms at the time for linear B-cell epitope prediction, BepiPred2 (Jespersen et al., 2017) and EpiDope (Collatz et al., 2021), for assessment of the antigenicity of our candidates. However, we found contradicting predictions by both methods for many proteins (data not shown). This prompted us to leave this approach aside for our selection scheme. However, the description of AlphaFold2 in late 2021 (Jumper et al., 2021) has changed the situation substantially, making the prediction of conformational epitopes feasible also for those putative antigens where only the primary amino acid sequence is known. Consequently, this resulted in the recent description of DiscoTope 3 (Høie et al., 2023), an algorithm that aims to identify conformational B-cell epitopes from AlphaFold2-predicted 3D protein structures. Therefore, we were interested to see retrospectively if our prioritization scheme would have been influenced by this and two other recently described methods. One is the 'Antigenic Protein and Peptide Ranker' (APRANK), aimed at prioritizing putative antigens of several pathogens, including *T. gondii*, based on *in silico* analyses (Ricci et al., 2021). The other is the recently improved BepiPred algorithm (V3) (Clifford et al., 2022). As explained in detail in Supplementary Figures 1A–E, our dataset of 95 candidates (Supplementary Table 1) showed that there is only little overlap (6 out of 95; Supplementary Figure 1D) where all three algorithms allowed the calculation of a epitope prediction score (cEPS), which allows the comparison of the algorithms (see Materials and Methods section). No significant correlations between the different scores could be observed (discussed in Supplementary Figure 1; and data not shown). In conclusion, we probably would have made the same (possibly false) decisions in case we had included Discotope 3, Bepipred 3 or EpiDope for our prioritization. Our results and those from other studies (Galanis et al., 2021; Batisti Biffignandi et al., 2023; Cia et al., 2023) indicate that despite recent improvements in the algorithms, making antigenicity predictions the basis for *a priori* selection of candidates for serological assays is of limited use. Their utility lies more in the verification of epitopes of known antigenic proteins.

There have been previous studies that reported on *T. gondii* antigen discovery using either protein (Liang et al., 2011; Felgner et al., 2015; Döşkaya et al., 2018) or peptide microarrays (Maksimov et al., 2012; Arranz-Solís et al., 2019; Arranz-Solís et al., 2021), but without a special emphasis on stage-specificity. None of the reactive polypeptides (exon products) of the protein arrays described by Döşkaya et al. (2018), which could still be retrieved from current ToxoDB (196 out of the previously reported 240 IDs) and that were probed with sera from mice either infected with tissue cysts or oocysts, met our selection criteria. Comparing the APRANK scores of these 196 proteins with those of our 95 candidates, also shows that their criteria (based on GO terms "outer membrane; heat-shock protein; chaperone; transport protein; integral membrane protein; transmembrane protein; lipoprotein or virulence associated protein" (Döşkaya et al., 2018)) resulted in inferior scores (Supplementary Figure 1E).

For specific diagnosis of oocyst-derived infections, herein a set of complementary *in silico* tools focused on the identification of predicted sporozoite- or oocyst/sporocyst wall-specific antigens. It resulted in a total of 32 candidates (POIs) that could be expressed and purified in sufficient amounts for further testing. We did not filter the candidates for being specific for *T. gondii* or other Coccidia (n= 48) (Supplementary Table 1), but rather chose to show their specificity during later steps of the experimental analysis once promising antigens would have been identified. Among those 32 proteins, five proteins tested in previous studies were included: TgERP, TgCCp5A, TgOWP1, TgSporoSAG and TgOWP8 (Crawford et al., 2010; Hill et al., 2011; Santana et al., 2015; Vieira et al., 2015; Burrells et al., 2016; Mangiavacchi et al., 2016; Liu et al., 2019).

We followed our very detailed and robust workflow to screen such antigens and develop an ELISA test able to identify oocyst-derived infections (Álvarez García et al., 2021). Accordingly, several limitations identified in earlier work were tackled systematically: i) the employment of different panels of reference serum samples from experimentally infected animals that were previously well-characterized through a battery of serological tests; ii) the use of a POI-based WB as a confirmatory test in combination with the development of a POI-based ELISA, and iii) the study of cross-reactions with the closely related Sarcocystidae parasite *N. caninum*. This restrictive criterion aimed at screening stage-specific immune responses in two relevant target species, pig and sheep. Sera from both are adequate reagents for testing the POI´s antigenicity since both species are relevant *T. gondii* IH. Moreover, pigs and humans share similar immunological features (Delgado Betancourt et al., 2019). This is the first study where sheep sera were used and where isolates of predominant archetypal types II and III were employed as inoculum in pigs. Moreover, up to now, studies that employed sera from experimental infections comparing both transmittable parasite stages are limited to one study carried out with pig sera (Hill et al., 2011) and three studies based on mouse sera (Crawford et al., 2010; Döşkaya et al., 2014; Santana et al., 2015). In addition, all serum panels used herein had been previously characterized, avoiding bias due to differences in diagnostic performance of tests (López-Ureña et al., 2023a; López-Ureña et al., 2023b).

In the initial screening, TgCCp5A and TgSR1 showed promising results with pig sera. However, only 1 or 2 out of up to 3 animals infected with oocysts from each panel detected the proteins (Table 1), indicating low immunogenicity. Accordingly, when TgCCp5A and TgSR1 were subjected to a more detailed analysis with the whole set of pig sera, the lack of stage-specificity and unspecific recognition by non-infected animals finally discredited these proteins as reliable source-attribution markers. In addition, all sera from *N. caninum*-infected sheep cross-reacted with TgCCp5A and TgSR1, which is not surprising given the marked conservation of both proteins across the Phylum Apicomplexa (Supplementary Table 1). Thus, testing cross-reactivity with closely related parasites is recommended in further

studies. While TgSR1 was evaluated for the first time, reactivity of sera from different species (pig, chicken, and humans) against TgCCp5A was previously evaluated by others (Santana et al., 2015; Liu et al., 2019). Importantly, none of these studies had used sera from pigs experimentally infected with both parasite stages, which is a crucial control. In a previous study, sera from naturally infected pigs were reported to show a significant increase in anti-TgCCp5A IgGs at 7 and 14 dpi (Santana et al., 2015). Others found 12% (11/90) of positive field pigs reacting with TgCCp5A (Liu et al., 2019). However, the time post-infection and the parasite stage involved was unknown since serum samples were from animals with natural infections. Further shortcomings were a lack of reference tests for sera characterization (Santana et al., 2015; Liu et al., 2019) and a higher IgM response with soluble tachyzoite antigen compared to TgCCp5A. Although TgCCp5A was recognized by human IgM and IgG in a toxoplasmosis outbreak, this was not seen by all sera (Santana et al., 2015). In addition, Liu et al. (2019) provided data of reactive sera from human patients with unknown clinical and serological history.

On the other hand, TgERP, TgSporoSAG, TgOWP1 and TgOWP8, reported in the literature as antigens able to serologically identify oocyst-driven infections, were excluded from the initial screening. TgERP (also called TgLEA850; Arranz-Solis et al., 2023), has been employed in several studies in humans and raised hopes about its source-attributing potential (Hill et al., 2011; Vieira et al., 2015; Burrells et al., 2016; Mangiavacchi et al., 2016; EFSA Panel on Biological Hazards et al., 2018). However, in the present study, TgERP showed limited antibody recognition and lack of stage-specificity. Other authors previously showed that TgERP was not exclusively recognized in oocyst-derived infections in humans (Hill et al., 2011), providing limited evidence for environmental contamination with oocysts. In the same study, pig serology indicated recognition of TgERP by sera from animals infected with oocysts *vs.* tissue cysts (Hill et al., 2011). This contrasts with the low reactivity and lack of stage specificity observed in our study with this protein. A low immunogenicity might be explained by the animals breed and age, but also by the antigens' time of exposure to the immune system as mentioned above. Moreover, a recent report has shown that TgERP is an intrinsically disordered protein, lacking a defined structure (Arranz-Solis et al., 2023), which might influence the immune response at the individual level (Uversky and Van Regenmortel, 2021).

In our hands, TgOWP1 and TgOWP8 also showed limited antibody recognition and lack of stage specificity. In contrast, two previous studies reported recognition of TgOWP1 by sera from free-range chickens as an indicator of oocyst-driven infections, although not for pigs infected with oocysts (Santana et al., 2015; Liu et al., 2019). In addition, TgSporoSAG was recognized by most infected pigs of panels 1 and 2, but it also failed to differentiate between oocyst- and tissue cyst-driven infections. A significant increase of anti-TgSporoSAG antibodies at 40 and 120 dpi in mice infected with oocysts was previously reported (Döşkaya et al., 2014), but the stage-specific response was not evaluated. This contrasts with a previous study which reported a lack of reactivity of TgSporoSAG when tested with sera from oocyst-infected mice or naturally infected humans (Crawford et al., 2010). The reasons for this discrepancy are unclear but could lie in the different expression systems employed to produce recombinant TgSporoSAG (bacteria *vs.* insect cells), possibly leading to differently folded protein forms.

Following the above-mentioned strict workflow, no antigen with source attributing value was identified. This could be due to the short time period before sporozoites cease to express stage-specific antigens and differentiate into tachyzoites and the non-replicative nature of the sporozoite itself, excluding "antigen amplification" and thus the boosting of the immune response (Fabian et al., 2021). A major strength of this work was the use of validated reference sera from experimentally infected animals, previously not available. Systematic step-by-step approaches, as the one followed here, are recommended for further studies before human serum samples can be used for final validation. Their drawback, as with any serum from natural infections, is that adults could have been exposed to different *T. gondii* stages during their lifetime, making analyses very complex. The ultimate goal would be the use of serological tests to detect human oocyst-driven infections (e.g. outbreaks, seroconversion in pregnant women) to prioritize intervention strategies.

