UNIVERSIDAD DE MURCIA

ESCUELA INTERNACIONAL DE DOCTORADO

Advances in the serological diagnosis of *Toxoplasma gondii* and *Neospora caninum* infections in the field of animal health

Avances en el diagnóstico serológico de las infecciones por *Toxoplasma gondii* y *Neospora caninum* en el campo de la sanidad animal

Dña. Ana Huertas López

2022

UNIVERSIDAD DE MURCIA

ESCUELA INTERNACIONAL DE DOCTORADO

Advances in the serological diagnosis of *Toxoplasma gondii* and *Neospora caninum* infections in the field of animal health

Avances en el diagnóstico serológico de las infecciones por *Toxoplasma gondii* y *Neospora caninum* en el campo de la sanidad animal

Dña. Ana Huertas López

2022

UNIVERSIDAD DE MURCIA

FACULTAD DE VETERINARIA

"Advances in the serological diagnosis of *Toxoplasma gondii* and *Neospora caninum* infections in the field of animal health"

"Avances en el diagnóstico serológico de las infecciones por *Toxoplasma gondii* y *Neospora caninum* en el campo de la sanidad animal"

Memoria presentada por la licenciada en Veterinaria

Ana Huertas López

Para optar al grado de Doctor en Ciencias Veterinarias con Mención Internacional

Directores:

José Joaquín Cerón Madrigal[1]
Carlos Martínez-Carrasco Pleite[2]
Gema Álvarez García[3]

Tutor:

José Joaquín Cerón Madrigal[1]

[1]Laboratorio Interdisciplinar de Análisis Clínicos (Interlab-UMU), Departamento de Medicina y Cirugía Animal, Facultad de Veterinaria, Campus de Excelencia Internacional "Mare Nostrum", Universidad de Murcia, España.

[2]Departamento de Sanidad Animal, Facultad de Veterinaria, Campus de Excelencia Internacional "Mare Nostrum", Universidad de Murcia, España.

[3]Departamento de Sanidad Animal, Facultad de Veterinaria, Universidad de Complutense de Madrid, España.

Acknowledgements

Tengo mucho que agradecer, y a muchas personas, que durante estos cuatro años “y pico” de tesis me han ayudado tanto. Sin todos vosotros, este trabajo no hubiera sido posible.

En primer lugar, a mis directores de tesis, José, Gema y Carlos. Tres personas muy diferentes y cada una me ha aportado una visión muy valiosa de este mundo académico y de investigación. Gracias a sus distintos puntos de vista esta tesis es mucho más completa, y me han mostrado el éxito de la colaboración entre grandes investigadores, a los cuales admiro muchísimo. José, tu ilusión por la investigación es contagiosa, y gracias por tratar siempre de buscar soluciones cuando todo parece perdido. Gema, gracias por tu infinita paciencia, en especial con la revisión. Gracias también por tus grandes ideas para resolver los problemas con los antígenos, y por tu ayuda para buscar muestras, hacer análisis, y lo que hiciera falta. Aunque hayamos estado en la distancia, te he sentido siempre cerca. Carlos, aún no sé qué fue lo que viste, que tenías tan claro que yo tenía que dedicarme a la investigación, cuando yo te decía “¡pero a mí lo que me gusta es la clínica!”. Pero gracias por verlo y por animarme a meterme en este “lío maravilloso” que ha sido la tesis. Y gracias por tu enorme apoyo y ayuda en todos estos años, y por esas sugerencias para dar clases, participar en concursos, proyectos de innovación, cursos y un largo etcétera. Eres un magnífico docente, me encanta tu forma de enseñar y espero algún día poder seguir tus pasos. Me has hecho ver todo el potencial del mundo académico e investigador y por ello te estaré eternamente agradecida.

También quiero agradecer a la profesora Silvia Martínez Subiela toda su ayuda y consejos durante el desarrollo de esta tesis. Ha sido como una cuarta directora, porque cuando había cualquier problema con la *fluo*, una llamada rápida y ahí estaba Silvia para solucionarlo. Y a todos mis compañeros y amigos que han pasado por Interlab a lo largo de estos años: Camila (mi roca), Ana Cantos (mi mentora, junto a Silvia, de la fluorometría), Damián (el consejero, tanto de laboratorio como de futuro), Marina (mi ejemplo de constancia y esfuerzo, y encima, más buena que el pan), Sandra (mi salvadora, que evitaba que colapsara con el Akta y los WB), Alberto (otro tanto con el

Bradford y los WB), Lorena (trabajadora incansable y la más rockera de la proteómica), Mariló (podemos competir en despistes, pero me ganas en lo "máquina" que eres), María José (mi fabulosa compañera de viaje por Ciudad Real), Luis RD (siempre de buen humor y el que trae las mejores galletas de República Dominicana), Alba, Asta, Gregorio (¡*team leish*!), Luis Pardo, Fran, Adrián, Carmen, Isa y Teresa. Y por supuesto, al Capitán del Pléyades, Ángel, y a Rocío y Angelita. Podría pasar días escribiendo todo lo que les tengo que agradecer a cada uno. ¡Muchísimas gracias a todos!

También quiero agradecer a todos los compañeros del Departamento de Sanidad Animal, Moisés, Clara, María Ortuño, Irene, Franky, Pablo, y a los profesores Laura del Río, Rocío Ruiz de Ybáñez, Francisco Alonso y Eduardo Berriatua, por su inestimable ayuda durante las prácticas y para formarme como docente. Y a todos los alumnos de Enfermedades Parasitarias de 3º de veterinaria, pues ha sido un placer darles clase, en especial a los de este curso 2021/2022 (que me conocen como la profesora McGonagall), y a mis alumnos de TFG Adrián y Ruth.

Al profesor Luis Miguel Ortega Mora de la Universidad Complutense de Madrid, por facilitarnos muestras para los estudios, y a los compañeros de Saluvet de la Universidad Complutense de Madrid, que me han ayudado enormemente en los diferentes estudios: Rober (mil gracias por toda tu ayuda con la "eterna" revisión), Nadia, Ángela, Carlos y Elvis. ¡Muchas gracias todos!

Muchas gracias al Dr. Francisco Javier Ibáñez de la Sección de Apoyo Estadístico del Área Científica y Técnica de Investigación de la Universidad de Murcia, por su valiosa ayuda en los meta-análisis y su paciencia para explicar los fundamentos estadísticos que empleamos. También me gustaría agradecer a los veterinarios D. Pedro Vallejo y Dña. María García, y al ganadero D. Antonio Martínez de "Las Pitufas" por su amabilidad y colaboración en la recogida de muestras durante esta tesis.

En las estancias en centros nacionales durante esta tesis conocí a personas maravillosas, que son también grandes investigadores: los profesores Ignacio García Bocanegra, Antonio Rivero y David Cano, y los doctorandos Sabrina, Débora y Javier, de la Universidad de Córdoba, así como al Dr. José de Jesús de la Fuente García y a la infatigable y tenaz doctora Marinela Contreras del IREC (Ciudad Real). Muchas gracias a

todos por enseñarme tanto, por vuestra paciencia conmigo y por hacerme sentir como una más del equipo. A través del grupo de la Universidad de Córdoba también tuve el honor de conocer "virtualmente" a los grandes expertos en *Toxoplasma* y *Neospora*, la profesora Sonia Almería y el profesor Jitender P. Dubey, a los que admiro y agradezco enormemente su ayuda y colaboración en el estudio de seroprevalencia.

My research stay in Thailand was one of the most incredible experiences in my life. I am grateful to Professor Woraporn Sukhumavasi (Aja Kwan), Babi Kyi Soe (Babi), Korrakod Pramsopee (Sky), Fai, Mai, Tu, My, Karlo, Dear, Mint, Dee and all the people at the Parasitology Unit (Chulalongkorn University). Aja Kwan, thank you for your warm hospitality during my stay. You taught me about toxoplasmosis, and also about Buddhism and your fascinating Thai culture. I fondly remember the days spent with you and your family ka. I am also grateful to my sister Babi, who share almost all her time with me during my stay. You helped me in my research, taking and processing samples, but we also travelled and spend our free time together (mainly at the Hot-Pot restaurant 5555). Babi, you were there even in the darkest moments and I love you for that. Sky, you were also always there for your Spanish friend, sharing wonderful moments at the "cafés" and museums, with the "Zumba" class or the Thai (culture and language) lessons, and I am very grateful for that. Khob khun na ka! I miss you all and I hope I will see you again soon.

También me gustaría agradecer a la Universidad de Murcia su apoyo, pues a través del Plan Propio ha financiado esta tesis (contrato predoctoral para la Formación del Profesorado Universitario R-1207/2017).

Durante estos años he recibido un incalculable apoyo de mis amigas. De Fuen, con la que he pasado momentos que voy a recordar toda mi vida. Desde tan increíbles como el descubrimiento del garbanzo hasta los más sencillos. Siempre estás ahí, pase lo que pase, y yo siempre estaré para ti. De mis amigas de la universidad, Eva, Isa, Ylva, Emilia, Alicia, Aranza y Marta, que, aunque estemos cada una por un lado, siempre aprovechamos cada oportunidad para vernos y ponernos al día. De mis chicas de la Cruz Azul, Fini y Ana, y a Juan, que me animaron a que siguiera estudiando, aunque tuviera que irme (cosa que me costó horrores, y por eso voy a verlos siempre que puedo). Y

también de Marta, con su optimismo y energía positiva. De Cristina y Antonio, que nos conocimos por casualidad, y por casualidad nos hicimos vecinos, y que son las personas más altruistas y generosas que conozco. De mi "cuñaica" Mery y mis "concuñados" Elena y Dani, que me han animado a seguir adelante como ellos hacen, sin rendirse. Os quiero mucho a todos.

Y como buena veterinaria, también tengo que agradecer a mis amigos animales, que me han acompañado toda mi vida: Whisky, Pongo, Mel, Nala, Shandy y Sira. Shandy, que has supervisado la escritura de la tesis de pe a pa, y me has ayudado a relajarme con tus ronroneos. Nala, que me acompañaste a lo largo de la carrera, soportaste estoicamente que practicase contigo y estuviste presente en los buenos y malos momentos. Has sido mi mejor amiga y siempre estarás conmigo. *Así que gracias por estar, por tu amistad y tu compañía. Eres lo mejor que me ha dado la vida.*

Camila, a ti te tengo que dedicar un párrafo aparte, y lo sabes. Bueno, también incluyo a Antonio, Figo (ay, meu Figo!) y Gora, que sois los cuatro maravillosos y os quiero. Minha irma, has sido mi gran apoyo durante estos años. Hemos compartido vivencias, alegrías y llantos. Siempre sabes calmarme y hacerme ver las cosas con perspectiva cuando más lo necesito. Y ya sabes cuánto admiro tu valentía, fortaleza y diligencia para todo lo que te propones. Gracias por ser tan maravillosa. ¡Cómo me alegro de que la tesis nos haya puesto en el mismo camino! Y de seguir recorriéndolo juntas, superando cualquier cosa que nos depare el futuro. Te quero muito!

A Tomás, mi compañero de vida, y sus padres, Tomás y Rosa, y a su hermana María. Tomás, desde que nos conocimos (allá por 2006) me has apoyado en todo lo que me he propuesto: en la carrera, el máster, el internado, la clínica, y ahora, en el doctorado. Tú me animaste a seguir mis sueños y siempre has creído en mí. Gracias por estar siempre, incluso en la distancia. Por recorrerte medio planeta para verme (esas 40 horas de viaje Perú -> Tailandia). Por aguantar mis ataques de estrés estos últimos meses. Y siempre con actitud positiva, viendo el lado bueno de las cosas. Me haces feliz cada día, y por eso te quiero, te amo, y te doy las gracias.

Y, por último, a mi maravillosa familia. Tengo la suerte de teneros, y si no fuera por vosotros, yo no estaría aquí. Os agradezco a todos, a "los Huertas" y a "los López",

a los que están y a los que se fueron, pero que tenemos presente. A mis titos Ana y Evaristo, Cecilia y Miguel, Paloma y Carlos y a mis primos Luis, Carlos, Miguel, María, Jorge Santiuste, Alejandro (Alo), Jorge Molina y Carlitos, que siempre os habéis alegrado cuando os enviaba un nuevo artículo publicado ("lectura ligera para el finde"). A mis titos Luis y Esther, por todas esas cenas y vuestra cálida acogida cuando más lo necesitaba, acompañadas siempre de muy buenos consejos. A mi madrina, Encarni, a la que quieroy admiro como la maravillosa profesora que es. Al tito Ramón, que además de ser un gran veterinario (porque, aunque esté jubilado, veterinario se es para siempre), lleva toda la vida apoyando a la "nenica", al igual que Isabel. A mi abuela Quiqui, que fuiste laprimera mujer de la familia en estudiar una carrera, y siempre he admirado tu entusiasmo por aprender, viajar y descubrir el mundo a cualquier edad. A mi abuelo Paco que, aunque al principio preferías que hiciera Medicina, cuando viste la ilusión queme hacía y las buenas notas que sacaba, te sentías orgulloso de mí y me lo demostrabascon cariño. Y a mi abuelica Encarna que, poniendo velas a los santos cada vez que teníaun examen, y llamándome casi todos los días, siempre estabas ahí para escucharme y animarme en lo que necesitara. Os echo de menos cada día. A mis padres, Toñi y Paco, que me han guiado toda mi vida, levantándome cada vez que tropezaba y enseñándomea valorar lo que de verdad importa. Sois mi ejemplo a seguir. Y muy en especial, a mi hermano Paco, la persona a la que más admiro y agradezco su ayuda durante estos años.Con tu paciencia para ayudarme en lo que necesitara, con cada problema que ha surgido, con tu entusiasmo cada vez que recibía una buena noticia, siempre estás ahí. ¡Manico! No sé qué haría sin ti. No sabéis lo afortunada que me siento de que seáis mi familia. Os quiero mucho.