The conclusion from this extensive work exploring source-attributing serology is that there is currently no antigen that allows robust estimates of the proportion of *T. gondii* infections acquired from oocysts by serological tests. This work provided solid new insights that can be used for further research and development of serological source-attributing approaches for *T. gondii*.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Ethics statement

The animal study was approved by Serum Panel 1: Ministry of Education, Youth and Sports from Czech Republic, PP 55/2016; Serum Panel 2: Animal Welfare Committee of the Community of Madrid from Spain, PROEX 293.7/20, PROEX 290.4/20 and PROEX 062/19; Serum Panel 3: Animal Ethic Committee from the Spanish National Research Council (CSIC), 1063/2021. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

N-ML-U: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Software, Validation, Visualization,

Writing – original draft, Writing – review & editing. RC-B: Funding acquisition, Resources, Supervision, Writing – review & editing. BK: Resources, Writing – review & editing. SC: Investigation, Methodology, Writing – review & editing. AP: Investigation, Methodology, Writing – review & editing. FT: Investigation, Methodology, Writing – review & editing. SK: Investigation, Methodology, Writing – review & editing. CS: Investigation, Methodology, Writing – review & editing. SJ: Investigation, Methodology, Writing – review & editing. PJ: Funding acquisition, Project administration, Resources, Writing – review & editing. JR-C: Resources, Writing – review & editing. L-MO-M: Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing. FSp: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. FSe: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. GÁ-G: Conceptualization, Formal Analysis, Funding acquisition, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpara.2023.1292322/full#supplementary-material

SUPPLEMENTARY TABLE 1
Bioinformatic data of 95 candidate proteins.

SUPPLEMENTARY TABLE 2
Primers used in this study.

SUPPLEMENTARY TABLE 3
Toxoplasma gondii-based serological techniques used for the characterization of the serum panels from pigs and sheep experimentally infected with *T. gondii* oocysts and tissue cysts.

SUPPLEMENTARY FIGURE 1
Epitope prediction of candidate proteins.

SUPPLEMENTARY FIGURE 2
Anti-*Toxoplasma* IgG kinetics in experimentally infected pigs with oocysts from type II and III isolates (panel 2) based on different enzyme-linked immunosorbent assays (IDScreen, PrioCHECK, Pigtype and TgSALUVET 2.0). Seroconversion was recorded from 14-21 days post-infection by all ELISA tests (significance not represented in the figures). Significant differences between experimental groups within sampling days are identified in the figures as follow for each ELISA: *= $P<$ 0.05, **: $P<$ 0.01, ***= $P<$ 0.001 and $P<$ 0.0001. Seroconversion was detected from 14-21 days post-infection and differences between pigs infected with oocysts from type II *versus* type III isolates were recorded only with TgSALUVET 2.0 at 42 days post-infection.

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Objective 2: To develop and validate a serological method that discriminate between *T. gondii* oocyst- *versus* tissue cyst-driven infections.

- Sub-objective 2.2: To develop and validate an enzyme-linked immunosorbent assay (ELISA) based on *T. gondii* sporozoite- or sporocyst/oocyst wall-specific proteins to differentiate between oocyst- *versus* tissue cyst-driven infections.

The outcomes from this sub-objective related to the determination of the **diagnostic value of TgERP for oocyst-attributing serological test** will be published as a short communication article **(paper No. 5)**:

- Authors: Nadia-María López-Ureña, Frank Seeber, Furio Spano, Bretislav Koudela, Rafael Calero-Bernal, Pikka Jokelainen, Luis-Miguel Ortega-Mora, Gema Álvarez-García.
- Title: **A thorough approach provides evidence for low diagnostic value of TgERP (TgLEA850) protein as a serological indicator for *Toxoplasma gondii* oocyst-derived infection**.

Abstract: The sporozoite-specific protein TgERP, also known as TgLEA850, was initially described as a useful antigen for oocyst-attributing serological tests. However, a recent rigorous screening of a wide panel of predicted oocyst-specific proteins did not find evidence for truly useful proteins for these purposes, including TgERP. To confirm this, herein were included two valuable rabbit positive serum controls: one against the recombinant protein (anti-rTgERP) and another against the native protein contained within sporulated oocysts lysate (anti-TgOocyst). Recombinantly histidine-tagged TgERP was expressed in *Escherichia coli*. Batch-to-batch variation between three distinct TgERP preparations were assayed. In addition, a conventional microplate system was compared with different commercial and in-house nickel-nitrilotriacetic acid (Ni-NTA) coated plates aiming at improving protein affinity and epitopes orientation. Specific anti-rTgERP IgG responses were also evaluated with reference sera from pigs experimentally infected oocysts and tissue cysts. Anti-rTgERP exhibited positive reactivity across all TgERP batches and plate systems. In contrast, positive reactivity with anti-TgOocyst was observed only with a specific TgERP batch and the conventional plate system (without Ni-NTA). No seroconversion

was recorded in experimentally infected pigs. These results provided evidence of the limited diagnostic value of TgERP to detect oocyst-derived infections.

The draft of the manuscript was included as an **Appendix 1** within this doctoral thesis.

These results have been presented as a poster communication in the annual meeting of the European Veterinary Parasitology College in Paris (June 2023).

CHAPTER VI: GENERAL DISCUSSION

The extent of the disease burden associated with oocyst-borne toxoplasmosis in humans is barely unknown. However, the infection is present in both vegetarians and non-vegetarians, evidencing that the foodborne infection is not exclusively associated to carnivorism (Dubey, 2010a). Moreover, in the last decades the relevance of oocyst-derived infections has notably increased, supported by numerous reports documenting the presence of *T. gondii* in diverse environmental matrices and several oocyst-borne outbreaks attributed to contaminated water or fresh produce in Brazil (Pinto-Ferreira et al., 2019a; Shapiro et al., 2019a). Accordingly, the present doctoral thesis has attempted to cover several gaps concerning the presence of *T. gondii* in environmental matrices and the possible limitations related to experimental design and methodologies (objective 1), as well as the identification of oocyst-specific antigens to develop and validate an oocyst-attributing serological test (objective 2).

For these purposes, first, a systematic review about the direct detection of *T. gondii* oocysts in soil, water, fresh produce, and bivalve mollusks was performed, considering worldwide published data until the end of 2020 (sub-objective 1.1, paper No. 1). Although previous studies reviewed, systematically or not, available data on *T. gondii* oocysts occurrence in these environmental matrices (Berrouch et al., 2020; Hohweyer et al., 2013; Kakakhel et al., 2021; Maleki et al., 2021; Nayeri et al., 2021; Shapiro et al., 2019a; Slana et al., 2021), this was the first systematic review that reported detection rates in all environmental matrices and covered the sampling strategies and detection methods followed, which is crucial for reporting robust, reliable and comparable results (Dumètre and Dardé, 2003).

Herein, soil, water, fresh produce, and bivalve mollusks samples were considered contaminated by *T. gondii* based on molecular or bioassay methods in all continents, except for Antarctic, where no studies had been conducted. The detection rates ranged between 0.09% (1/1109) and 100% (8/8), with all type of matrices testing positive to *T. gondii* worldwide, encompassing a diverse range of fresh produce. Most of the studies were performed in America, specifically in Brazil. However, a high heterogeneity among the different studies was found, mainly related to the diverse sampling strategies and diagnostic methods followed. Environmental variables were scarcely documented although it is widely known that rainfalls, humidity and temperature notably influence

the oocysts distribution with, e.g., an increase of *T. gondii*-positive samples during rainy seasons in some studies conducted on soil, water, fresh produce and bivalve mollusks (Ajmal et al., 2013; Gao et al., 2016; Hernandez-Cortazar et al., 2017; Miller et al., 2008; Shapiro et al., 2019a; Wells et al., 2015). Seasonality can additionally affect the efficiency/efficacy of the recovery methods by altering the sample composition as evidenced with water (Shaw et al., 2008), as well as the survival/viability of oocysts in the case of dry and warm environments (de Wit et al., 2020; Lélu et al., 2012). Similarly, the presence of felines or feline's feces in the sampling areas was barely specified in selected studies, even though it has been documented that soil contamination with oocysts declines with increasing distance from the core areas of cat home ranges (Gotteland et al., 2014) and that high detection rates (19%) in vegetables has been associated with areas where actively oocyst-shedding cats were previously identified (6%) (Lilly and Webster, 2021). Moreover, previous studies referred that the type of samples influenced on the efficiency of oocyst recovery and/or detection with, e.g., higher rates of recovery in soil samples that had lowest sand content (Lélu et al., 2011), membrane saturation in turbid water (Blaizot et al., 2020), more or less abundance of PCR inhibitors (e.g., polysaccharides), adhesion force and presence of foam-forming substances based on the type of fresh produce (e.g., saponins in spinach) (Slana et al., 2021), and higher or lower detection rates based on the type of tissue/organ analyzed from bivalve mollusks (Arkush et al., 2003; Palos-Ladeiro et al., 2015; Shapiro et al., 2015). However, the characteristics of collected samples were barely described, not just regarding the type of samples (like tap or ground water, or baby or adult greens, among others), but also the origin, the treatment if applicable, among others. Furthermore, the outcomes from this systematic review emphasize the necessity of controlling and determining the specific critical point in the food chain where contamination occurs, as ready-to-eat products have tested positive to *T. gondii* in different studies (Caradonna et al., 2017; Marques et al., 2020; Paraoan et al., 2023).

In terms of methodology, various oocyst recovery processes were applied, including washing, sedimentation, flotation, and filtration. Nevertheless, a frequent practice was a combination of them. Polymerase chain reaction methods were the most used ones, mainly based on the 529 RE and B1 molecular markers. Nevertheless, relevant

limitations were found in relation to the detection methods, including lack of standardized techniques and of internal amplification controls, as well as the lack of viability confirmation, among other facts extensively discussed by Slana et al. (2021). Since no basic guidelines are still available for the detection of oocyst in environmental matrices, it is recommended to include comprehensive information in publications, encompassing experimental design and methodology details. Harmonized protocols (e.g., standard operating procedures) are also highly needed.

Regardless of the multiple limitations and the improvement opportunities detected within this systematic review, it also highlights the remarkable level of environmental contamination. Unfortunately, there was a lack of multidisciplinary researches and so the contribution of *T. gondii*-positive environmental samples to human toxoplasmosis could not be determined, except for very few studies linked to outbreaks, most of them from South America (e.g., Blaizot et al., 2020; Coutinho et al., 1982; De Moura et al., 2006; Pinto-Ferreira et al., 2019b), which represents a major gap for developing and implementing proper intervention strategies, especially in those highly endemic regions with frequent outbreaks. Based on that, objective 2 was aimed on the development and validation of a serological test that could specifically identify oocyst-driven infections.

Previous attempts have been made to develop a serological test that discriminate between oocyst- and tissue cyst-driven infections (Burrells et al., 2016; Crawford et al., 2010; Döşkaya et al., 2014; Hill et al., 2011; Liu et al., 2019; Mangiavacchi et al., 2016; Santana et al., 2015; Vieira et al., 2015), but two relevant issues were identified among them: i) the high variability in experimental designs, and ii) the lack of well-characterized reference sera derived from animals experimentally infected with oocysts and tissue cysts. In response to those limitations, a step-by-step workflow (Álvarez-García et al., 2021) was followed in this doctoral thesis, which started with the characterization of serum panels from pigs and sheep experimentally infected with different *T. gondii* stages and isolates, employing a wide range of in-house and commercial serological tests. This task primarily involved validating the most widely used serological tests in domestic pigs and small ruminants (sub-objective 2.1, papers No. 2 and 3)

To achieve this, first, in the absence of a consensus on WB interpretation for pigs and small ruminant, an exhaustive analysis was performed using different infection groups

to identify immunodominant antigens (IDAs) and define a criterion of seropositivity based on IDAs frequency and intensity of recognition in an in-house tachyzoite-based WB (TgSALUVET WB). In the case of small ruminants, cross-reactivity with anti-*N. caninum* IgGs was also evaluated for this purpose. Surprisingly, six out of eight and two out of five IDAs were recognized prior to the infection in pigs and small ruminants, respectively. In the case of pigs, this could be related to cross-reactivity with closely related parasites since it is already known that there are conserved or similar proteins (antigens) among cyst-forming apicomplexan parasites (Gondim et al., 2017; Lorenzi et al., 2016), and antibodies against other parasites were not tested in these pigs prior to the infection (e.g., against *Sarcocystis* spp.). The 30 kDa antigen resulted the most frequently cited IDA in WB tests performed in these host species (Basso et al., 2013; Conde et al., 2001; Hebbar et al., 2022; Olsen et al., 2022; Pardini et al., 2012; Wastling et al., 1994), and in some cases it had been considered as criterion of positivity together with at least other antigen (Basso et al., 2013; Olsen et al., 2022; Pardini et al., 2012). However, herein it was recognized prior to the infection, but also by anti-*N. caninum* IgGs. Contrary, the 9–10 kDa IDA resulted highly specific as it was recognized only after the infection by animals infected with *T. gondii.* The 9–10 kDa antigen was not described in the criterion of positivity employed for pigs (Al-Adhami and Gajadhar, 2014; Basso et al., 2013; Olsen et al., 2022; Pardini et al., 2012), although it was considered as an IDA in goats (Hebbar et al., 2022; Wastling et al., 1994). Thus, to avoid false positive results, the defined criterion of seropositivity for pigs comprised the recognition of three out of eight IDAs with medium or high intensity (9–10, 19, 25, 28, 30, 33–35, 43–45 and 69 kDa). In small ruminants, a more restrictive criterium was established due to the cross-reactivity detected with anti-*N. caninum* IgGs and it consisted of the recognition of the 9–10 and/or 24–26 kDa antigens together with two other IDAs (18–20, 30 and 37–40 kDa) with medium-high intensity. After this, TgSALUVET WB was regarded as a reference test to develop and preliminary validate a novel lyophilized tachyzoite-based ELISA test (TgSALUVET ELISA 2.0) for pigs, sheep and goats, and an immunofluorescence antibody test (TgSALUVET IFAT) for pigs, both showing good initial diagnostic performance.

Next, these in-house tests were included in the comparative study together with up to four commercial ELISA tests (IDScreen, PrioCHECK, Pigtype and IDEXX). All serological

tests showed good to excellent diagnostic performance and agreement with sera from pigs and sheep experimentally infected with *T. gondii*, except for PrioCHECK, which showed low diagnostic specificity with pig serum samples, parameter that improved after a cutoff readjustment. This similarity on performance was reflected in the kinetics of anti-*T. gondii* IgGs since seroconversion was recorded between two and three weeks post-infection in both pigs and sheep, as documented in previous studies conducted in domestic pigs with TgSALUVET ELISA 2.0, PrioCHECK and IDScreen (Kauter et al., 2022; Largo-de la Torre et al., 2022) and in small ruminants with PrioCHECK (Glor et al., 2013) and other in-house ELISA tests (Castaño et al., 2019, 2014; Sánchez-Sánchez et al., 2019), although these studies conducted the infections following different experimental designs. Moreover, higher levels of anti-*T. gondii* IgGs were recorded in all tests in pigs infected with type III isolates, probably due to the higher virulence and earlier dissemination observed in some type III isolates compared to type II isolates (Fernández-Escobar et al., 2021, 2020b; Largo-de la Torre et al., 2022).

Similar results were obtained in the comparative study when analyzing samples from pigs and sheep naturally infected with *T. gondii*, except for PrioCHECK and TgSALUVET WB, which showed low specificity and sensitivity with pig serum samples, respectively. These results pinpoint the relevance of including sera from natural infections during the validation of diagnostic assays since they better represent the infection dynamics in the field, as suggested by the World Organization for Animal Health (WOAH, 2023). Again, cutoff readjustments allowed an improvement in ELISA tests diagnostic parameters and a data harmonization. Nonetheless, no improvement was possible for TgSALUVET WB, consequently it is not recommended for routinary diagnosis in domestic pigs although it proved to be useful as a reference test for the initial validation of serological assays when using pig sera from experimental infections.

The excellent performance showed by TgSALUVET ELISA 2.0 was similar to that reported in a study that used lyophilized *Besnoitia besnoiti* tachyzoites in an ELISA test (García-Lunar et al., 2017), probably due to the type of antigen. Regarding TgSALUVET IFAT, it is very likely that the outstanding performance with all pig serum panels was in part due to the consensus criterion between two experienced operators since significant discordances have been recorded in previous studies conducted on *N. caninum*

(Campero et al., 2018). Contrary to the outcomes from commercial ELISA tests, previous studies that employed sera from naturally infected pigs reported better performance for PrioCHECK (Basso et al., 2013), but worst performance for IDScreen and Pigtype (Steinparzer et al., 2015). In the case of naturally infected small ruminants, similar diagnostic performance to the reported herein was recorded for IDEXX (Mainar-Jaime and Barberán, 2007; Opsteegh et al., 2010), but better for PrioCHECK (Glor et al., 2013) and worse for IDScreen (Mangili et al., 2009), and no data was available for Pigtype. Nevertheless, even they were the same test analyzed herein, performances reported between studies might not be comparable due to the different experimental designs/approaches followed (e.g., serum samples and reference test(s) used, among others) (WOAH, 2023).

It is worth noting that the issue of cross-reactivity observed with TgSALUVET WB was corroborated with the ELISA tests since all of them yielded a notable number of false positive results when assessing sheep *N. caninum*-positive sera. Hence, although the cutoffs for ELISA tests used in pigs were adjusted solely to enhance diagnostic sensitivity and specificity values and harmonized the data, in the case of small ruminants it is advisable to consider further cutoff readjustments based on the specific epidemiological context to avoid false positive results. While this is not a novel research subject, as other studies have previously documented this issue (Bjerkas et al., 1994; Gondim et al., 2017; Huertas-López et al., 2021; Nishikawa et al., 2002; Sánchez-Sánchez et al., 2021), it appears to be an aspect that is seldom considered during the development and validation of serological tests. This poses a substantial challenge for the livestock industry due to the similar clinical signs caused by both closely related pathogens in small ruminants (Moreno et al., 2012), as well as to the possibility of co-infections in these species (Rossi et al., 2011; Villagra-Blanco et al., 2019). In fact, this is considered as one task within the serological test validation (WOAH, 2023) and must be taken into consideration in future studies. Based on these outcomes, our recommendation for the serodiagnosis of *T. gondii* in small ruminants is to apply both readjusted cutoffs, the one aimed to improve the diagnostic sensitivity and specificity and the one aimed to avoid false positive, considering as doubtful those samples with results between both cutoffs, which should be reanalyzed by a highly specific technique such as WB.