Por todo lo que recibí

estar aquí vale la pena.

Pau Donés

A mi familia.

Happiness can be found,

even in the darkest of times,

if one only remembers to turn on the light.

Albus Dumbledore

INDEX

Abstract

Abstract

Introduction: toxoplasmosis and neosporosis are two parasitic diseases caused by the Apicomplexan parasites *Toxoplasma gondii* and *Neospora caninum*, respectively, which are globally distributed. Toxoplasmosis is a zoonosis and a main cause of reproductive failure in small ruminants with the subsequent high economic impact. *Neospora caninum* is not a zoonotic pathogen, but it is a major cause of abortion in cattle and is also associated to reproductive failures in sheep. Currently, diagnosis of these diseases is commonly based on serological methods, but it could be improved by the application of a more sensitive technique such as Time-resolved fluorescence immunoassays (TRFIA).

Material and methods: this PhD thesis comprises three main activities: (1) a systematic review and a meta-analysis on the development of new serological methods to diagnose toxoplasmosis in humans and animals, focused on their One Health approach followed and the critical analysis of the validation process; (2) a study of the seroprevalences rates and risk factors associated to *T. gondii* infection in dogs and cats from an anthropized area (Bangkok, Thailand); and (3) the development and validation of new TRFIAs for detection of anti-*T. gondii* antibodies in cat and goat sera, and anti-*N. caninum* antibodies in sheep serum and milk samples. In cats, several recombinant and a recombinant chimeric antigens were evaluated by this technique. Analytical validation consisted of estimating of the inter- and intra-assay precision, analytical sensitivity (Se), accuracy and cross-reactivity with closely related pathogens. Diagnostic validation was assessed by the evaluation of the positive/negative discriminative potential of the technique (Mann Whitney U test), a ROC analysis (for determination of the optimal cut-off, Se, specificity -Sp- and area under the curve), the agreement (using the *kappa* value) and the Spearman's correlation coefficient.

Results: there is a lack of implementation of the One Health approach in the studies on serological diagnosis of toxoplasmosis, especially in those conducted in humans and by

physician-based research teams. The validation process of the newly developed methods is widely heterogeneous and the exclusive evaluation of the diagnostic performance of the assays, without considering the analytical characteristics, is frequent. The seroprevalence rates of *T. gondii* infection in cats and dogs from Bangkok were 18.7% and 7.9%, respectively. A TRFIA was developed to detect anti-*T. gondii* antibodies in cats, with the chimeric antigen TgSAG1-GRA8 providing a better diagnostic performance in comparison with other antigens. In addition, a TRFIA based on the same *T. gondii* chimeric antigen was developed to diagnose *T. gondii* infection in goats with excellent results in the analytical Se, and high Se and Sp in the ROC analysis. However, the cross-reactivity detected with specific anti-*N. caninum* antibodies limits the use of this technique. Finally, a TRFIAs based on recombinant NcGRA7 antigen was successfully developed and validated to detect anti-*N. caninum* antibodies in ovine blood sera and full-cream milk samples, with high analytical and diagnostic performances.

Discussion and conclusions: this thesis provides new and up-to-dated knowledge on the development of serological methods to diagnose *T. gondii* infection in animals and humans,highlighting the need of implementation of the One Health approach and of a close following of the different steps of the validation process recommended by the OIE. The high seroprevalence of *T. gondii* found in dogs and catsfrom Bangkok should be considered a public health concern in this anthropized area. In addition, the new TRFIA developed in this thesis increase the Se of the current diagnostic methods for the detection of *T. gondii* and *N. caninum* infections and was adapted to non-invasive samples, although future research should include the evaluation by TRFIA of new *T. gondii* antigens avoiding the cross-reactivity with *N. caninum*.

Abstract

Resumen

Resumen

a) Introducción

En la actualidad, la importancia del enfoque One Health está cada vez más presente en todas las áreas de investigación relacionadas con la salud (Gibbs, 2014; Rabinowitz et al., 2017). Este enfoque se centra en el estudio de las enfermedades teniendo en cuenta los componentes relacionados con el ser humano, los animales domésticos y silvestres, así como el medio ambiente que comparten (Okello et al., 2011). Esta estrecha interrelación hace necesaria una aproximación interdisciplinar para estudiar y controlar las enfermedades (Okello et al., 2011; Horton and Lo, 2015; Rabinowitz et al., 2017; Krecek et al., 2020). El enfoque interdisciplinar de One Health cobra una mayor importancia en las zoonosis, especialmente en áreas antropizadas, donde la coexistencia de humanos y otros animales puede facilitar la transmisión de este tipo de enfermedades infecciosas y parasitarias (Thompson, 2013; Schmiege et al., 2020).

Por otro lado, la detección de individuos positivos es esencial para llevar a cabo medidas de prevención y control efectivas y, de esta forma, poder controlar la expansión de la enfermedad (Erdman and Kain, 2008; Gilman et al., 2012; Gutiérrez-Expósito et al., 2017). Además, los estudios epidemiológicos son necesarios para saber la distribución de un patógeno en un área geográfica concreta, así como para identificar los posibles factores de riesgo para las personas y/o animales (Thrusfield et al., 2018). Para todo ello, es fundamental contar con técnicas de diagnóstico fiables que permitan realizar muestreos en poblaciones amplias y obtener un diagnóstico temprano, y así poder tomar las medidas de control precisas (Ince and McNally, 2009; Gutiérrez-Expósito et al., 2017; Thrusfield et al., 2018). En este sentido, se suelen emplear las técnicasserológicas por su facilidad de uso, su habitual alta fiabilidad y su excelente relación calidad/precio (Liu et al., 2015; Wyrosdick and Schaefer, 2015; Thrusfield et al., 2018).

En los últimos años existe la tendencia de desarrollar técnicas automatizadas y

altamente sensibles para mejorar la detección de individuos verdaderamente positivos, y evitar los falsos positivos (Ince and McNally, 2009; Wyrosdick and Schaefer, 2015). También se está optando por usar muestras no invasivas, como la leche o la saliva, que tienen numerosas ventajas sobre las muestras invasivas: recolección más simple, evitar el estrés y el dolor en el muestro, así como reducir los costes en los programas de detección (Pritchard, 2001; Robertson and Nicholson, 2005; Samaranayake, 2007; Brinkhof et al., 2010; Khaitan et al., 2015; Valinetz and Cangelosi, 2021). Sin embargo, debido a la menor cantidad de anticuerpos que hay en este tipo de muestras en comparación con la sangre (Pritchard, 2001; Khaitan et al., 2015), se necesitan técnicas altamente sensibles para evitar errores en el diagnóstico.

Centrándonos en las enfermedades parasitarias estudiadas en esta tesis doctoral, la toxoplasmosis y la neosporosis son dos enfermedades distribuidas globalmente que tienen un gran impacto económico y sanitario. Están causadas por los parásitos Apicomplexa *Toxoplasma gondii* y *Neospora caninum*, respectivamente (Dubey and Schares, 2011; Dubey, 2016). Ambos protozoos están filogenéticamente relacionados, y tienen un genoma de tamaño similar y grupos de proteínas ortólogos (Lorenzi et al., 2016; Gondim et al., 2017). Los hospedadores definitivos de *T. gondii* son los félidos, y pueden tener un amplio rango de hospedadores intermediarios, desde aves a mamíferos, incluyendo al ser humano (Dubey, 2016; Attias et al., 2020). En el caso de *N. caninum*, los cánidos son los hospedadores definitivos, y tienen, hasta el momento, un menor número de especies identificadas como hospedadores intermediarios (bóvidos, ovejas, cabras, caballos, gatos y otras especies domésticas y silvestres) (Dubey and Schares, 2011; Lindsay and Dubey, 2020).

La toxoplasmosis tiene una gran importancia para salud pública, al ser una zoonosis mundialmente distribuida, lo que implica un elevado coste médico para su diagnóstico y tratamiento (Rahmanian et al., 2020). En humanos, *T. gondii* puede producir abortos, malformaciones fetales, y también problemas oculares y neurológicos, sobre todo en niños infectados congénitamente y pacientes inmunocomprometidos (Dubey, 2016). Además, la toxoplasmosis está asociada a un alto impacto económico en las granjas, especialmente en las de pequeños rumiantes, en los que puede producir

fallos reproductivos (Dubey, 2016; Dubey et al., 2020b; 2020c). En el hospedador definitivo, la infección por *T. gondii* suele ser asintomática, pero los gatitos infectados congénitamente o aquellos gatos adultos inmunodeprimidos pueden padecer problemas respiratorios, digestivos, cutáneos, oculares y neurológicos (Dubey et al., 2020a). Por lo que respecta a la neosporosis, no es una zoonosis, y su impacto está asociado principalmente a las cuantiosas pérdidas económicas causadas por el fallo reproductivo en rumiantes. De hecho, *N. caninum* es la principal causa de abortos en bovinos (Dubey et al., 2007), y también se ha detectado un aumento en la presencia de este parásito en granjas de pequeños rumiantes (Rodrigues et al., 2020; Romanelli et al., 2021).

El diagnóstico de ambos parásitos normalmente se basa en técnicas serológicas, probablemente por su disponibilidad comercial y la facilidad de uso para estudios en grandes poblaciones (Liu et al., 2015; Sinnott et al., 2017; Ybañez et al., 2020b). Las técnicas más empleadas para la detección de anticuerpos anti-*T. gondii* y anti-*N. caninum* son las técnicas de aglutinación, la técnica de inmunofluorescencia indirecta (IFAT) y el ensayo inmunoenzimático (ELISA) (Wyrosdick and Schaefer, 2015; Sinnott et al., 2017). Estas técnicas suelen basarse en antígenos nativos (Liu et al., 2015; Wyrosdick and Schaefer, 2015; Sinnott et al., 2017), que son difíciles de estandarizar y tienen un elevado coste y una preparación laboriosa (Rostami et al., 2018; Ybañez et al., 2020b). Por ello, hay un aumento reciente del desarrollo de técnicas basadas en antígenos recombinantes y recombinantes quiméricos (en adelante, solo quiméricos) (Liu et al., 2015; Sinnott et al., 2017; Rostami et al., 2018; Ybañez et al., 2020b).

Las técnicas serológicas presentan limitaciones. Por ejemplo, existe una gran variabilidad en la sensibilidad y especificidad en los ELISAs dependiendo del antígeno usado, siendo normalmente mayor con antígenos nativos o con el uso combinado de antígenos recombinantes que con un solo antígeno recombinante (Liu et al., 2015; Sinnott et al., 2017). Por otra parte, el desarrollo de técnicas como ELISA e IFAT a veces está limitado por la falta de conjugados específicos de especie y la presencia de reactividad cruzada con otros parásitos Apicomplexa (Dubey and Lindsay, 1996; Wyrosdick and Schaefer, 2015; Sinnott et al., 2017). Otra desventaja del IFAT es la

inherente subjetividad en la interpretación de sus resultados (Dubey and Lindsay, 1996; Liu et al., 2015; Wyrosdick and Schaefer, 2015; Sinnott et al., 2017). Por otro lado, las técnicas de aglutinación, que no requieren conjugados específicos de especie, tienen una sensibilidad variable, y también pueden ser subjetivas, dependiendo su interpretación de la experiencia del personal que la emplea (Seefeldt et al., 1989; Sroka et al., 2008; Wyrosdick and Schaefer, 2015).

Por tanto, los objetivos de esta tesis son:

1. Revisar sistemáticamente y meta-analizar la bibliografía científica sobre el desarrollo de técnicas serológicas para el diagnóstico de la infección por *T. gondii*. Dentro de este punto, se persiguen dos subobjetivos:
    a. Describir y comparar las características de los artículos de investigación llevados a cabo en los campos de la salud humana y animal, averiguando si el desarrollo de técnicas serológicas para diagnosticar la infección por *T. gondii* se realiza desde una perspectiva One Health.
    b. Analizar las características técnicas y la validación analítica y diagnóstica de las técnicas serológicas evaluadas en los artículos publicados, de acuerdo con la guía de validación planteadas por la Organización Mundial de Sanidad Animal (OIE) para animales, identificando posibles lagunas y proponiendo mejoras para futuros estudios.
2. Estudiar la seroprevalencia y los factores de riesgo asociados a la infección por *T. gondii* en perros y gatos de Bangkok (Tailandia), un área urbana donde estas especies antropogénicas y los humanos coexisten estrechamente.
3. Desarrollar y validar una nueva técnica altamente sensible, la Fluorometría en tiempo retardado (TRFIA) para la detección de anticuerpos anti-*T. gondii* en muestras de suero sanguíneo de gatos y cabras.
4. Desarrollar y validar una nueva TRFIA altamente sensible para la detección de anticuerpos anti-*N. caninum* en suero sanguíneo y leche de ovejas.

b) Capítulo 1

En este capítulo se realizó una revisión sistemática y meta-análisis sobre el desarrollo de técnicas serológicas para el diagnóstico de toxoplasmosis en humanos y

animales.