Once the reference serum panels were well-characterized, the next step consisted in searching for predicted sporozoite- or oocyst/sporocyst wall-specific proteins for later testing their usefulness for source-attributing serological diagnosis (sub-objective 2.2, paper No. 4). The similarity between the porcine and human immune systems, exceeding 80%, underscores the significance of employing this panel of sera (Delgado Betancourt et al., 2019; Pabst, 2020) in the development and validation of source-attributing serological tools whose ultimate goal is to be used in humans. Thirty two out of 95 identified *in silico* POIs from *T. gondii* omics data were successfully cloned, recombinantly expressed, and purified. The identified POIs were absent in accessible tachyzoite and bradyzoite proteome datasets (Álvarez García et al., 2021) and should be either secreted or surface-exposed, as well as putative abundant in oocysts based on proteomic and transcriptomic data (Fritz et al., 2012a, 2012b). Among these POIs were all previously described antigens with source-attributing potential: TgERP, TgCCp5A, TgSporoSAG, TgOWP1, and TgOWP8 (Burrells et al., 2016; Crawford et al., 2010; Döşkaya et al., 2014; Hill et al., 2011; Liu et al., 2019; Mangiavacchi et al., 2016; Santana et al., 2015; Vieira et al., 2015). The restrictive step-by-step workflow followed herein (Álvarez-García et al., 2021) resulted operationally manageable and enabled the generation of robust/ feasible results since, although only two (TgCCp5A and TgSR1) out of the 32 POIs passed the screening for pigs by inducing seroconversion and showing stage-specificity in WB, in a later step, when all samples from both pig serum panels were analyzed by both WB and ELISA, three issues were uncovered: i) just a few animals seroconverted, ii) both POIs lacked stage-specificity, iii) both POIs showed unspecific reactivity with non-infected animals. In addition, both POIs cross-reacted with anti-*N. caninum* IgGs. Furthermore, none of the 32 POIs passed the screening when testing sheep serum samples. These results underscore the importance of employing well-characterized serum samples from experimental infections conducted with diverse parasite stages to ascertain the stage-specificity of the proteins, as well as the antibody kinetics. Controversially, only one previous study employed sera from pigs experimentally infected with both parasite stages (Hill et al., 2011), whereas three of them employed sera from mice infected with both stages (Crawford et al., 2010; Döşkaya et al., 2014; Santana et al., 2015). Indeed, in some cases, the conclusion regarding the utility of certain antigens in distinguishing between transmission routes was contingent upon the findings derived from human sera

(Burrells et al., 2016; Mangiavacchi et al., 2016; Vieira et al., 2015), who can be reinfected with different parasite stages through their lifetime.

Herein, the low reactivity against TgCCp5A and TgSR1 was not an issue related to a failure in inducing infection after inoculation since all serum panels presented a significant increase of anti-*T. gondii* IgGs by multiple tachyzoite-based serological tests in the comparative studies (sub-objective 2.1, papers No. 2 and 3). In fact, seroconversion was also confirmed by a sporulated oocysts soluble antigen-based WB test in pigs infected with oocysts and tissue cysts, recording similar antigen reactivity in all experimental groups compared to TgSALUVET WB (unpublished, Appendix 2). Hence, the most relevant limitation in this task might be the short time-lapse available for the immune system to recognize sporozoite- and sporocysts/oocyst wall- specific antigens before sporozoites turn to fast-replicating tachyzoites, in addition to the non-replicative capacity of sporozoites for boosting the immune response (Fabian et al., 2021).

To finish, despite TgERP did not pass the screening, an extra effort was done to confirm or rule out its usefulness for such purposes as it was the most widely used protein for source-attributing assays in the literature, demonstrating promising results (Burrells et al., 2016; Hill et al., 2011; Mangiavacchi et al., 2016; Vieira et al., 2015) (sub-objective 2.2, paper No. 5, unpublished, Appendix 1). To accomplish this task and obtain more robust data, serum samples from a rabbit subcutaneously inoculated with the recombinant (anti-rTgERP) or native antigen contained within sporulated oocysts lysate (anti-TgOocyst) were employed as positive controls. In addition, non-previously evaluated variables were considered, such as batch-to-batch antigenicity variation and different plate systems, which included conventional ones and different commercial and in-house Ni-NTA coated plates following the protocols from previous studies (Cressey et al., 2008; Paborsky et al., 1996). The goal of Ni-NTA coated ELISA plates was to improve protein purity, affinity and epitope exposure. The anti-rTgERP serum showed high reactivity with all batches and plate systems, whereas anti-TgOocyst antibody reacted positively only with one protein batch and when using the conventional plate system (without Ni-NTA). This is a relevant outcome since, although both the recombinant and the native antigens were administrated subcutaneously, via that presumably facilitates the immune system´s recognition of the antigen compared to the natural oral route of

infection with oocysts, lower reactivity was observed with the anti-TgOocyst serum *vs.* the anti-rTgERP serum even with the conventional ELISA plate system. This difference in reactivity may be attributed to conformational disparities between the native and the recombinant TgERP or their different abundance in the inoculated preparations. Consistently, when the pig serum panels from experimental infections were tested with the selected protein batch and ELISA test conditions, no IgG seroconversion was recorded. A similar scenario was observed when attempting to detect specific IgA antibodies. Our results contrast with published data conducted with TgERP using sera from pigs infected with oocysts and tissue cysts, where exclusively reactivity was observed by oocyst-infected pigs, with detectable antibody until 8 months post-infection (Hill et al., 2011). Nevertheless, limited conclusions could be drawn from the rest of studies that used TgERP since they were exclusively conducted with human sera (Burrells et al., 2016; Mangiavacchi et al., 2016; Vieira et al., 2015). Curiously, when the selected batch was employed a year later in the ELISA test, no reactivity or lower reactivity was observed with serum samples that previously tested positive with the same batch, including positive controls (data not shown). These outcomes could be explained by the fact that TgERP is an intrinsically disordered protein, lacking a defined structure (Arranz-Solís et al., 2023), a characteristic that could impact the immune response at an individual level (Uversky and Van Regenmortel, 2021), but also the epitope exposure as the conformational structure might change.

The inclusion of anti-TgOocyst antibody is recommended in further POIs screening since it could simplify the restrictive workflow employed herein based on the lack of reactivity against TgCCp5A or TgSR1 and the recognition of TgERP in WB (Appendix 3). Unfortunately, this reagent was not available at the beginning of this doctoral thesis, but the same conclusions would have been reached.

In summary, this doctoral thesis has offered valuable insights into the environmental transmission route and serodiagnosis of *T. gondii*. First, high level of contamination in different environmental matrices and multiple improvement opportunities for enhancing the experimental design and implementation of future studies were identified. Second, updated data on the diagnostic performance of the most widely used serological tests in the diagnosis of *T. gondii* infections in domestic pigs and small

ruminants was provided based on the most comprehensive comparative studies performed to date, along with recommendations on cutoff readjustments to harmonize data or avoid false positive results in the presence of anti-*N. caninum* IgGs. These comparative studies included new in-house serological tests and allowed to determine a restrictive criterion of seropositivity for WBs for pigs, sheep and goats. Third, despite the extensive and exhaustive work carried out to identify a wide range of oocyst-specific proteins and develop an oocyst-attributing serological tool, which included the employment of valuable well-characterized sera from experimental infections performed orally with oocysts and tissue cysts or subcutaneously with one of the recombinant antigens or sporulated oocysts lysate, no useful antigen could be found to estimate the relative importance of oocyst-driven infections through serology.

CHAPTER VII: CONCLUSIONS

Objective 1: To determine the relevance of the environmental route in *T. gondii* transmission and the potential limitations related to the sampling strategies and detection methods employed with different environmental matrices.

- **First:** *Toxoplasma gondii* oocysts exhibit a widespread presence in soil, water, fresh produce, and bivalve mollusks matrices globally, posing a potential threat to animal and public health. Nevertheless, a scarcity of conducted investigations persists, and certain regions of the world remain understudied. Furthermore, future surveys should be conducted following the One Health approach.

- **Second:** Caution should be advised in data interpretation given the high heterogeneity recorded between studies, mainly related to the different experimental designs and detection methodologies employed. Basic guidelines are required to obtain robust and harmonized results and to set up a validated standard operating procedure. Key points related to sampling strategies, such as sample size, origin, season, presence of felines, among others, as well as the implementation of artificial oocyst contamination procedures to determine the performance characteristics of the detection methods, should be properly addressed.

Objective 2: To develop and validate a serological method that discriminate between *T. gondii* oocyst- *versus* tissue cyst-driven infections.

- **Third**: An exhaustive criterion of seropositivity has been established for a Western blot test based on tachyzoites (TgSALUVET WB) for pigs, sheep, and goats. This criterion not only considered the frequency and intensity of antigen recognition, but also cross-reactivity between *T. gondii* antigens and anti-*N. caninum* IgGs for small ruminants. The test exhibited high specificity, proving valuable as a reference test for the initial validation of serological assays, and proving to be suitable for discarding false positive results in sheep. However, TgSALUVET WB is not recommended for routine diagnosis in domestic pigs.

- **Fourth:** The newly developed and validated lyophilized tachyzoite-based ELISA tests (TgSALUVET ELISA 2.0) are accurate for routine diagnosis as they showed excellent diagnostic performance for both pigs and small ruminants. The

immunofluorescence antibody test (TgSALUVET IFAT) validated herein for pigs also showed excellent diagnostic performance. However, it is not recommended for routine diagnosis, unless the results are interpreted by two experienced operators.

- **Fifth:** The evaluated commercial ELISA tests employed in pigs proved to be well-validated, except PrioCHECK that showed lower diagnostic specificity values. Thus, PrioCHECK required a cutoff value readjustment to obtain more reliable and comparable data. This readjustment should be considered by diagnostic laboratories.

- **Sixth:** The evaluated commercial ELISA tests used in small ruminants showed good to excellent diagnostic performance, although diagnostic parameters could be improved with a cutoff readjustment. However, cross-reactivity with anti-*N. caninum* IgGs was recorded in all ELISA tests, including TgSALUVET ELISA 2.0. Thus, additional cutoff readjustments were necessary to avoid false positive results. Depending on the epidemiological scenario, it may result useful to apply both readjusted cutoffs and re-analyze the doubtful samples (with results in between cutoffs) with a confirmatory test.

- **Seventh:** A pioneer *in silico* approach led us to identify a high number of *T. gondii* predicted sporozoite- and sporocyst/oocyst wall-specific proteins from omics available data. The unprecedented effort pursued led us to successfully obtain a total of thirty-two recombinant proteins, including the five oocyst-specific proteins previously used in source-attributing serological tests (TgERP, TgSporoSAG, TgCCp5A, TgOWP1 and TgOWP8).

- **Eighth:** The restrictive step-by-step workflow followed herein resulted useful to screen all predicted sporozoite- and sporocyst/oocyst wall-specific proteins. Only two proteins, TgCCp5A and TgSR1, exhibited promising results during the screening step with a limited set of pig sera. However, later, when these proteins were thoroughly tested by WB and ELISA, they were discarded for the same reasons as the other proteins: lack of stage-specificity, low antigenicity, and unspecified reactivity with non-infected animals.

- **Nineth:** In a last attempt to re-evaluate the source attributing value of TgERP, which included the evaluation of different TgERP batches and ELISA plate systems to improve epitope exposition, the results confirmed its limited diagnostic value in identifying oocyst-driven infections.
- **Tenth:** The rabbit polyclonal antibody obtained against sporulated oocysts lysate (anti-TgOocyst) stands as a valuable reagent that, if included in the workflow as an additional reference control, will markedly improve and simplify the process for identifying proteins of interest, as well as for developing and validating source-attributing serological tests.

CHAPTER VIII: BIBLIOGRAPHY

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APPENDIXES

Appendix 1. A thorough approach provides evidence for low diagnostic value of TgERP (TgLEA850) protein as a serological indicator for *Toxoplasma gondii* oocyst-derived infection (article draft, paper No. 5).

A thorough approach provides evidence for low diagnostic value of TgERP (TgLEA850) protein as a serological indicator for *Toxoplasma gondii* oocyst-derived infection

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Abstract

The importance of the environmental route on *T. gondii* transmission is increasing in recent years. The sporozoite-specific protein TgERP, also known as TgLEA850, was initially described as suitable for oocysts-attributing serological tests. However, a recent exhaustive screening of a wide panel of proteins predicted to be oocyst-specific, including TgERP, did not find evidence of their usefulness as oocyst-specific markers for serological diagnosis. Thus, our aim was to re-evaluate the robustness of TgERP as a serological indicator for oocyst-derived infections. For this, two valuable well-characterized positive serum controls derived from rabbits inoculated with the recombinant or native TgERP, anti-rTgERP and anti-Oocyst, respectively, were employed. Batch-to-batch variation between three distinct TgERP preparations was assayed by a conventional enzyme-linked immunosorbent assay (ELISA) microplate system. After the selection of a TgERP batch, a conventional microplate system was compared with different commercial and in-house nickel-nitrilotriacetic acid (Ni-NTA) coated plates aiming at improving protein affinity and epitope exposure. Finally, specific anti-TgERP IgG responses were evaluated by using reference sera from pigs experimentally infected with oocysts and tissue cysts. High reactivity was observed with the anti-rTgERP serum with all batches and plate systems, whereas high reactivity with the anti-TgOocyst serum was only observed with one TgERP batch in a conventional ELISA plate system. Moreover, no seroconversion was recorded with pig sera. The fact that TgERP behaves as an intrinsically disordered protein could explain these poor reactivities and the differences between tested batches. These preliminary results provide evidence for the low diagnostic value of TgERP to serologically detect oocyst-derived infections.

Keywords: TgERP/TgLEA850, *Toxoplasma gondii,* ELISA, oocyst-driven infections, diagnosis.

1. Introduction

Toxoplasma gondii is a widely distributed foodborne apicomplexan parasite that infects any warm-blooded animal, including humans. It is the cause of toxoplasmosis, a disease linked to reproductive failure in pregnant woman and livestock, especially small ruminants (Dubey et al., 2020b, 2020a; Innes et al., 2009; Rico-Torres et al., 2016), as well as to severe neurological and respiratory diseases in immunocompromised hosts (Barratt et al., 2010), becoming a disease of relevance in both human and animal health.

Toxoplasma gondii has three infective stages: sporozoites (contained within sporulated oocysts), bradyzoites (contained within tissue cysts) and tachyzoites (Attias et al., 2020), responsible for the environmental, meatborne and maternal-fetal transmission routes, respectively. Although the contribution of each route of transmission remains unknown, the environmental one might be more relevant than previously thought, causing 44.1% (15/34) of worldwide human toxoplasmosis outbreaks until 2018 (Pinto-Ferreira et al., 2019), with more than 900 laboratory confirmed cases (Minuzzi et al., 2021). Nevertheless, one of the main limitations in identifying the source(s) of infection is that: i) the incubation time is up to 30 days post-infection (Dubey, 2021) and, therefore, at that moment the environmental source could no longer be contaminated nor exist; ii) there is a lack of standardized methods to detect *T. gondii* in environmental matrices. In this scenario, source-attributing serological tools could help to design adequate intervention strategies. Several attempts have been made with proteins predicted to be sporozoite- or sporocysts/oocysts wall-specifics, with reported promising results in many cases (reviewed by Álvarez-García et al., 2021). Among them, the *T. gondii* late embryogenesis-related protein, TgERP, also known as LEA850 (Arranz-Solís et al., 2023), has been the most cited and employed one, and its usefulness has been documented under different scenarios (Burrells et al., 2016; Hill et al., 2011; Mangiavacchi et al., 2016; Vieira et al., 2015).

The TgERP protein was identified by Hill et al. (2011) by gel electrophoresis, mass spectroscopy and Western blot test (WB) and resulted to be abundantly expressed in sporozoites compared to tachyzoites and bradyzoites. Its usefulness to serologically discriminate between oocyst- *vs.* tissue cyst-driven infections was confirmed with the exclusive TgERP-positive reactivity observed with serum samples from pigs (up to 8 months post-infection) and mice experimentally infected with oocysts compared to those infected with tissue cysts (Hill et al., 2011), as well as with human serum or saliva samples from presumed oocyst-derived infections (Burrells et al., 2016; Hill et al., 2011; Mangiavacchi et al., 2016; Vieira et al., 2015). In contrast, a recently published article that aimed to confirm the serological utility of previously described proteins predicted to be sporozoite- or sporocysts/oocysts wall-specific, including TgERP, as well as newly identified ones, failed in finding antigens able to serologically detect oocyst-driven infections (López-Ureña et al., 2023b). The robustness of those results relied on the exhaustive step-by-step workflow followed that included the employment well-characterized reference serum panels from pigs and sheep experimentally infected with

oocysts and tissue cysts (López-Ureña et al., 2023b).

Based on this, the objective of this study was to re-evaluate the usefulness of TgERP as an antigen for oocyst-attributing serological tests, using two additional valuable positive serum controls (against recombinant and native TgERP) and evaluating further variables, such as batch-to-batch variation and different enzyme-linked immunosorbent assay (ELISA) plate systems.

2. Materials and methods

2.1. Experimental design

The workflow followed herein included the: a) characterization of two positive serum controls produced against the recombinant and native TgERP, which were employed in the ELISAs, b) the evaluation of batch-to-batch variations between a newly produced TgERP batch *vs.* two previously produced batches using a conventional TgERP-ELISA and the positive serum controls, c) comparison of a conventional TgERP-ELISA using Maxisorp plates (Nunc™, Thermo Fisher Scientific, Massachusetts, U.S.A.) *vs.* two in-house nickel-nitrilotriacetic acid (Ni-NTA) coated Maxisorp (Nunc™, Thermo Fisher Scientific, Massachusetts, U.S.A.) and Corning (Corning Life Sciences, Maine, U.S.A.) plates and one commercial Ni-NTA coated ELISA plate (Pierce™, Thermo Fisher Scientific, Massachusetts, U.S.A.) aiming to improve TgERP purification and the epitopes exposure, using also the positive serum controls, d) validation of TgERP-ELISA with serum samples from pigs orally inoculated with oocysts and tissue cysts.

2.2. Production of native antigens

Toxoplasma gondii tachyzoites were cell-cultured, purified, preserved and prepared for WB as indicated by López-Ureña et al. (2023a).

To produce a lysate of sporulated oocysts for rabbit inoculation and to obtain a polyclonal anti-sporulated oocysts lysate antibody (see section 2.4.1), a pellet of 2.03 x 10^8 recently obtained and purified *T. gondii* sporulated oocysts from TgShSp1 strain (Type II, ToxoDB genotype #3) (Sánchez-Sánchez et al., 2019) was resuspended in 5 mL of cold PBS and subjected to four cycles of freezing and thawing in liquid nitrogen. Next, the content underwent sonication in cycles lasting 50 seconds at 20% amplitude at 4 °C. A total of ten sonication cycles were required until complete oocyst, sporocysts and sporozoites structures were no longer observed under light microscopy. The protein concentration in whole oocysts lysate extract was determined using Bradford (Bio-Rad, California, U.S.A.), following manufacturer´s instructions, and the preparation was kept in small aliquots at –80 °C until use.

A similar approach was followed for the obtention of sporulated oocysts soluble antigen for WB, but an additional step was included prior to the protein quantification, which involved the centrifugation of the whole lysate extract at 11450 rpm for 30 min at 4 °C and the subsequent recovery of the supernatant.

2.3. TgERP expression, purification and quantification

TgERP was expressed, purified and quantified as specified by Arranz-Solís et al. (2023) and López-Ureña et al. (2023b), but with some slight modifications, as specified below.