Material y métodos

La revisión sistemática y meta-análisis se llevó a cabo siguiendo las pautas establecidas en el informe PRISMA-P (Page et al., 2021). En primer lugar, se llevó a cabo una amplia búsqueda en bases de datos científicas (Web of Science, Scopus y PubMed) con los términos MeSH "toxoplasma OR toxoplasm*" y "diagnosis". Se seleccionaron artículos publicados entre 2014 y 2019 sobre el desarrollo de técnicas de diagnóstico serológico de la infección por *T. gondii* en humanos y animales domésticos y silvestres. Tras aplicar los criterios de inclusión, los artículos restantes fueron sometidos a una revisión de calidad mediante la herramienta QUADAS 2 (Whiting, 2011). Los artículos con riesgo bajo o medio de sesgo fueron sometidos a la extracción de datos y meta-análisis. Se analizaron variables relacionadas con datos bibliométricos, objetivos del estudio, población de estudio, técnicas evaluadas y de referencia, características de las técnicas evaluadas y proceso de validación. El análisis estadístico fue realizado con el programa R y se consideró significación estadística cuando $p > 0.05$.

Resultados

Tras aplicar los criterios de inclusión, se obtuvo un total de 191 artículos. De entre ellos, solo un 38.2% (73/191) de artículos tuvo un riesgo bajo o medio en la revisión de calidad mediante la herramienta QUADAS 2 y fueron finalmente seleccionados para continuar la revisión. Los artículos presentaron una gran heterogeneidad. En concreto, en relación a las características bibliométricas, objetivos del estudio y técnicas evaluadas y de referencia, hubo muchas diferencias entre los artículos realizados por investigadores médicos y veterinarios, y también entre aquellos llevados a cabo en muestras dehumanos y en muestras de animales. Además, solo un artículo aplicó la perspectiva One Health, desarrollando una técnica de diagnóstico en animales domésticos y silvestres.

Médicos, profesionales diferentes a médicos y veterinarios, y equipos interdisciplinares, así como estudios realizados en seres humanos, usaban con mayor frecuencia técnicas comerciales como referencia. Al contrario, los estudios realizados por veterinarios y en animales solían usar técnicas no comerciales. Nuestros resultados

sugieren que el concepto “gold standard” es malinterpretado por los autores, especialmente en el caso de investigadores médicos y otros profesionales. Se detectaron varias áreas de mejora en relación a las características de las técnicas evaluadas, especialmente relacionadas con la falta de algunos datos importantes en una proporción considerable de los estudios, que pueden limitar su reproducibilidad. Por otro lado, también se detectó una elevada heterogeneidad en el proceso de validación, especialmente entre artículos realizados en humanos y animales. Muy pocos estudios llevaron a cabo una validación analítica de las técnicas, mientras que la mayoría determinaron la sensibilidad y especificad diagnóstica. Los estudios hechos en animales determinaron con mayor frecuencia la concordancia entre la técnica evaluada y la técnica de referencia, mientras que en los realizados en humanos usaron el coeficiente de correlación en su lugar.

c) Capítulo 2

El objetivo de este estudio fue estimar la seroprevalencia y los factores de riesgo asociados a la infección por *T. gondii* en perros y gatos de un área antropizada como Bangkok (Tailandia).

Material y métodos

Se analizaron muestras de plasma sanguíneo de 318 perros y 321 gatos para detectar anticuerpos anti-*T. gondii* mediante la técnica de aglutinación modificada (MAT), con un punto de corte de 1:25. Además, se realizó un estudio longitudinal en 18 perros y 20 gatos durante junio y julio de 2019.

Resultados

La seroprevalencia detectada fue de 7,9% (25/318; 4,9-10,8% 95% CI) en perros y de 18,7% (60/321; 14,4-23.0% 98% CI) en gatos. En perros, se identificaron como factores de riesgo las razas mestizas y vivir totalmente en exterior. En gatos, solo el aumento de edad se determinó como factor de riesgo. En el estudio longitudinal, no se detectó ninguna seroconversión, y los títulos de los animales positivos se mantuvieron constantes.

d) Capítulo 3

En este capítulo se evaluaron varios antígenos recombinantes (TgGRA7, TgGRA7 truncado, TgSAG2 y TgSAG2 truncado) y uno quimérico (TgSAg1-GRA8) para el desarrollo de una TRFIA para la detección de anticuerpos frente a *T. gondii* en gatos. Además, se desarrolló y validó una TRFIA basada en un antígeno quimérico TgSAG1-GRA8 (TgSAG1-GRA8-TRFIA) para la detección de anticuerpos anti-*T. gondii* en cabras.

Material y métodos

Para la detección de anticuerpos anti-*T. gondii* en plasma de gatos, se emplearon 21 muestras seropositivas y 80 seronegativas de las analizadas en el capítulo anterior mediante MAT, y confirmadas mediante un ELISA comercial. Se desarrollaron 5 TRFIAs, uno para cada antígeno (cuatro recombinantes y uno quimérico), y los resultados de cada uno de ellos fue comparado con los obtenidos con MAT y ELISA como referencia. Se analizó el potencial discriminatorio entre positivos y negativos mediante un test U de Mann Whitney, y se calcularon los puntos de corte óptimo, sensibilidad y especificidad mediante análisis ROC para cada una de las técnicas desarrolladas, así como el coeficiente de correlación de Spearman con los resultados del ELISA comercial. Por último, todas las muestras fueron analizadas mediante un IFAT para la detección de anticuerpos anti-*N. caninum*, y aquellas muestras positivas fueron excluidas del análisis estadístico para evitar el efecto de una posible reactividad cruzada.

En cabras, se desarrolló la técnica TRFIA basada en el antígeno quimérico TgSAG1-GRA8 empleando 205 muestras de suero (100 seropositivas y 105 seronegativas). Se llevó a cabo una validación analítica de la técnica, estudiando la precisión intra- e inter-ensayo, la sensibilidad analítica (a través del cálculo del límite de detección y el límite de cuantificación), la exactitud (mediante la linealidad bajo dilución y el test de recuperación) y la reactividad cruzada con *N. caninum* (usando otros 31 sueros de ovejas seropositivas a *N. caninum* y seronegativas a *T. gondii*). Posteriormente, se evaluó el potencial para discriminar entre muestras positivas y negativas y se calculó el punto de corte óptimo, la sensibilidad y la especificidad respecto a un ELISA *in house* y un WB usados como referencia. También se analizó la concordancia (kappa) y la correlación de Spearman con las técnicas de referencia.

Resultados

En el estudio realizado en gatos, la TRFIA basada en el antígeno quimérico TgSAG1-GRA8 (TgSAG1-GRA8-TRFIA) mostró una mayor discriminación entre las muestras positivas y negativas ($p < 0,001$), así como una mejor sensibilidad (93,6%) y especificidad (89,5%) para el punto de corte óptimo (293803 cuentas por segundo o CPS), en comparación con los otros TRFIAs basados en antígenos recombinantes. Además, TgSAg1-GRA7-TRFIA tuvo una concordancia considerable (kappa = 0,78) y una correlación moderada significativa (coeficiente de Spearman = 0,62; $p < 0,001$) comparando con las técnicas de referencia. Por último, cuatro gatos tuvieron un resultado positivo en el IFAT para el diagnóstico de neosporosis.

Respecto a la TgSAG1-GRA8-TRFIA desarrollado en cabras, mostró una alta precisión, sensibilidad analítica y exactitud. El análisis ROC obtuvo un punto de corte óptimo de 217,4 unidades de fluorometría para *T. gondii* (UFT), con un 92% de sensibilidad y un 90,48% de especificidad. Esta técnica tuvo una elevada concordancia con las técnicas de referencia (*kappa* = 0,83), así como una correlación positiva y significativa (coeficiente de Spearman = 0,75; $p < 0,001$). Sin embargo, la TgSAG1-GRA8-TRFIA tuvo reacciones cruzadas con anticuerpos anti-*N. caninum*. Esta reactividad cruzada del antígeno TgSAG1-GRA8 fue confirmada mediante WB, así como la presencia de reactividad con anticuerpos anti-*N. caninum* usando el antígeno recombinante TgSAG1.

e) Capítulo 4

En el último capítulo de esta tesis, se desarrolló y validó analítica y diagnósticamente una TRFIA basada en el antígeno recombinante NcGRA7 (NcGRA7-TRFIA) para la detección de anticuerpos anti-*N. caninum* en suero sanguíneo y leche entera de ovejas.

Material y métodos

Para el desarrollo y validación de la técnica NcGRA7-TRFIA en suero ovino, se empleó un total de 346 muestras de suero sanguíneo (208 provenientes de ovejas infectadas experimentalmente, 117 de un rebaño de ovejas de leche con una historia clínica previa de abortos asociados a *N. caninum* y 21 sueros negativos). Se analizaron

otras 22 muestras de suero de ovejas infectadas por *T. gondii* y seronegativas a *N. caninum* para estudiar la reactividad cruzada de la técnica. Para la técnica NcGRA7-TRFIA en leche, primero se emplearon 47 muestras de leche de ovejas seropositivas y 26 seronegativas a *N. caninum* para seleccionar el mejor protocolo de desnatado, comparar la NcGRA7-TRFIA usando leche desnatada y entera, y realizar la validación analítica. Después, se analizaron muestras de leche entera de 23 ovejas seropositivas y 75 seronegativas para la validación diagnóstica de la técnica.

La validación analítica de los nuevos ensayos para cada tipo de muestra se realizó mediante la evaluación de la precisión intra- e inter-ensayo, de la sensibilidad analítica (mediante la estimación del límite de detección y el límite de cuantificación), de la exactitud (mediante la linealidad bajo dilución y el test de recuperación) y de la reactividad cruzada con *T. gondii*.

Para continuar la validación diagnóstica de la NcGRA7-TRFIA en suero, las muestras del rebaño de ovejas se emplearon para calcular el punto de corte, la sensibilidad y la especificidad diagnósticas mediante un análisis ROC, y se evaluó la concordancia (valor *kappa*) y la correlación (coeficiente de Spearman) entre la NcGRA7-TRFIA y dos ELISAs, uno comercial y otro *in house*. Además, se comparó la cinética de anticuerpos en las ovejas con infección experimental por *N. caninum* entre la NcGRA7-TRFIA y el ELISA *in house* mediante la prueba de Wilcoxon para pares relacionados.

En el caso de la validación diagnóstica de la NcGRA7-TRFIA en leche, se estimaron los mismos parámetros (punto de corte, sensibilidad y especificidad mediante análisis ROC; kappa y coeficiente de correlación de Spearman) usando como referencia los resultados de la NcGRA7-TRFIA y el ELISA *in house* en suero.

Resultados

La técnica GRA7-TRFIA mostró una adecuada precisión, sensibilidad analítica y exactitud para detectar anticuerpos anti-*N. caninum* en suero y leche entera de ovejas. No se detectó reactividad cruzada con *T. gondii*.

El punto de corte establecido para la NcGRA7-TRFIA en suero fue de 62,68 Unidades de Fluorometría para *N. caninum* (UFN), con una sensibilidad del 100% y un

95,35% de especificidad. Por otro lado, esta técnica detectó la seroconversión hasta 7 días antes que con el ELISA *in house*. La técnica NcGRA7-TRFIA en suero tuvo una concordancia casi perfecta con el ELISA comercial (*kappa* = 0,96) y una correlación positiva y estadísticamente significativa con el ELISA *in house* (coeficiente de Spearman = 0,81; $p < 0,0001$).

El punto de corte, sensibilidad y especificidad de la NcGRA7-TRFIA en leche entera fue diferente en función de si la técnica de referencia era la NcGRA7-TRFIA en suero (punto de corte óptimo 84,9 UFN; 100% de sensibilidad y 98,1% de especificidad), el ELISA *in house* en suero (punto de corte óptimo 123,9 UFN; 90,7% de sensibilidad y 91,3% de especificidad), o los resultados coincidentes de ambas técnicas en suero (punto de corte óptimo 97,9 UFN; 100% de sensibilidad y 100% de especificidad). Además, la NcGRA7-TRFIA en leche tuvo una concordancia casi perfecta con la técnica en suero (*kappa* = 0,98) y con los resultados coincidentes de ambas técnicas de referencia (*kappa* = 1,00), mientras que también fue considerable con el ELISA *in house* (*kappa* = 0,69). La correlación de Spearman fue mayor con el GRA7-TRFIA en suero (coeficiente de Spearman = 0,82; $p < 0,0001$) que con el ELISA *in house* (coeficiente de Spearman = 0,52; $p < 0,0001$).

f) Discusión general

Los resultados de la revisión sistemática evidencian que el enfoque One Health necesita ser integrado en la investigación sobre el diagnóstico de toxoplasmosis, con la participación en esos estudios de distintas disciplinas científicas. Esto sería esencial para el diseño y la aplicación efectiva de medidas de para el control de la toxoplasmosis. Además, esta revisión destaca la necesidad de desarrollar un protocolo consensuado y estandarizado para la validación de técnicas serológicas para la detección de anticuerpos anti-*T. gondii* en humanos y animales.

El segundo capítulo de esta tesis pone de manifiesto la circulación de este parásito en un área donde existe un estrecho contacto entre humanos y perros y gatos, especialmente por la práctica de cuidado comunitario de perros y gatos callejeros, muy extendida en la ciudad de Bangkok (Jittapalapong et al., 2007; Savvides, 2013; Toukhsati et al., 2015). Dada la posible liberación de ooquistes a lo largo de la vida de los gatos (si

se dan ciertas condiciones de inmunosupresión) (Dubey et al., 2020a), y el papel como vector mecánico de ooquistes de los perros (ya sea en su pelaje o tras su ingestión y liberación en sus heces) (Frenkel et al., 2003; Schares et al., 2005; Cong et al., 2018), la considerable presencia de *T. gondii* detectada en áreas urbanas y peri-urbanas de esta ciudad puede suponer un riesgo para la salud pública.

A pesar de que la secuencia empleada para producir los antígenos recombinantes TgGRA7 y TgSAG2 (y los obtenidos tras su truncado) fueron seleccionadas para evitar la posible reactividad cruzada con *N. caninum* (Goodman, 2006), la escasa discriminación entre positivos y negativos, así como su baja sensibilidad y especificidad, limita el uso de estos antígenos para el diagnóstico de toxoplasmosis felina. Estos antígenos fueron evaluados en otros estudios con mejores resultados (Huang et al., 2002; Cai et al., 2015; Abdelbaset et al., 2017; Salman et al., 2018). El peor rendimiento de los antígenos producidos en esta tesis puede deberse al uso de un Sistema de expresión diferente, ya que este sistema puede afectar a la antigenicidad y al desempeño diagnóstico de los antígenos (Letourneur et al., 2001; Marti et al., 2002; Ybañez et al., 2020b). Sin embargo, esta es la primera vez que se evalúa el antígeno quimérico basado en epítopos de las proteínas TgSAG1 y TgGRA8 para la detección de anticuerpos anti-*T. gondii* en gatos, con resultados prometedores. Con respecto a los cuatro gatos seropositivos al IFAT de *N. caninum*, solo hay un estudio previo que buscara estos anticuerpos en gatos en Tailandia, no encontrando ninguno positivo (Arunvipas et al., 2012). Por tanto, esta es la primera vez que se detectan anticuerpos anti-*N. caninum* en gatos en Tailandia.

Los resultados de validación diagnóstica de la TgSAG1-GRA8-TRFIA para el diagnóstico de la toxoplasmosis caprina fue similar a otros ELISAs basados en antígenos quiméricos y desarrollados previamente para la detección de anticuerpos anti-*T. gondii* en ovejas (Holec-Gąsior et al., 2019), pero fue mayor que otras técnicas basadas en el recombinante TgSAG1 o en la mezcla de TgSAG1 y TgGRA7 (Velmurugan et al., 2008; Bachan et al., 2018). Asimismo, esta es la primera vez que una técnica serológica ha sido validada para detectar anticuerpos anti-*T. gondii* en cabras infectadas de forma natural (Dubey et al., 2020b). Sin embargo, dada la reactividad cruzada detectada con laTgSAG1-

GRA8-TRFIA, este antígeno no debería ser empleado para el diagnóstico de toxoplasmosis caprina, a menos que se realizase el serodiagnóstico de la infección por *N. caninum* de forma paralela. Por otro lado, y dado que ambos parásitos tienen una relación filogenética estrecha que puede favorecer la presencia de estas reacciones cruzadas inespecíficas (Gondim et al., 2017), los resultados de este estudio ponen de manifiesto la importancia de evaluar la reactividad cruzada entre *T. gondii* y *N. caninum* en las técnicas serológicas empleadas para el diagnóstico de estas enfermedades en el campo de la sanidad animal.

Por último, en esta tesis se han desarrollado dos TRFIA basados en el antígeno recombinante NcGRA7 (NcGRA7-TRFIA) para la detección de anticuerpos anti-*N. caninum* en suero y leche entera de oveja. Los excelentes resultados de sensibilidad y especificidad obtenidos en suero son similares a otros estudios en los que desarrollaron ELISAs para el diagnóstico de neosporosis ovina (Andreotti et al., 2009; Pinheiro et al., 2015) y bovina (Aguado-Martínez et al., 2008). Además, no se detectó reactividad cruzada en los sueros de ovejas seropositivas a *T. gondii* y seronegativas a *N. caninum*. Por otro lado, uno de los resultados más llamativos de este estudio fue la detección más temprana, con hasta 7 días de antelación, de la seroconversión en ovejas experimentalmente infectadas con *N. caninum* usando la técnica NcGRA7-TRFIA comparando con un ELISA *in house* basado en extracto a partir de taquizoítos enteros (antígeno nativo).

Estos excelentes resultados, incluyendo una elevada sensibilidad analítica y diagnóstica, llevaron a desarrollar la técnica NcGRA7-TRFIA para la detección de anticuerpos anti-*N. caninum* en leche de ovejas, debido a que normalmente este tipo de muestras presenta menor cantidad de anticuerpos que las muestras de suero (Pritchard, 2001; Khaitan et al., 2015). Además, la TRFIA no se afecta por la presencia de lipemia o hemólisis en la muestra (Parra and Cerón, 2007). Esta es la primera vez que se valida una técnica serológica para el diagnóstico de neosporosis en leche entera de ovejas. Solo hay un estudio previo en el que desarrollaron una técnica para detectar anticuerpos anti-*N. caninum* en ovejas, pero emplearon leche desnatada (Tamponi et al., 2015). En vacas sí se ha desarrollado un ELISA para muestras de leche entera, pero obtuvieron una baja sensibilidad (Byrem et al., 2012). Por tanto, la NcGRA7-TRFIA desarrollada en este

estudio no solo tiene las ventajas inherentes al uso de una muestra no invasiva, como la simplicidad de recolección, evitar dolor y estrés en los animales muestreados, o reducir el coste (Pritchard, 2001; Robertson and Nicholson, 2005; Samaranayake, 2007; Brinkhof et al., 2010; Khaitan et al., 2015; Valinetz and Cangelosi, 2021), sino que también evita el proceso de desnatado que puede alargar el tiempo y el coste del estudio.

g) Conclusiones

1. Los resultados obtenidos en la revisión sistemática y meta-análisis sobre el desarrollo de técnicas serológicas para detectar anticuerpos anti-*T. gondii* en humanos y animales destaca la necesidad de realizar una evaluación de la calidad de los estudios antes de llevar a cabo cualquier análisis estadístico para minimizar la introducción de errores y sesgos en los análisis. Así, se identificaron sesgos en la selección de los sujetos de estudio, en la especificación del punto de corte de la técnica evaluada y en el flujo del estudio mediante la herramienta QUADAS 2, y un total de 61,8% de estudios evaluados fueron descartados.
2. Hay margen de mejora para la aplicación de la perspectiva One Health en los estudios sobre el desarrollo de técnicas serológicas para diagnosticar la infección por *T. gondii*, lo cual es esencial para diseñar y ejecutar medidas efectivas de intervención para el control de la toxoplasmosis. En este campo solo hubo un 42,5% de colaboración entre investigadores pertenecientes a distintas ramas del conocimiento, y los veterinarios tuvieron más en cuenta la necesidad de abordar el estudio de la toxoplasmosis desde un punto de vista One Health que los médicos.
3. El proceso de validación de las técnicas serológicas para la infección por *T. gondii* mostró una elevada heterogeneidad tanto en medicina humana como en veterinaria, y no siguió la guía planteada por la OIE. La descripción de los protocolos y reactivos usados en las técnicas evaluadas podría mejorarse, especialmente en los casos en los que el tipo de antígeno, el isotipo de anticuerpo, el uso de controles y el análisis de muestras por duplicado no se especificaba; la sensibilidad analítica apenas fue estudiada y el concepto de “gold standard” fue malinterpretado.
4. La considerable seroprevalencia de *T. gondii* en gatos y perros en un área

antropizada como Bangkok (Tailandia) indica que este parásito zoonótico circula en ambientes urbanos donde existe un estrecho contacto entre humanos y estas especies. Por tanto, nuestros resultados evidencian la existencia de un riesgo para la salud pública y puede servir de base para diseñar estrategias de intervención apropiadas en el área de estudio y en otras áreas sinantrópicas con factores de riesgo epidemiológico similares. Además, el biobanco de suero obtenido sirvió para el desarrollo de una nueva técnica serológica en gatos.

5. Se desarrollaron nuevas Fluorometría en tiempo retardado (TRFIAs) para el diagnóstico serológico de las infecciones por *T. gondii* y *N. caninum* en hospedadores relevantes, tales como gatos, cabras y ovejas, y mostraron buen rendimiento diagnóstico. Para seguir un enfoque One Health, en el caso del serodiagnóstico de la infección por *T. gondii*, las TRFIAs se emplearon en hospedadores definitivos e intermediarios que pueden ser fuente de infección para los humanos.
6. La detección de anticuerpos anti-*T. gondii* mediante TRFIA en gatos mostró que no todos los antígenos (TgSAG1-GRA8, TgGRA7, TgGRA7 truncado, TgSAG2 y TgSAG2 truncado) funcionan tan bien como se espera. Por otro lado, la TRFIA basada en el antígeno quimérico TgSAg1-GRA8 tuvo una excelente sensibilidad analítica y rendimiento diagnóstico para la detección de anticuerpos anti-*T. gondii* en gatos y cabras.
7. La reactividad cruzada detectada entre TgSAG1 y los anticuerpos anti-*N. caninum* limita el uso de técnicas serológicas basadas en este antígeno para el diagnóstico de la infección por *T. gondii* al menos en cabras. Esta reactividad cruzada debería ser considerada cuando se evalúe la utilidad diagnóstica de cualquier antígeno en aquellas especies en las que ambos parásitos Apicomplejos hayan sido descritos.
8. La TRFIA basada en el antígeno recombinante NcGRA7 (NcGRA7-TRFIA) obtuvo resultados destacables en la validación analítica y diagnóstica para la detección de anticuerpos anti-*N. caninum* en suero ovino, sin reactividad cruzada con anticuerpos anti-*T. gondii* y con una detección más temprana de la seroconversión de ovejas infectadas experimentalmente comparando con un

ELISA basado en extracto a partir de taquizoítos enteros.

9. La NcGRA7-TRFIA también mostró una elevada sensibilidad para la detección de anticuerpos anti-*N. caninum* en muestras de leche enterade ovejas. Esta técnica tiene las ventajas asociadas al uso de muestras no invasivas que son fáciles y rápidas de recoger, permitiendo su uso para estudios serológicos a gran escala. En el futuro, esta técnica podría ser adaptada para otros rumiantes hospedadores, como cabras o vacas.

General introduction

General introduction

a) The One Health approach for zoonotic diseases

Nowadays, the importance of the One Health approach is gradually more considered in all the research areas related to health (Gibbs, 2014; Rabinowitz et al., 2017). This approach is focused on the relationship between humans, domestic and wild animals and the environment they share (Okello et al., 2011) (Figure 1). In this regard, animal, human and environmental health are interconnected, so an interdisciplinary approach is necessary in order to better design appropriate intervention strategies against diseases (Okello et al., 2011; Horton and Lo, 2015; Rabinowitz et al., 2017; Krecek et al.,2020).

Figure 1. Interacting health domains in One Health: humans, wildlife, domestic animals, and environment.

In the wake of globalization, climate change, environment degradation and human population increase, a higher number of emerging infectious diseases has been recently detected (Keusch et al., 2009; Okello et al., 2011; Horton and Lo, 2015), as we have experienced with the coronavirus disease 2019 (COVID-19) pandemic, the Severe Acute Respiratory Syndrome coronavirus (SARS-CoV) and Middle East Respiratory Syndrome (MERS) in the last years (Schmiege et al., 2020). Some of these emerging diseases are zoonoses (Feldmann et al., 2002; Kruse et al., 2004) and have a crucial importance in public health,such as COVID-19, West Nile virus infection, avian influenza, MERS, Ebola, H1N1 influenza and AIDS, among others (Hemelaar, 2012; Rabinowitz et al., 2017; Schmiege et al., 2020). Other non-emerging zoonoses, including infections by bacteria (e.g.,*Mycobacterium* spp., *Brucella* spp., *Salmonella* spp., *Campylobacter jejuni* and *Escherichia coli*), viruses (e.g., rabies) and parasites (e.g., *Plasmodium* spp., *Leishmania* spp., *Trichinella* spp., *Echinococcus* spp., *Giardia* spp. and *Toxoplasma gondii*) have alsoa huge impact on public and animal health worldwide (Okello et al., 2011; Thompson, 2013; Ding et al., 2018; EFSA and ECDC, 2018; Wang et al., 2021).

Zoonoses can be transmitted from animals to humans through different ways: by direct contact (airborne transmission; close contact with domestic animals or exposure to wildlife), by indirect contact (when animals contaminate the environment with pathogens, including soil, water and fomites; iatrogenic transmission), by arthropod vectors (vector-borne diseases) and by consumption of animal products or of contaminated water or vegetables (food-borne diseases) (Schweon and Vitale, 2020). The interdisciplinary One Health approach becomes particularly significant in this type of infectious diseases, especially in anthropized areas, where the coexistence of humans and other animals can facilitate the transmission of zoonoses (Thompson, 2013; Schmiege et al., 2020). For example, the community animal-keeping in urban and peri-urban areas could suppose a risk for public health. This practice consists in feeding stray dogs and cats without adopting them, so these animals have not usually a veterinary control with the subsequent risk of the spread of zoonotic infections. The practice is common in Buddhist countries such as Thailand (Savvides, 2013; Toukhsati et al., 2015), and is also frequent to take care of cat and dog feral colonies in cities from developed countries, with the involved risk of getting zoonotic infections (de Wit et al., 2020;

Genchi et al., 2021).

b) The importance of an accurate serological diagnosis in infectious diseases

Accurate serological diagnostic techniques are necessary to know the real distribution of pathogens in a specific geographical area, to identify potential risk factors for humans and/or animals, to detect potential carriers of pathogens and for an early diagnosis. Thus, serological results can facilitate the design and implementation of appropriate intervention strategies. In this way, the spread of the disease can be avoided, and subsequent control of the disease can be achieved (Erdman and Kain, 2008; Ince andMcNally, 2009; Gilmanet al., 2012; Gutiérrez-Expósito et al., 2017; Thrusfield et al., 2018). Serological techniques are commonly performed in human and veterinary medicine due to their easiness of use, normally high reliability and their cost-effectiveness (Liu et al., 2015; Wyrosdick and Schaefer, 2015; Thrusfield et al., 2018). These techniques detect antibodies against the studied pathogen in biological samples from individuals, and thusprovide information about the present or past contact with the pathogen (Thrusfield etal., 2018).