BL21 *Escherichia coli* strain expressing pQE90S encoded TgERP-6His tag was cultured in 500 mL LB medium containing the appropriate antibiotic for TgERP selection. The culture was incubated at 37 °C at 225 rpm until reaching an OD_{600nm} of 0.5–0.7, moment when TgERP expression was induced by adding isopropyl-β-D-1-galactopyranoside (1mM final concentration). Then, the culture was incubated overnight at 28 °C at 225 rpm and, after that, cells were pelleted at 4800 rpm for 20 min at 4°C.

For TgERP purification, cells pellets were resuspended in 10 mL of lysis buffer (50 mM sodium phosphate, 300 mM NaCl, 10 mM imidazole, pH 8.0) that contained cOmplete™ protease inhibitor (Roche, Basel, Switzerland) and HS-nuclease (MoBiTec, Goettingen, Germany). The content was sonicated and subjected to a wet boiling treatment at 95 °C for 5 min aiming to improve the purification process since TgERP is known to be heat-stable (Arranz-Solís et al., 2023) and would remain in the supernatant when centrifuged at 8000 rpm for 10 min at 4 °C, while contaminants precipitate. The recovered supernatant was filtered through a 0.45-µm pore membrane and purified using PureCube 100 INDIGO Ni-Agarose (Cube Biotech, Monheim, Germany) as specified by the manufacturer. The unbounded content was discarded using Column PD10-Empty (Cytiva, Massachusetts, U.S.A.) and washing buffer (50 mM sodium phosphate, 300 mM NaCl, 20 mM imidazole, pH 8.0). Then, elution buffer (50 mM sodium phosphate, 300 mM NaCl, 500 mM imidazole, pH 8.0) was added to recover bounded TgERP. The integrity of recovered TgERP was confirmed by SDS-PAGE Coomassie staining and WB, using as control an anti-6His Tag antibody (Qiagen, Venlo, Netherlands). To finish, the buffer was changed to PBS using VivaSpin®2 columns (Sartorius, Göttingen, Germany) and the protein was quantified using Qubit protein assay kit (Invitrogen™, Thermo Fisher Scientific, Massachusetts, U.S.A.), following in both cases the manufacturers´ instructions.

This production of TgERP was regarded as the most recently obtained batch (named as B1). Two additional boiled TgERP batches were employed. One of these batches had been produced a few months earlier (named as B2), while the other had been produced two years before (named as B3).

2.4. Serum panels

2.4.1. Serum samples used as controls to develop TgERP-ELISA

A crude serum from an experimentally inoculated rabbit with the recombinant form of TgERP (named as anti-rTgERP in the following sections) was obtained by Preclinics (Germany) for previous studies (Fabian, 2021).

The polyclonal antibody targeting *T. gondii* oocysts derived from a rabbit experimentally inoculated with sporulated oocysts lysate (named as anti-TgOocyst in the following sections). For this, an aliquot of the recently obtained sporulated oocysts lysate (section 2.2), which protein concentration was approximately 1 mg/mL, was shipped on dry ice to Rekom Biotech S.L. (Granada, Spain), where the rabbit inoculation was carried out, making use of the animal facilities of the Institute of Parasitology and Biomedicine López Neyra – CSIC. The rabbit was subcutaneously inoculated five times, with each injection administered approximately every

fifteen days. Each injection consisted of 1 mL of lysate, which contained 500 µL of adjuvant. Serum samples were collected prior to the first immunization and after the second (28 days post-inoculation [dpi]) and fourth (59 dpi) boosters to confirm reactivity against *T. gondii* antigens using an ELISA performed by Rekom Biotech S.L. (Granada, Spain) based on the inoculated lysate and *T. gondii* recombinant antigens SAG1 and GRA7. Total rabbit bleeding was conducted 76 dpi, starting with an initial blood collection from the central artery of the ear and concluding with a cardiac puncture. The collected samples were purified by Rekom Biotech S.L. (Granada, Spain), although no isotypes were characterized. The purified anti-Oocyst was shipped to SALUVET research group and characterized using tachyzoite-based and sporulated oocysts soluble extract-based WB tests, as well as sporulated oocyst-based immunofluorescence antibody tests (IFAT).

Two samples were regarded as negative controls: a rabbit serum obtained prior to the immunization (named as pre-immune in the following sections), to have the same host species as the positive serum controls, and a commercially available *T. gondii*-negative pig serum (Sigma/Merck, Darmstadt, Germany) (named as Neg.-pig in the following sections) in order to have the background from the species that was used to validate the TgERP-ELISA.

In addition, an anti-histidine antibody (Qiagen, Venlo, Netherlands) was used (diluted at 1/2000) in all ELISA plate systems to confirm if the TgERP attachment happened from the 6His-tag terminal in those plates with Ni-NTA.

2.4.2. Serum samples used to validate TgERP-ELISA

TgERP-ELISA test validation was performed using two serum panels from pigs experimentally infected with oocysts and tissue cysts from different strains of *T. gondii* (Dámek, 2023; Largo-de la Torre et al., 2022). These serum panels were characterized using different tachyzoite-based conventional serological tests (López-Ureña et al., 2023c, 2023a) and were used in the previous study that found no specific-oocyst protein useful for source-attributing serology (López-Ureña et al., 2023b).

In summary, Panel 1 corresponded to prepubertal sows that were orally inoculated with 400 *T. gondii* oocysts (n= 3) or 10 tissue cysts (n= 3) from type II isolate (CZ-Tiger strain, ToxoDB RFLP genotype #3), and 400 *T. gondii* oocysts (n= 3) or 10 tissue cysts (n= 2) from type III isolate (CZ-Šimková strain, ToxoDB #2), bled at 0, 2, 4 and 6 weeks post-infection (wpi) (n= 44) (Dámek, 2023). Panel 2 corresponded to three-months-old female pigs inoculated orally with 1000 oocysts from the isolates TgShSp1 (type II, ToxoDB #3, n= 5) and TgShSp24 (type III, ToxoDB #2, n= 5), as well as non-infected pigs (n= 3), bled at -4, 0, 4, 7, 14, 21, 28 and 42 dpi (n= 103) (Largo-de la Torre et al., 2022).

2.5. Serological tests

2.5.1. Sporulated oocyst-based immunofluorescence antibody tests

A total of 300 sporulated oocysts from TgShSp1 strain (Type II, ToxoDB genotype #3) (Sánchez-Sánchez et al., 2019), previously inactivated at 60 °C for 1 min, was fixed per 4-mm well and acetone permeabilized at –20 °C for 30 min. After a wash with Milli-Q water, 8 µL/well of anti-TgOocyst antibody diluted at 1/100, 1/500, 1/1000, 1/2000

and 1/4000 in PBS was added and incubated in a dark chamber for 30 min at 37 °C. Then, three washes were performed with PBS, each of 10 min, and 8 µL/well of fluorescein isothiocyanate (FITC)-conjugated secondary antibody (IgG) diluted at 1/80 in 0.2% Evans Blue Dye PBS was added (Sigma/Merck, Darmstadt, Germany). The slides were incubated and washed under the same conditions, including an additional wash with Milli-Q water. The slides were dried in dark at room temperature and cover slides were fixed with Fluoroshield™ (Sigma/Merck, Darmstadt, Germany). In addition, a previously described agarose-based IFAT was followed to immobilize oocysts (Fabian et al., 2021), diluting serum samples at 1/10, 1/25, 1/50 and 1/100, $Alexa_{568nm}$-conjugated anti-rabbit IgG (Invitrogen™, Thermo Fisher Scientific, Massachusetts, U.S.A) at 1/500 and a FITC-conjugated *Maclura pomifera* lectine at 1/100 (CiteAb, Bath, England). Pre-immune serum was included as negative control.

2.5.2. Tachyzoite- and sporulated oocysts soluble antigen-based WB tests

Tachyzoite-based WB test was performed as indicated by López-Ureña et al. (2023a). A similar approach was followed for the sporulated oocysts soluble antigen-based WB, but using 100 µg/single-well comb of the antigen, also under reducing conditions. Peroxidase conjugated anti-rabbit antibody (IgG) was employed at 1/1000 (Sigma/Merck, Darmstadt, Germany) and the 4-chloro-1-naphthol reaction (Thermo Fisher Scientific, Massachusetts, U.S.A.) was stopped with Milli-Q water based on the colorimetric reactivity of the anti-TgOocyst antibody.

2.5.3. TgERP-ELISA tests

The conventional TgERP-ELISA was performed as detailed by López-Ureña et al. (2023b), with minor modifications. For coating, 0.2 µg/well of TgERP was employed and the reaction was stopped when the anti-rTgERP serum (diluted at 1/2000) reached an optical density at 620 nm (OD_{620nm}) of 0.4–0.5 based on pilot experiments (data not shown).

For the Ni-NTA-TgERP-ELISA tests, two previously in-house published protocols were merged (Cressey et al., 2008), and two different ELISA plates were tested, Maxisorp (Nunc™, Thermo Fisher Scientific, Massachusetts, U.S.A.) and Corning (Corning Life Sciences, Maine, U.S.A.). The protocol was as follows: the wells were coated overnight with 100 µL/well of 10 mM N,N-bis [carboxymethyl] lysine (NTA) (Merck, Darmstadt, Germany) diluted in 0.1 M sodium dihydrogen phosphate monohydrate solution (Carl Roth, Karlsruhe, Germany), pH 8. After three washed with 0.05% PBS Tween 20 (PBS-T), the plates were blocked at room temperature with 300 µL/well of 3% bovine serum albumin (BSA) PBS-T (Carl Roth, Karlsruhe, Germany). Then, the plates were washed with a series of buffers: i) 500 mM imidazole PBS-T (Merck, Darmstadt, Germany), ii) PBS-T, iii) 100 mM EDTA PBS-T (Carl Roth, Karlsruhe, Germany), pH 8.0, and iv) PBS-T. Later, 200 µL/well containing 10 mM nickel sulfate (Fluka, North Carolina, U.S.A.), diluted in MilliQ water, was added and incubated at room temperature for 20 min. Subsequently, the plates were washed with PBS-T followed by two washes with PBS, pH 7.4. After this, the plates were ready to be coated with TgERP. The following steps were the same used for the conventional TgERP-ELISA.