On the other hand, in recent years there is a trend to develop automated and highly sensitive diagnostic techniques in order to avoid subjectivity in the interpretation of results; thus, the aim is to improve the detection of true-positive animals and avoid false-positive results (Ince and McNally, 2009; Wyrosdick and Schaefer, 2015). In addition, there is a current tendency to use non-invasive samples, like milk or saliva, that have several advantages over the invasive ones, such as simplicity of collection, less stressful and painful sampling (Pritchard, 2001; Robertson and Nicholson, 2005; Samaranayake, 2007; Khaitan et al., 2015; Valinetz andCangelosi, 2021) or the reduction of the costs of the screening programs (Pritchard, 2001; Brinkhof et al., 2010; Khaitan et al., 2015). However, diagnostic techniques performed on non-invasive samples sometimes show less sensitivity (Se) than those carried out on invasive samples, having low agreement between both type of samples (Valinetz and Cangelosi, 2021). This could be due to thefact that antibody concentrations are lower in milk and saliva samples than in blood sera (Pritchard,2001; Khaitan et al., 2015). Therefore, the development of highly sensitive techniques (Samaranayake, 2007; Khaitan et al., 2015), as well as an accurate

optimization processto adapt the technique to the type of sample, and an appropriate validation process including the use of a well-coded reference panel of samples (Kaufman and Lamster, 2002) is required for the implementation of the use of non-invasive samples in the serological diagnosis.

c) Toxoplasmosis and neosporosis: general concepts

Toxoplasmosis andneosporosis, the targeted parasitic diseases of this PhD thesis, are worldwide distributed diseases associated to a high economic and health impact. They are caused by the Apicomplexan parasites *Toxoplasma gondii* and *Neospora caninum*, respectively (Dubey and Schares, 2011, Dubey, 2016). Both protozoa are included in the Sarcocystidae family and are phylogenetically closely related, with similar total genome size (62-65 Mb) and orthologous groups of proteins, such as the family of surface proteins (SAG) called SRS family (Lorenzi et al., 2016; Gondim et al., 2017).

Life cycle and transmission routes

Regarding the life cycle of *T. gondii* (Figure 2) and *N. caninum* (Figure 3), both have sexual reproduction exclusively in their respective definitive hosts, whereas they reproduce asexually in both the intermediate and the definitive hosts (Attias et al., 2020; Sokol-Borrelli et al., 2020). There are three infective stages during the life cycle of *T. gondii* and *N. caninum* (Dubey et al., 2002, 2007; Freppel et al., 2019; Attias et al., 2020; Lindsay and Dubey, 2020):

i. Sporozoite: after sexual reproduction, oocysts (with an approximate diameter 12 µm) are excreted by the definitive host through the feces and constitute a source of infection for other host after the sporulation, when two sporocysts, each of them containing four sporozoites, are formed inside oocysts.
ii. Tachyzoite: rapidly dividing intracellular stage by asexual multiplication.
iii. Bradyzoite: slowly dividing intracellular stage by asexual multiplication inside tissue cysts.

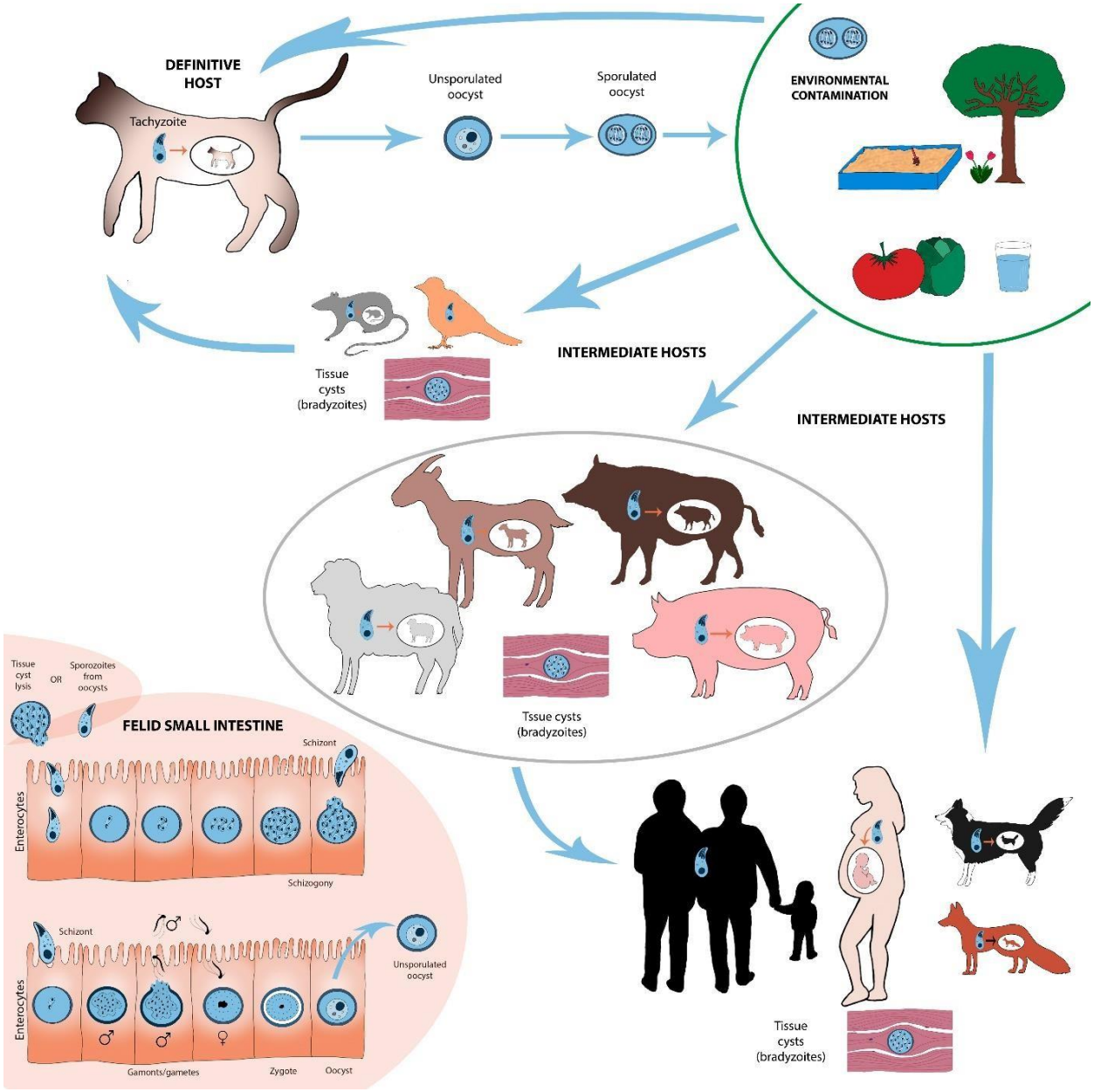


Figure 2. Toxoplasma gondii life cycle.

The definitive hosts of *T. gondii* are felids (family Felidae), while it could have a wide range of warm-blood vertebrates as intermediate hosts, including birds and mammals (Dubey, 2016; Attias et al., 2020). Dogs have been also described as mechanical carriers of *T. gondii*, due to their behaviour of rolling on cat's feces, carrying the sporulated oocysts in their fur (Frenkel et al., 2003), or even by their coprophagous behaviour. In this way, sporulated oocysts can remain intact through the digestive tract of the dog and continue being viable after passing in the dog's feces (Lindsay et al., 1997;

Schares et al., 2005; Cong et al., 2018). In the case of *N. caninum*, canids (dog, coyote, wolf and dingo) act as definitive hosts, with a narrower range of species identified as intermediate hosts, such as cattle, sheep, goats, horses, cats and other domestic and wild species (Dubey and Schares, 2011; Donahoe et al., 2015; Lindsay and Dubey, 2020).

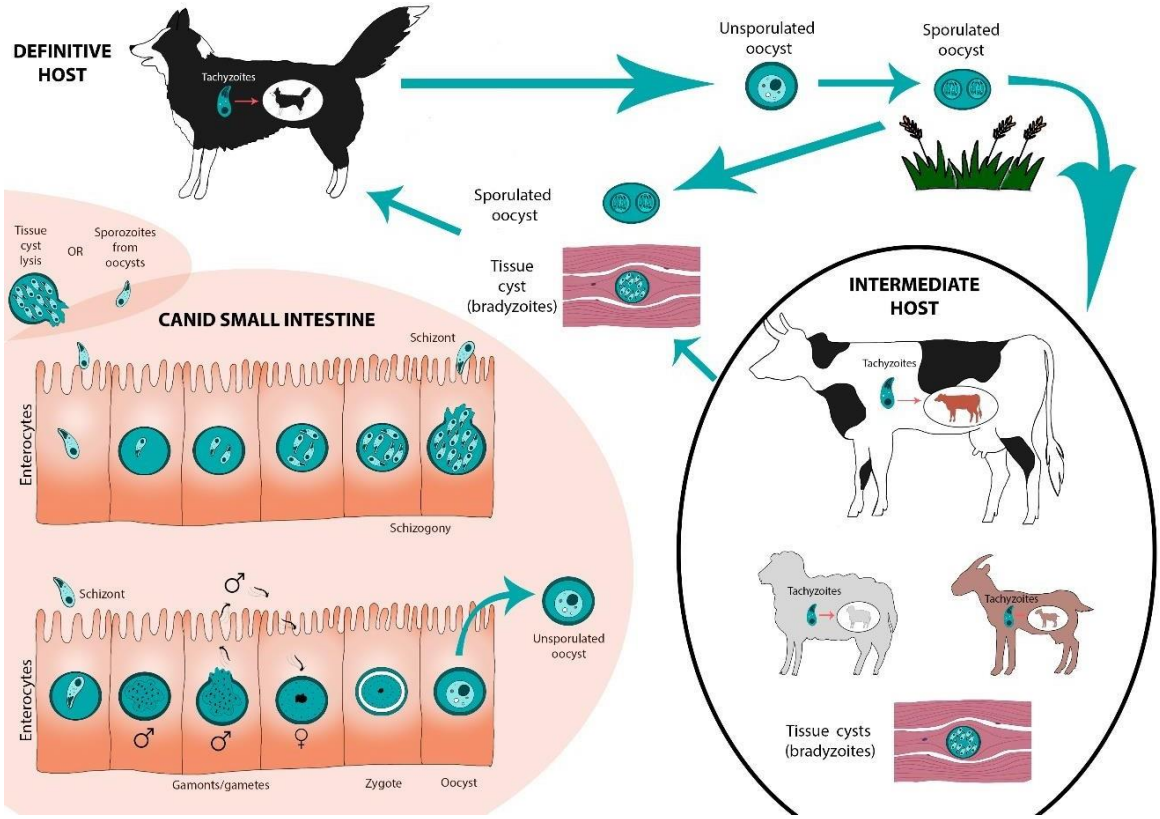


Figure 3. Neospora caninum life cycle.

In brief, the definitive and the intermediate hosts can be infected by the ingestion of cysts present in the tissues of other intermediate host (Figure 4), or by sporulated oocysts contaminating food or environment (Dubey et al., 2007; Attias et al., 2020). Vertical transmission of tachyzoites from the mother to the foetus or the neonate is also possible during gestation and peripartum period in both definitive and intermediate host (Dubey et al., 2007; Attias et al., 2020). Transplacental transmission of *T. gondii* and *N. caninum* could be endogenous, after the re-activation of the bradyzoites in the persistent infected mother's tissue cysts during pregnancy, or exogenous, after the consumption of sporulated oocysts during the pregnancy (Trees and Williams, 2005). However, there are some differences in these transmission routes

between *T. gondii* and *N. caninum*. Endogenous transplacental transmission is the most effective route of *N. caninum* infection in cattle, with fetal transmission ratesup to 95%, and it is responsible of the maintenance of the infection within cattle flocks (Williams et al., 2009; Donahoe et al., 2015). *Neospora caninum* remains for life in the tissues of the infected cows, and can be transmitted to the foetus over the consecutive pregnancies (Donahoe et al., 2015). On the contrary, transmission of *T. gondii* infection from persistent infected mothers is less frequent, being exogenous transplacental transmission the main route of vertical transmission of this parasite in sheep (Innes et al., 2009) and humans (Trees and Williams, 2005). On the other hand, although not being frequent, *T. gondii* infection can also be postnatally acquired via milk (Attias et al., 2020); whereas lactogenic transmission of *N. caninum* has not been confirmed (Dubey et al., 2007). Finally, another significant difference between *T. gondii* and *N. caninum* life cycles is the frequency of oocysts shedding by the definitive host. In this regard, felids can shed large amounts of *T. gondii* oocysts during some time after primoinfection (normally less than 21 days) and, less frequently, after a re-infection or under immunosuppressive conditions (Lappin, 2010; Dubey, 2016). Quite the opposite, canidscan shed *N. caninum* oocysts for a shorter period, 5 days of more, after primary infection(Dubey et al., 2007).