In the case of the commercial Ni-NTA plates (Pierce™), the manufacturer´s instructions were followed.

2.6. Data analyses

Significant differences between and within TgERP batches or ELISA plate systems were determined by comparing the mean OD values of three to four replicates of the serum controls in a two-way ANOVA, followed by a Tukey's multiple comparison test if applicable (GraphPad Prism software, version 8.0.1). The kinetics of specific IgG antibodies against TgERP, as well as the capacity of TgERP to discriminate between oocyst- *vs.* tissue cyst-driven infections, were thoroughly examined with the newly developed TgERP-ELISA using serum Panels 1 and 2. To discern seroconversion, the mean OD value from each experimental group was compared between sampling weeks/days using a two-way ANOVA with repeated measures, followed by a Dunnett's multiple comparison test when applicable. The same approach was followed to determine differences between experimental groups within sampling weeks/days, and Tukey's multiple comparison test was employed when necessary. Sphericity was not assumed, and the Geisser-Greenhouse correction was automatically applied to ensure the accuracy of the results. Differences were considered significant when the *P* values were lower than 0.05.

3. Results

3.1. Anti-TgOocyst showed reactivity against *T. gondii* oocysts and tachyzoites

Rekom Biotech S.L. (Granada, Spain) reported seroconversion in the rabbit inoculated with sporulated oocysts lysate at 28 dpi onwards based on sporulated oocysts lysate-, SAG1- and GRA7-based ELISA tests. These results were confirmed by our laboratory using the conventional oocyst-based IFAT (without agarose). With this IFAT, IgG titers of 1/500 and 1/1000 were recorded at 28 and 59 dpi with the crude serum, respectively. After its purification, the anti-TgOocyst showed high reactivity at 1/20 dilution in a *T. gondii* tachyzoite-based and sporulated oocysts soluble antigen-based WB tests **(Figure 1A-B)**, and titers of 1/1000 or 1/10 in a conventional or agarose oocyst-based IFAT, respectively **(Figure 1C).**

3.2. Recognition of TgERP by the anti-TgOocyst antibody was dependent on the TgERP batch

Anti-rTgERP detected all TgERP batches whereas anti-TgOocyst only showed a positive reaction against B1. Significant differences in reactivity (expressed as OD values) were found for B1 *vs.* B2 or B3 with both anti-rTgERP and anti-TgOocyst **(Figure 2A)**. Differences in OD values between anti-TgOocyst and pre-immune serum were recorded only with B1 ($P < 0.0001$), while differences in OD values between anti-rTgERP and pre-immune serum were recorded with all TgERP batches ($P < 0.0001$).

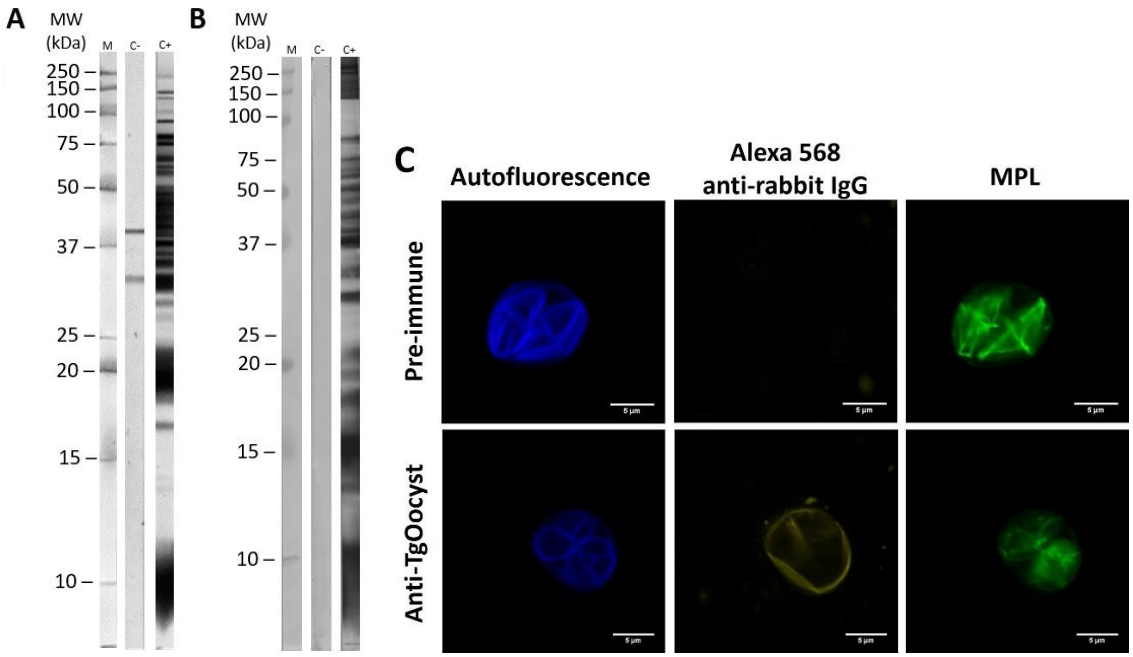

Figure 1. *Toxoplasma gondii* antigen recognition with the anti-TgOocyst antibody in tachyzoite-based **(A)** or sporulated oocysts soluble antigen-based **(B)** Western blot tests. Agarose oocyst-based immunofluorescence antibody test using anti-TgOocyst antibody **(C)**. MW: molecular weight, M: molecular weight marker, C-: pre-immune serum control, C+: anti-TgOocyst antibody control, MPL: fluorescein isothiocyanate-conjugated *Maclura pomifera* lectine. Scale bar: 5 µm.

Similarly, differences in OD values between anti-TgOocyst and anti-rTgERP were recorded with all batches, but were less significant in the case of B1 (B1: P= 0.0155, B2 and B3: P< 0.0001). Based on this, TgERP B1 was selected for the following experiments.

3.3. The employment of Ni-NTA-based ELISA plates did not improve the recognition of TgERP by anti-TgOocyst antibody

Anti-rTgERP showed significant OD differences in reactivity in conventional ELISA plates (Maxisorp without Ni-NTA) *vs.* any Ni-NTA-based ELISA plates, and in Ni-NTA Maxisorp plates *vs.* Ni-NTA Corning plates, while anti-TgOocyst showed significant difference in reactivity only between the conventional ELISA plate and the Ni-NTA-based ELISA plates **(Figure 2B)**. Differences in OD values between the pre-immune serum and the anti-rTgERP serum were recorded with the conventional ELISA plates (P< 0.0001), Ni-NTA Corning plates (P< 0.0001) and Ni-NTA Pierce plates (P= 0.0045). However, significant differences between the OD values obtained with pre-immune serum and anti-TgOocyst antibody were only observed when the conventional ELISA plates were used (P< 0.0001). Positive reactivity with the anti-histidine antibody was only observed in the conventional ELISA plates. Based on this, the conventional ELISA plate system was selected for the following assays.

3.4. No seroconversion was recorded in TgERP-ELISA test with sera from experimentally infected pigs

A non-significant increase in IgG levels against TgERP was observed after the infection when all serum samples from Panels 1 and 2 were analyzed by the newly developed TgERP-ELISA test **(Figure 2C)**.