Figure 4. Dog (definitive host of Neospora caninum) eating placental tissues from cows (intermediate host), as potential source of infection. Photograph courtesy of Mr. Pedro J. Vallejo Mateo (DVM).

In the definitive host, the tissue cyst wall dissolute in the small intestine and the bradyzoites colonize the enterocytes (Dubey, 2016; Sokol-Borrelli et al., 2020). With

regards to *T. gondii*, after formation of several asexual generations of the parasite (schizogony), the gamogony is formed. Microgametes (male) and macrogametes (female) are then produced and the fertilization occurs when they are mature. Later, the oocyst containing the zygote disrupt the enterocyte wall and is excreted via feces (Dubey, 2016). Although little is known about the sexual cycle of *N. caninum* inside the enterocytes, it is supposed to happen similarly to *T. gondii*, and unsporulated *N. caninum* oocysts are also shed through feces (Sokol-Borrelli et al., 2020). In the environment, sporulation of *T. gondii* oocysts occurs within 1 to 5 days (Dubey, 2016), whereas *N. caninum* oocysts sporulate within 1 to 3 days (Lindsay et al., 1999). When the infection occurs by ingestion of sporulated oocysts of *T. gondii* or *N. caninum*, both in the definitive or the intermediate host, the ingested oocyst liberates the sporozoites which invade the lamina propria of the small intestine and differentiate to tachyzoites (Dubey, 2016; Sokol-Borrelli et al., 2020). Horizontal transmission by ingestion of tissue cysts is also possible in carnivorous intermediate hosts and, in this case, the bradyzoites liberated in the small intestine also differentiate to tachyzoites. Then, the tachyzoites rapidly multiplicate and spread through the organism, producing the acute infection. Tachyzoites are also able to cross the placental barrier, infecting the foetus (Sokol-Borrelli et al., 2020). Finally, the tachyzoites differentiate to bradyzoites, with slow asexual multiplication, and they form tissue cysts usually located in the nervous system and the skeletal or cardiac muscle (Dubey et al., 2007; Sokol-Borrelli et al., 2020).

Impact of toxoplasmosis and neosporosis in animal and public health

Toxoplasmosis has greater importance for public health because it is a globally distributed zoonosis that involves high medical costs worldwide regarding diagnosis and treatment (Rahmanian et al., 2020). In humans, *T. gondii* can produce abortions and fetal malformations, and also ocular and neurological disorders more commonly found in congenitally infected children and immunocompromised patients (Dubey, 2016). This parasite is considered to infect one third of the human population, with seroprevalences ranging from 30% to 60% in different geographical areas (Rahmanian et al., 2020).

Moreover, toxoplasmosis is associated to a high economic impact in farms, especially in small ruminant flocks, where this parasite is one of the main causes of reproductive failure (Dubey, 2016; Dubey et al., 2020b, 2020c). Seroprevalence in sheep

flocks is variable between countries and it increases with the age of the animal. For example, it ranged from 17.7% in France and 27.8% in The Netherlands, to 72.8% in Austria and 75.7% in Belgium, during nationwide screenings (Dubey et al., 2020c). In goats, nationwide serosurveys showed 5.1% of prevalence in Korea, 13.3% in The Netherlands, 17% in Norway and 73.3% in Serbia (Dubey et al., 2020b).

In the definitive host, *T. gondii* infection is usually asymptomatic, but congenitally infected kittens and immunocompromised cats could suffer from respiratory, digestive, cutaneous, ocular and neurological disorders (Calero-Berna and Gennari, 2019; Dubey et al., 2020a). The seroprevalence in cats is widely variable depending on the geographical area and the serological technique used for screening; for instance, it resulted in 7.2% in the USA by enzyme-linked immunosorbent assay (ELISA) and 41% in Norway by modified agglutination test (MAT) during nationwide surveys. Moreover, *T. gondii* seroprevalence in cats increases with the age of the animaland, in addition, it is normally higher in those cats living outdoors that can prey on intermediate hosts, mainly rodents and birds (Dubey et al., 2020a).

In dogs, clinical toxoplasmosis is normally associated to immunosuppressive conditions, such as co-infections with other pathogens or immunosuppressive drugs (Calero-Berna and Gennari, 2019; Dubey et al., 2020d). The most frequent clinical manifestations in this species are neurological, ophthalmological and cutaneous signs (Calero-Berna and Gennari, 2019). A high variability is also found in dogs when analyzing the seroprevalence of anti-*T. gondii* antibodies in different parts of the world (Dubey et al., 2020d). For example, it reaches a 70% in Brazil, estimated by indirect fluorescent antibody test (IFAT) (de Paula Dreer et al., 2013; Ruffolo et al., 2016), whereas in China, several studies performed by indirect hemagglutination assay (IHA) found less than 20% of prevalence (Li et al., 2012; Qian et al., 2015), although one study detected 52.9% of seropositive dogs by ELISA (Jiang et al., 2015).

On the other hand, neosporosis is not a zoonosis, so its impact is mainly related to the economic losses caused by the transmissible reproductive failure in ruminants, and especially in cattle farms. In fact, *N. caninum* is considered the main cause of abortions in cattle (Dubey et al., 2007). The overall seroprevalence of this parasite in cattle herds differs between continents: 8% in Oceania, 13% in Africa, 15% in Europe,

18% in Asia and 24% in America (Ribeiro et al., 2019). In addition, *N. caninum* provokes reproductive failure in small ruminants (Arranz-Solís et al., 2016; Romanelli et al., 2021), although it could have been underestimated and/or misdiagnosed in these host species over the years due to the cross-reactions with *T. gondii* (Gondim et al., 2017). Recent studies showed an overall *N. caninum* seroprevalence of 12% in sheep (Romanelli et al., 2021) and 5.99% in goats (Rodrigues et al., 2020).

In dogs, the definitive host of *N. caninum*, the infection may mostly cause neurological disorders, such as rigid hyperextension of the hind limbs, progressive paralysis or difficulty of swallowing (Dubey and Lindsay, 1996). According to a recent systematic review and meta-analysis, the estimated seroprevalence of *N. caninum* in dogs worldwide is 17.1% (Anvari et al., 2020).

d) Toxoplasmosis and neosporosis: diagnosis in humans and animals

Diagnosis of Toxoplasma gondii infection in humans

The detection of *T. gondii* infection in humans are normally performed by serological, molecular and imaging techniques (Wyrosdick and Schaefer, 2015; Ybañez et al., 2020b). Imaging techniques, such as computed tomography, magnetic resonance imaging, nuclear imaging and ultrasonography can be used to diagnose both cerebral and ocular toxoplasmosis in humans (Rostami et al., 2018). Molecular techniques, such as polymerase chain reaction (PCR), are commonly used to confirm the presence of *T. gondii* DNA in individuals with clinical toxoplasmosis (e.g., in amniotic fluid, cerebrospinal fluid and aqueous humour samples) (Wyrosdick and Schaefer, 2015; Rostami et al.,2018). There has been a recent increase in the development of molecular techniques, for example, Real Time-PCR (RT-PCR) or Loop-mediated isothermal amplification (LAMP) (Liu et al., 2015; Rostami et al., 2018). On the other hand, serological methods are normally preferred for screening during pregnancy and epidemiological studies. They can also be used for diagnosis of clinical toxoplasmosis, normally associated with a molecular method, because serological techniques are not always able to detect the infection in the foetus or brain of congenitally infected and immunocompromised people (Liu et al., 2015; Rostami et al., 2018).

Sabin-Feldman dye test and mice or cats bioassay are considered the gold standard techniques for detection of anti-*T. gondii* antibodies and for the parasite isolation, respectively (Liu et al., 2015; Wyrosdick and Schaefer, 2015; Ybañez et al., 2020b). However, their high cost and laborious proceduremakes these techniques more suitable for research purposes than for large epidemiological screening (Liu et al., 2015; Uddin et al., 2021). So, the most commonly employed serological technique for detection of anti-*T. gondii* antibodies in humans is ELISA, over latex agglutination test (LAT) or IFAT (Wyrosdick and Schaefer, 2015).

Diagnosis of Toxoplasma gondii infection in animals

Although molecular and histological methods are preferred to confirm the presence of *T. gondii* in cases of abortions in ruminants, or clinical toxoplasmosis in cats and dogs (Calero-Berna and Gennari, 2019; Dubey et al., 2020d; Lindsay and Dubey, 2020), serological methods are widely used in epidemiological studies and also in *T. gondii* control programs in domestic and wild animals (Dubey et al., 2020a, 2020c, 2020d; Hatam-Nahavandi et al., 2021).

Nowadays, cat or mice bioassays are also considered the gold standard techniques for confirming *T. gondii* infection (Liu et al., 2015). In general, MAT, ELISA and IFAT are the most frequently used serological techniques for the diagnosis of neosporosis in animals (Wyrosdick and Schaefer, 2015).

With regards to small ruminants, most of the epidemiological studies performed in these species, which were reviewed by Dubey (2020), were carried out by MAT, ELISA and IFAT. However, there is not any serological technique that has been accurately validated regarding a reference standard for the diagnosis of caprine and ovine toxoplasmosis (Dubey et al., 2020b; 2020c).

In cats, MAT and IFAT are also frequently used in large-scale screenings. MAT is highly specific and do not present cross-reactivity with other parasites. Other agglutination tests have shown less Se and specificity (Sp) than MAT for thediagnosis of feline toxoplasmosis. In addition, ELISA kits and rapid tests are also commercially available, with variable Se and Sp (Dubey et al., 2020a; Uddin et al., 2021).

Serodiagnosis of canine toxoplasmosis is normally performed by MAT and IFAT, with highly specific results. Moreover, ELISAs based on recombinant antigens, WB and immunocromatography (ICT) have been also developed (Dubey et al., 2020d).

Diagnosis of Neospora caninum infection in animals

The definitive diagnosis of abortions caused by *N. caninum* infection in ruminants and dogs can be made by the histological analysis of fetal tissues. The infection can be also detected by molecular methods, which are highly sensitive and specific, and can be used on fetal tissues, cerebrospinal or amniotic fluid, blood, etc. (Silva and Machado, 2016; Sinnott et al., 2017; Lindsay and Dubey, 2020). Several molecular methods have been developed for the diagnosis of neosporosis in ruminants, such as conventional PCR, RT-PCR, nested PCR and multiplex PCR (Sinnott et al., 2017). Quantitative RT-PCR and LAMP have also been described in dogs (Mahittikorn et al., 2017).

Regarding the serological diagnosis of bovine neosporosis, IFAT was initially considered the reference technique (Sinnott et al., 2017), although some authors argue that Western Blotting (WB) is a more sensitive and specific technique, so it should be used to confirmuncertain results (Dubey, 2003; Hu et al., 2011; Álvarez-García et al., 2013). Other serological tests, such as ELISAs based on the tachyzoite lysate (TLA), are commercially available, and several ELISAs based on recombinant antigens have been also developed (Atkinson et al., 2000; Aguado-Martínez et al., 2008; Sinnott et al., 2017). The Se obtained by these newly developed techniques varied regarding the antigen used (Sinnott et al., 2017). Nevertheless, other study comparing commercial ELISA kits for detection of bovine neosporosis found Se and Sp values over 95% (Álvarez-García et al., 2013). ELISA and IFAT are also commonly used in epidemiological studies on *N. caninum* infection in sheep and goats (Dubey andSchares, 2011; Rodrigues et al., 2020), although the variety of techniques and the number of available commercial assays are lower comparing with cattle.

In dogs, the most frequently used serological techniques are IFAT and ELISA. As for cattle, IFAT was initially considered the gold standard technique for the serodiagnosis of canine neosporosis. However, WB is recommended to confirm doubtful results (Silva and Machado, 2016).

e) Limitations of the current diagnostic techniques for toxoplasmosis and neosporosis in animals

Although PCR has a relatively high Sp values in the detection of *T. gondii*, its Se values are sometimes unproven (Wyrosdick and Schaefer, 2015). PCR is also a highly sensitive andspecific method for the detection of *N. caninum*, but its high cost has limited a wider use in some countries (Sinnott et al., 2017).

In addition, the most used serological methods for the detection of anti- *T gondii* and *N. caninum* antibodies have also limitations. In particular, in the case of ELISA technique (Atkinson et al., 2000; Liu et al., 2015; Wyrosdick and Schaefer, 2015; Gondim et al., 2017; Sinnott et al., 2017; Dubey et al., 2020a) the main limitations are:

- Variable Se and Sp values depending on the antigen used. Higher performance is usually obtained with TLA or with a combination of recombinant antigenscompared with the use of a single recombinant antigen.
- Lack of species-specific conjugates for wildlife.
- Presence of cross-reactivities between antigens and specific antibodies against other Apicomplexan parasites.

In the case of IFAT technique, the limitations that have been described (Atkinson et al., 2000; Dubey and Lindsay, 1996; Liu et al., 2015; Wyrosdick and Schaefer, 2015; Gondim et al., 2017; Sinnott et al., 2017) are as follows:

- Lack of species-specific conjugates for wildlife.
- Presence of cross-reactivities reactivities between antigens and specific antibodies against other Apicomplexan parasites.
- It is subjective, and its results interpretation could depend on the experience expertise and training of the staff.