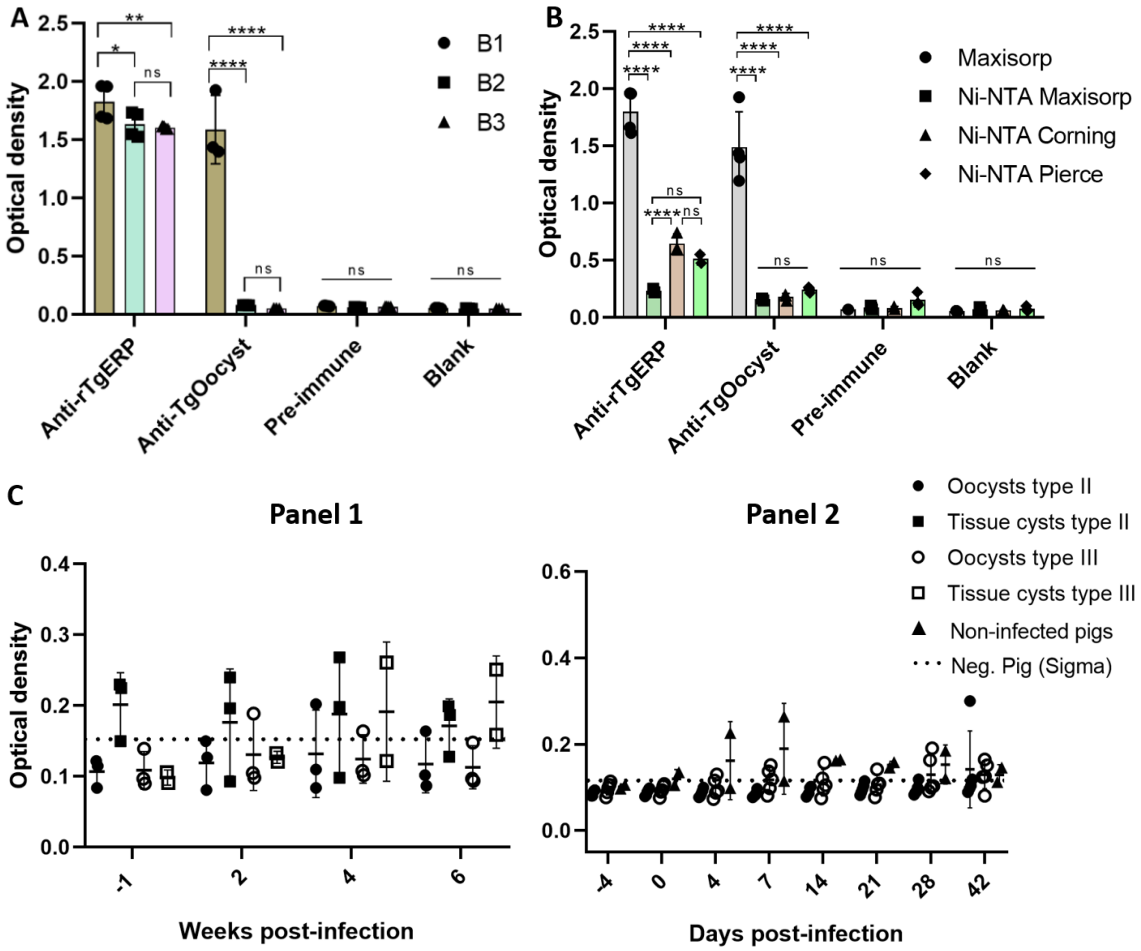

Figure 2. TgERP-ELISA tests based on different TgERP batches **(A)** and ELISA plate systems **(B)**, and kinetics of specific IgG antibodies against TgERP using sera from pigs experimentally infected with oocysts and tissue cysts from type II and type III isolates **(C)**. Significant differences were identified as follows: *= $P< 0.05$, **= $P< 0.01$, ***= $P< 0.001$ and ****= $P< 0.0001$. Ns: not significant. One negative control from Panel 2 showed almost twice higher optical density prior to the infection respect to 42 dpi and was excluded for the statistical analysis and the antibody kinetics graphs.

4. Discussion

This is the first study where additional specific sera controls and ELISA variables were used to re-evaluate the robustness of TgERP as a specific serological indicator for oocyst-derived infections. In this last attempt, two well-characterized positive serum controls derived from rabbits subcutaneously inoculated with the recombinant and native antigen, TgERP or sporulate oocysts lysate, respectively, were

obtained, characterized and used. Furthermore, additional ELISA variables were tested, including the analysis of different batches of TgERP and different ELISA plate systems (with and without Ni-NTA). Unfortunately, the outcomes of this study corroborated the lack of oocyst-attributing serological value of TgERP, as previously pointed out by López-Ureña et al. (2023b) after following an exhaustive workflow and the use of well-characterized reference pig and sheep serum panels.

In this previous study, TgERP was identified as a sporozoite-specific protein though an *in silico* approach, but after a first screening with selected reference sera, TgERP was not selected as a suitable marker of oocyst-derived infections due to its low antigenicity and lack of stage specificity (López-Ureña et al., 2023b).

Interestingly, different reactivity against TgERP was observed herein with the anti-rTgERP and anti-TgOocyst controls. The rabbit anti-TgERP serum showed high reactivity with all batches and plate systems, whereas the anti-TgOocyst antibody only showed a positive reaction against the recently produced batch when using the conventional plate system (without Ni-NTA coating). This is a relevant outcome since, although both the recombinant and the native antigens were administrated subcutaneously, via that presumably facilitates the immune system´s recognition of the antigen compared to the natural oral route of infection with oocysts, lower reactivity was observed with the anti-TgOocyst serum *vs.* the anti-rTgERP serum even with the conventional ELISA plate system. The abundance of TgERP in the preparations inoculated to the rabbits could explain, at some extend, differences in reactivity within ELISA conditions between anti-rTgERP and anti-TgOocyst, but not the differences between batches and ELISA plate systems for a specific serum control. These differences are more likely to be related to the possible conformational variation during TgERP expression in procaryotic cells or to the fact TgERP is an intrinsically disordered protein, lacking a defined structure (Arranz-Solís et al., 2023), which could impact the immune response on an individual level (Uversky and Van Regenmortel, 2021), but also the epitope exposure.

Accordingly, these results give evidence of the importance of including the anti-Oocyst antibody as positive control, which can help in the screening of oocysts-specific proteins with putative source attribution value. Despite the promising results obtained with B1 and conventional ELISA plates, no IgG seroconversion was recorded with pig serum samples (Panels 1 and 2). A similar scenario was observed when attempting to detect specific IgA antibodies (data not shown). These results corroborate the results published by López-Ureña et al. (2023b), but contradict other studies also conducted with TgERP employing sera from pigs experimentally infected with oocysts and tissue cysts, in which positive reactivity was exclusively observed in oocyst-infected pigs, with detectable antibodies until 8 months post-infection (Hill et al., 2011). Nevertheless, limited conclusions could be drowned from the rest of studies that used TgERP in serological tests since they were exclusively conducted with human sera (Burrells et al., 2016; Mangiavacchi et al., 2016; Vieira et al., 2015), who could be reinfected with different *T. gondii* stages through their lifetime.

Variation between different recombinant protein batches and ELISA plate systems were not tested in previous studies (Burrells et al., 2016; Hill et al., 2011; Mangiavacchi et al., 2016; Vieira et al., 2015) and was not observed with a *T. gondii* recombinant multiepitope peptide antigen in a serological test developed for pigs (Song et al., 2021). Curiously, when the selected TgERP batch was employed a year later in the ELISA test, no reactivity or lower reactivity was observed with serum samples that previously tested positive with the same batch, including positive controls (data not shown). Unfortunately, the variation between and within batches hampers results reproducibility. Again, these outcomes could be explained by the intrinsically disordered characteristic of TgERP, lacking a defined structure (Arranz-Solís et al., 2023).

In conclusion, TgERP lacks value to identify oral oocyst-derived infections. It is highly recommended to include an anti-TgOocyst antibody as a positive control in step-by-step workflows, as the followed herein, for future studies aimed at developing and validating oocyst-attributing serological tests.

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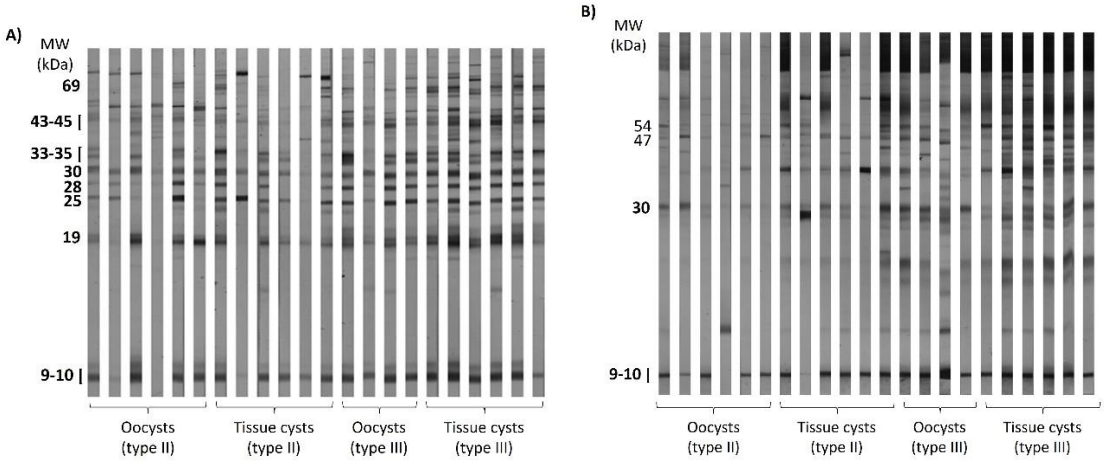
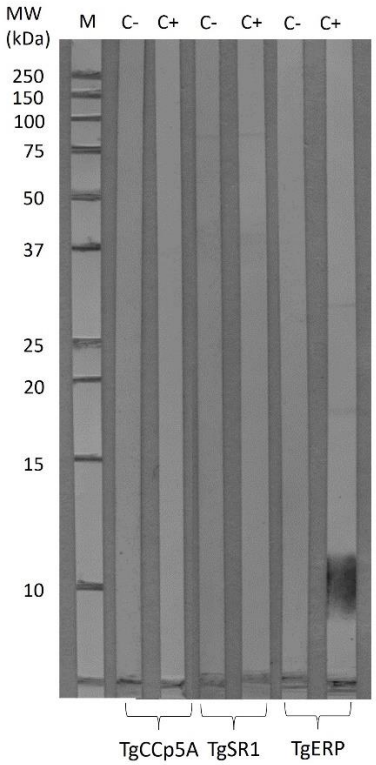
Appendix 2. Reactivity of anti-*Toxoplasma gondii* IgGs on Western blot tests based on *Toxoplasma gondii* tachyzoites **(A)** or sporulated oocysts soluble extract **(B)** in pigs experimentally infected with oocysts or tissue cysts from type II and type III isolates at six weeks post-infection.



Note: the sporulated oocysts soluble extract-based Western blot test needs to be improved to dissolved more efficiently wall structures and insoluble antigens.

Appendix 3. Reactivity of the rabbit polyclonal antibody produced against *Toxoplasma gondii* sporulated oocysts lysate (anti-TgOocyst) in a Westen blot tests based on TgCCp5A, TgSR1 and TgERP proteins.



MW: molecular weight, M: molecular weight marker, C-: pre-immune serum control, C+: rabbit polyclonal antibody produced against sporulated oocysts lysate (anti-TgOocyst). Predicted molecular weight of each protein: 50-62 kDa for TgCCp5A, 62-70 kDa for TgSR1 and 11-13 kDa for TgERP.