Finally, with regard to agglutination tests, the limitations described (Seefeldt et al., 1989; Sroka et al., 2008; Wyrosdick and Schaefer, 2015) are:

- Variable Se values, being more sensitive MAT than LAT and IHA for the

detection ofanti-*T. gondii* antibodies.

- MAT is also subjective and its results interpretation could depend on the expertise and training of the staff.

f) Recombinant and recombinant chimeric antigens: a way to improve serological assays?

Serological methods such as IFAT, MAT and ELISA are often based on native antigens, which are directly obtained from the parasite, such as TLA or formalin fixed tachyzoites (Liu et al., 2015; Wyrosdick and Schaefer, 2015; Sinnott et al., 2017). Nevertheless, they are difficult to standardize and have a costly and time-consuming preparation (Holec-Gąsior et al., 2014; Liu et al., 2015; Rostami et al., 2018; Ybañez et al., 2020b), and keeping cultures of live tachyzoites are needed for their production (Zhou et al., 2019). Moreover, these native antigens can present cross-reactivity with other Apicomplexan parasites (Gondim et al., 2017; Sinnott et al., 2017).

Recombinant antigens are specific proteins obtained from the parasite after a process of encoding, cloning and expression. The most often used recombinant antigens are surface antigens (SAG), dense granule antigens (GRA), rhoptry antigens (ROP), matrix antigen (MAG) and microneme antigens (MIC) (Liu et al., 2015; Uddin et al., 2021). A particular type of recombinant antigens is the recombinant chimeric antigens (henceforth, chimeric antigens), which are composed by different antigenic epitopes from selected proteins (Uddin et al., 2021). Recombinant and chimeric antigens are easier to standardize and do not require the use of cultures of live tachyzoites (Liu et al., 2015; Rostami et al., 2018; Ybañez et al., 2020b), and thus, there is a recent increase in the development of serological techniques based on these types of antigens.

TgSAG1, TgSAG2 and TgGRA7 are the most frequently used *T. gondii* recombinant antigens for serodiagnosis in animals (Ferra et al., 2020; Uddin et al., 2021). In fact, TgSAG1 is commonly employed in commercial kits (Liyanage et al., 2021). However, there are many *T. gondii* recombinant and chimeric antigens have been tested in different animal species:

- In sheep (Ferra et al., 2020; Uddin et al., 2021):
  - Recombinant antigens: TgH11, which is a polypeptide part of the

TgGRA4 antigen (Tenter et al., 1994), showed 34%-43% Se and 89-100% Sp by ELISA, TgH4 by ELISA had 79% Se and 100% Sp, TgSAG1 by ELISA had 96% Se and 100% Sp, TgSAG2 by ELISA had 81.3-100% Se and 85.7-100% Sp, and TgGRA1 and TgROP1 by ELISA with 98-100% Se and 100% Sp.

- Mixture of recombinant antigens: TgH4 + TgH11, TgSAG1 + TgMIC1 + TgMAG1, TgSAG2 + TgGRA1 + TgROP1, TgGRA1 + TgGRA2 + TgGRA6, TgGRA1 + TgROP1, TgGRA1 + TgSAG2, TgSAG2 + TgROP1, TgGRA1 + TgSAG2 + TgROP1 have been evaluated by ELISA with good results (Se and Sp over 90% except from the mixture TgSAG1 + TgMIC1 + TgMAG1 that showed 77.9% Se).
- Chimeric antigens: MIC1-MAG1-SAG1S, MIC1-MAG1-SAG1L, SAG2-GR1-ROP1S, SAG2-GR1-ROP1L, GRA1-GRA2-GRA6, AMA1N-SAG2-GRA1-ROP1, AMA1C-SAG2-GRA1-ROP1, AMA1-SAG2-GRA1-ROP1 and SAG2-GRA1-ROP1-GRA2 by ELISA showed > 90% Se and Sp.

- In goats (Ferra et al., 2020; Uddin et al., 2021):
  - Recombinant antigens: TgSAG1 by ELISA obtained 92.7% Se and 90.7% Sp, and TgSAG2 by ELISA had 82.1% Se and 91.4% Sp.
  - Chimeric antigens: AMA1N-SAG2-GRA1-ROP1, AMA1C-SAG2-GRA1-ROP1, AMA1-SAG2-GRA1-ROP1, SAG2-GRA1-ROP1-GRA2 by ELISA showed variable Se (88.9%, 95.6%, 95.6% and 57.8%, respectively) and excellent Sp (>95% in all cases).

- In cattle (Ferra et al., 2020; Uddin et al., 2021):
  - Recombinant antigens: TgGRA7 by ELISA showed >95% of Se and Sp, whilst TgSAG2 by ELISA obtained 80-87.1% Se and 85.7-91.4% Sp.

- In cats (Ferra et al., 2020; Uddin et al., 2021):
  - Recombinant antigens: TgH4 by ELISA with 93-100% Se and 100% Sp and TgH11 by ELISA with 50-64% Se and 100% Sp, TgSAG1 by rapid diagnostic test (RDT) with 100% Se and 99.2% Sp, TgSAG2 by ELISA

with 91.9% Se and 88.1% Sp, TgGRA2 by ELISA with 27% Se and 96.5% Sp, TgGRA7 by ELISA and ICT with 35.1-100% Se and 89.9%-100% Sp, TgGRA6, TgGRA15 and TgMIC10 by ELISA with <90% Se and Sp.

- Mixture of recombinant antigens: TgH4 + TgH11 by ELISA, TgSAG1 + TgSAG2 + TgGRA6 by RDT and TgSAG1 + TgSAG2 by dynamic flow ICT (DFICT) have been evaluated, with Se and Sp over 90%. By ELISA, the mixtures GRA6 + GRA7, GRA2 + GRA7 and GRA2 + GRA6 + GRA7 + GRA15 showed Se and Sp <90%, while SAG2 + GRA7 and SAG2 + GRA6 had >90% Se and <90% Sp, and SAG2 + GRA2 + GRA6 + GRA7 + GRA15 had 89.2% Se and 95.4% Sp.
- Chimeric antigens: GRA2-GRA6-GRA7-GRA15 by ELISA obtained 70.3% Seand 86.1% Sp, while SAG2-GRA2-GRA6-GRA7-GRA15 by ELISA had 89.2%Se and 95.4% Sp.

- In dogs % (Dubey et al., 2020d; Ferra et al., 2020; Uddin et al., 2021):
  - Recombinant antigens: TgSAG1, TgGRA7 and TgMAG1 havebeen tested by ELISA with Se and Sp over 90%, whilst TgGRA1 by ELISA showed 81% Se and 95.4% Sp.
  - Mixture of recombinant antigens: TgSAG1 + TgSAG2 by DFICT had 96.2% Se and 98.6% Sp.

On the other hand, NcSRS2 and NcGRA7 recombinant antigens are often employed for serodiagnosis of *N. caninum* infection, especially in cattle and dogs (Sinnott et al., 2017), although several *N. caninum* recombinant and chimeric antigens have also been evaluated in animals:

- In cattle:
  - Recombinant antigens: NcSAG1, NcSRS2, Ncp40, NcSUb1, NcGRA2 and NcGRA7 have been evaluated by ELISA with high Se (>90%) and variable Sp (80-100%), when Se and Sp data were shown; NcSAG1 has been studied by LAT without providing Se and Sp data, while NcGRA6 by LAT showed 60% Se and 100% Sp; NcSAG1 by antigen print immunoassay (APIA) had 85% Se and 96% Sp; and NcSAG4 has been

also evaluated by WB (without providing Se and Sp data) (Sinnott et al., 2017).

- Chimeric antigens: a chimeric protein designed with 8 epitopes from two proteins (NCBI protein code: XP_003885442.1 and XP_003884964.1) wasevaluated by ELISA and showed 96.6% Se and 97.0% Sp (Pereira et al., 2020).

- In sheep (Sinnott et al., 2017):
  - Recombinant antigens: NcSRS2 by ELISA showed 100% Se and 94.5% Sp.
- In goats (Sinnott et al., 2017):
  - Recombinant antigens: NcMIC10 by ELISA had 90% Se and 100% Sp (onlywith 10 caprine sera).
- In dogs (Sinnott et al., 2017):
  - Recombinant antigens: NcSRS2 by ELISA obtained 100% Se and 93.3% Sp; NcGRA6 by LAT showed 60% Se and 100% Sp; and NcGRA2, NcGRA7 andNcPF were evaluated by ELISA but the studies did not provide Se and Sp data.

Justification

Justification

The main goal of this thesis has been the development of new serological techniques for the detection of anti-*T. gondii* and anti-*N. caninum* antibodies that offer advantages over the already available techniques. The choice of these parasitic infections is justified because they are worldwide distributed with significant impact in animal health. Moreover, toxoplasmosis is a relevant zoonosis and whether a One Health approach is being implemented in the serodiagnosis of this shared pathogen between humans and both domestic and wild animals should be elucidated.

For this purpose, the first step undertaken in this doctoral dissertation was to perform a deep literature research to identify and describe: (a) current trends in serological diagnostic techniques used to detect anti-*T. gondii* antibodies, and (b) the gaps in the implementation of the One Health strategy and the validation procedure, for the improvement of further development of diagnostic tests. In this regard,systematic reviews and meta-analyses have proven to be a useful tool to synthetize andhighlight the main findings, make comparisons and determine the gaps in knowledge that are needed to be filled (Ferreira González et al., 2011). This type of review consists on the answering of a question review through a wide literature search on scientific databases, the selection of potentially eligible articles based on the selection criteria, the quality assessment after which only the high-qualityarticles are chosen, the data extraction, the statistical analysis of the data and the interpretation of results (Ferreira González et al., 2011; Page et al., 2021). For diagnostic accuracy systematic reviews, the PRISMA statements are followed (Page et al., 2021), and quality assessment are commonly performed with QUADAS 2 tool (Whiting, 2011).

The second part of this PhD was focused on *T. gondii* infection to evaluate the seroprevalence and associated epidemiological risk factors in cats and dogs in an anthropized area,Bangkok (Thailand), where the common practice of community cat and dog keeping could suppose an additional public health risk (Savvides, 2013; Toukhsati et al., 2015). Seroprevalence of *T. gondii* infection had been previously estimated in cats

(Jittapalapong et al., 2007, 2010; Sukhumavasi et al., 2012) and dogs (Jittapalapong et al., 2007, 2009) from Bangkok. However, there was not any updated information on the serological status of *T. gondii* infection in these species studied at the same time. Regarding their special close relationship with humans, as well as the practice of community cat and dog keeping in this city, updated epidemiological studies on these species could serve to know the current state of the urban and peri-urban life cycle of *T. gondii* and, indirectly, to evaluatethe potential risk of infection for humans. Moreover, we employed MAT, that is one of the most used serological techniques in animals. The samples obtained in this study were also employed as areference panel of sera for the subsequent development and validation of a TRFIA to detect anti-*T. gondii* antibodies in cats in this PhD thesis.

In the third part of this thesis several TRFIAs were developed to increase sensitivity of current techniques for the detection of anti-*T. gondii* and anti-*N. caninum* antibodies. TRFIA has proven to be an accurate technique for diagnosis of other parasitic diseases, such as leishmaniosis, with high analytical Se values (Cantos-Barreda et al., 2017a, 2017b). This technique is based on the detection of the fluorescence emitted by a lanthanide chelate, europium^{3+}, which occurs when there is a complete union between the antigen, the primary antibody from the sample and the europium labeled-secondary antibody (Parra et al., 2004). In addition to its high Se, the results of this technique are not interfered with lipemia or hemolysis (Parra and Cerón, 2007). The use of TRFIA based on recombinant and/or chimeric antigens of *T. gondii* and *N. caninum*, which are easier to standardize (Liu et al., 2015), could be a new interesting approach for the diagnosis of toxoplasmosis and neosporosis in the field of animal health. On the other hand, the highly sensitive TRFIA could be a promising technique for its use on non-invasive samples for the diagnosis of *T. gondii* and *N. caninum* infections.

The new TRFIAs developed in this thesis were optimized and validated for the diagnosis of feline and caprine toxoplasmosis in serum samples, and ovine neosporosis in serum and milk samples. Cats were studied because of their key importance in the life cycle of *T. gondii*, as they are definitive hosts and can spread the disease via oocyst shedding (Dubey, 2010). In this regard, cats' serostatus is a public health concern because of the close contact of this felid with humans (Hatam-Nahavandi et al., 2021).

The choice of the goat as a study target in this PhD thesis was because *T. gondii* has a wide economic impact on small ruminant flocks; in addition, clinical signs are particularly severe in goats (Dubey et al., 2020b), where *T. gondii* is one of the main etiological causes of reproductive failure, and goats can also suffer fromrepeated abortions in successive pregnancies (Dubey, 1982; Sánchez-Sánchez et al., 2018), unlike sheep, that develops an immune response after primo-infection that protects it from subsequent abortions (Innes et al., 2009). Moreover, as previously mentioned in the introduction, there is a lack of validated serological techniques for thediagnosis of caprine toxoplasmosis in naturally infected goats (Dubey et al., 2020b). Finally, regarding the diagnosis of neosporosis, although this parasite has a greater economic impact in cattle farms, bovine neosporosis has been widely studied and thereare numerous serological and molecular techniques available (Lindsay and Dubey, 2020). However, the attention on ovine neosporosis has recently increased because of the economic losses associated to the reproductive failure caused by *N. caninum* (Arranz- Solís et al., 2016; Romanelli et al., 2021; Sánchez-Sánchez et al., 2021). In addition, it could have been misdiagnosed over the years, due to the cross-reactions with the closely related and highly prevalent *T. gondii* (Gondim et al., 2017), so specific serological methods are still necessary. The use of non-invasive samples, such as milk, for serodiagnosis of *N. caninum* infection in sheep would allow to perform large screenings in lactating animals, as this type of samples are easier and cheaper to collect, and are less painful and stressful for the animal (Pritchard, 2001; Robertson and Nicholson, 2005; Samaranayake, 2007; Brinkhof et al., 2010; Khaitan et al., 2015; Valinetz and Cangelosi, 2021). Moreover, due to the high Se of this technique, it could be adapted for samples from milk tanks in the future, similar to how it is done in cattle (Varcasia et al., 2016).

The new TRFIAs were based on the recombinant antigens TgGRA7 and TgSAg2 for anti-*T. gondii* antibodies detection, and on NcGRA7 to detect anti-N. *caninum* antibodies. These recombinant antigens were selected regarding their frequent use for serodiagnosis in animals, and also the good diagnostic performance normally obtained by different serological techniques (Sinnott et al, 2017; Ferra et al, 2020; Uddin et al; 2021) as previously mention in the introduction. Additionally, the chimeric antigen

TgSAG1-GRA8, which contains epitopes from TgSAG1 and TgGRA8, was evaluated by TRFIA for serodiagnosis of *T. gondii* infection.

On the other hand, the development of new serological techniques always needs a validation process to ensure the accuracy and reliability of the new test. In this respect, a validation protocol including analytical and diagnostic validation (Jacobson, 1998; OIE, 2019a) has been followed in this PhD thesis. For the former, inter- and intra-assay precision, analytical Se (based on the detection limit and the limit of quantification of the technique), the analytical accuracy (estimated by the linearity under dilution and the spike and recovery test) and the analytical Sp (based on the estimation of potential cross-reactivities with other pathogens) were assessed (Jacobson, 1998; Andreasson et al., 2015). Concretely, the close relationship between *T. gondii* and *N. caninum* sometimes cause that *T. gondii* seropositive animals could give a positive result in a technique for the detection of anti-*N. caninum* antibodies, or vice versa (Gondim et al., 2017). So, this potential cross-reactivity that could lead to misdiagnosis was studied during the analytical validation of the new serological techniques. On the other hand, for the diagnostic validation, a broad panel of well-characterized samples was measured with the new technique, taking one or more previously validated techniques as reference (OIE, 2019a). A Receiver-Operating Curve (ROC) analysis was used to determine the optimal cut-off value of the technique that provides the highest diagnostic Se and Sp values (Jacobson, 1998; Gardner and Greiner, 2006; Alemayehu and Zou, 2012; Polo and Miot; 2020). In addition, other assessments such as agreement (based on *kappa* value) (McHugh, 2012) and correlation (Schober and Schwarte, 2018) with the reference technique were analyzed.

Objectives

Objectives

The main objectives of this doctoral thesis were:

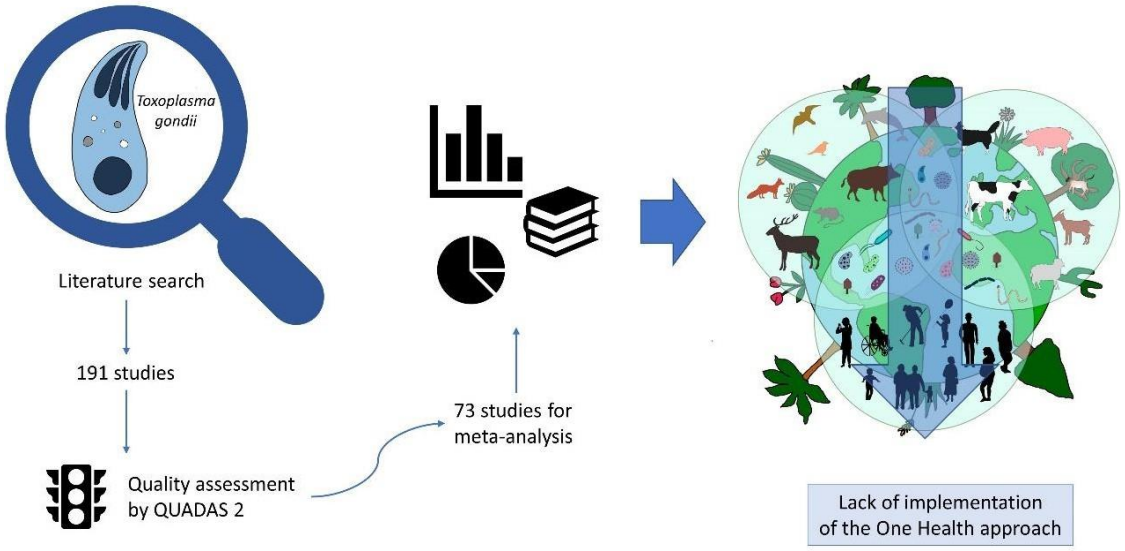
1. To systematically review and meta-analyze the scientific literature on the development of serological techniques for the diagnosis of *Toxoplasma gondii* infection. Two sub-objectives were pursued:
    a. To describe and compare the characteristics of the research articles conducted in the field of human and animal health, to find out if the development of serological tests to diagnose *T. gondii* infection was addressed following a One Health approach.
    b. To analyze the technical characteristics and the analytical and diagnostic validation of the serological techniques developed in the published studies, according to the validation guide provided by the World Organization of AnimalHealth (OIE) for animals and to identify potential gaps for further improvements.
2. To study the seroprevalence and the risk factors associated to *T. gondii* infection in dogs and cats in Bangkok (Thailand), an urban area where these anthropogenic species and humans closely coexist.
3. To develop and validate a new highly sensitive technique, the Time-resolved fluorescence immunoassay (TRFIA) for the detection of anti-*T. gondii* antibodies in serum samples from cats and goats.
4. To develop and validate a new highly sensitive TRFIA for the detection of anti-*N. caninum* antibodies in serum and milk samples from sheep.

Chapter 1. Literature review on the serological diagnosis of *Toxoplasma gondii*

Introduction: systematic review and meta-analysis on the serological diagnosis of *Toxoplasma gondii* in human an animals

Although reviews on the techniques for serological diagnosis of *T. gondii* infection have been previously done in both animals and humans (Liu et al., 2015; Wyrosdick and Schaefer, 2015; Ybañez et al., 2020b; Uddin et al., 2021), they were normally focused on the description of the currently available techniques, and sometimes on the use of different types of antigens. However, they did not critically analyse whether these studies were conducted from a One Health perspective, or if they followed an appropriate validation process. For this reason, and due to the importance of *T. gondii* as a zoonotic pathogen (Dubey, 2016), a systematic review and meta-analysis was performed in this chapter, focusing on the serological diagnosis of this parasite and following the PRISMA statement (Page et al., 2021). Based on this review, two manuscripts have been generated. The first one aimed to determine if the articles recently published on the development of serological techniques for detection of anti-*T. gondii* antibodies in humans and animals followed a One Health approach (Chapter 1a). On the other hand, the second review analysed the technical characteristics of these developed techniques and their validation process (Chapter 1b).

Chapter 1a: The systematic review and meta-analysis on the serological diagnosis of *Toxoplasma gondii* infection highlight the lack ofa One Health approach



Huertas-López, A.[a], Sánchez-Sánchez, R.[b], Cantos-Barreda, A.[c,d], Álvarez-García, G.[b], Ibáñez-López, F. J.[e], Martínez-Subiela, S.[a], Cerón, J. J.[a], and Martínez-Carrasco, C[d]. The systematic review and meta-analysis on the serological diagnosis of *Toxoplasma gondii* infection highlight the lack of a One Health approach. Submitted to Animal Health Research Reviews Journal.

[a] Interdisciplinary Laboratory of Clinical Analysis, Interlab-UMU, University of Murcia – Regional Campus of International Excellence "Campus Mare Nostrum", 30100 Espinardo, Murcia, Spain

[b] Animal Health Department, Complutense University of Madrid, Ciudad Universitaria s/n, 28040 Madrid, Spain

[c] Department of Biochemistry and Molecular Biology-A, University of Murcia - Regional Campus of International Excellence "Campus Mare Nostrum", 30100 Espinardo, Murcia, Spain

[d] Animal Health Department, University of Murcia - Regional Campus of International Excellence "Campus Mare Nostrum", 30100 Espinardo, Murcia, Spain

[e] Statistical Support Section (SAE), Scientific and Research Area (ACTI), University of Murcia - Regional Campus of International Excellence "Campus Mare Nostrum", 30100 Espinardo, Murcia, Spain

Abstract

Toxoplasma gondii is a globally distributed food-borne zoonotic parasite that can infect a wide range of warm-blooded hosts. The control of this zoonosis partially depends on the serological diagnosis and, therefore, numerous techniques have been developed for humans and animals. A systematic review and a meta-analysis were performed to analyse and compare the One Health approach followed in articles published between 2014 and 2019 focused on the development and evaluation of serological techniques for the diagnosis of *T. gondii* infection in humans and domestic and wild animals. After an exhaustive search on scientific databases, a quality assessment was performed on 191 articles by QUADAS 2 tool, and 73 articles were finally selected. A total of 18 variables were extracted and analysed from these articles, including data regarding bibliometric characteristics, study aims and methodology. Articles were widely heterogeneous, in particular between studies conducted by physicians and veterinarians, as well as between techniques developed for the detection of anti-*T. gondii* antibodies in humans and animals. The results evidenced that the One Health approach followed in the diagnosis of *T. gondii* still needs further integration between scientific disciplines, which is essential to design and implement effective intervention measures to control toxoplasmosis.

Keywords: meta-analysis, One Health, serological techniques, systematic review, *Toxoplasma gondii*

1. Introduction

Toxoplasma gondii is a protist included in the subphylum Apicomplexa with a wide host range, including birds and mammals (Dubey, 2016; Attias et al., 2020). It is the causative agent of toxoplasmosis, one of the most relevant zoonoses worldwide. The European Food Safety Authority (EFSA) identified *T. gondii* as one of the three potentially most important foodborne parasites in Europe. However, currently it is not routinely controlled either in food or water (EFSA and ECDC, 2018). This lack of attention contrasts with the evidence indicating that toxoplasmosis is the third leading food-borne zoonosis in humans and the second cause of mortality associated to foodborne zoonoses in USA according to the Centers for Disease and Control (CDC) (Hoffmann et al., 2012).

Humans can become infected by the ingestion of *T. gondii* cysts present in raw or undercooked meat from infected animals, and also by ingestion of oocysts excreted in cat faeces contaminating fruits, vegetables and water, and less frequently by congenital infection, inhalation of oocysts, consumption of raw milk, transfusions or even organ transplantations (Cook et al., 2000; Koutsoumanis et al., 2018; Aguirre et al., 2019; Attias et al., 2020). The multiple sources of infection, including meat and environmental routes, and the wide parasite host range explain how *T. gondii* is so broadly spread worldwide. In humans, *T. gondii* may cause abortions and foetal malformations, as well as ocular and neurological disorders; in domestic animals, for example, the economic impact of toxoplasmosis in small ruminant flocks due to reproductive failure is very important but probably infra-estimated (Dubey, 2016; Dubey et al., 2020b). Its relevance in other domestic and wild bird and mammal species lies in their contribution to the maintenance of the *T. gondii* life cycle (Dubey, 2016). So, as a globally distributed zoonosis, the control of toxoplasmosis should involve joint and coordinated research at least by physicians and veterinarians (Wyrosdick and Schaefer, 2015; Aguirre et al., 2019).

In this context, accurate diagnosis is a key component in the control of toxoplasmosis, usually based on serological techniques (Dabritz and Conrad, 2010; Liu et al., 2015; Ybañez et al., 2020b). These techniques are frequently used, in both humans

and animals, for *in vivo* diagnosis of the infection and for epidemiological studies, with a wide variety of techniques such as enzyme-linked immunosorbent assays (ELISA), indirect fluorescent antibody tests (IFAT), Western blot and agglutination assays (e.g., modified agglutination assay or MAT, indirect haemagglutination assay or IHA) (Liu et al., 2015; Wyrosdick and Schaefer, 2015; Ybañez et al., 2020b).

The recent coronavirus disease 2019 (COVID-19) pandemic has highlighted the need for greater interdisciplinary collaboration to address prevention measures from a One Health perspective (Schmiege et al., 2020; Tiwari et al., 2020). The general trend among studies focusing on the diagnosis of *T. gondii* is to target a single host species, being reduced the number of studies that have performed the serological diagnosis in several host species (Yin et al., 2015; Sun et al., 2017; Zhuo et al., 2017). In addition, the simultaneous study of the definitive host along with other intermediate hosts is very infrequent (Al-Adhami and Gajadhar, 2014). Indeed, there are only a few studies addressing the research on *T. gondii* with a One Health approach (Suijkerbuijk et al., 2018; Aguirre et al., 2019) and, if so, these approaches have not been critically evaluated. Therefore, the objective of this study was to perform a systematic review and meta-analysis of articles on serological diagnosis of *T. gondii* infection in humans and domestic and wild animals, analysing whether the approach of these studies is carried out from the One Health perspective.

2. Material and methods

The present systematic review and meta-analysis was performed in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA-P) statement (Page et al., 2021).

2.1 Search strategy

A systematic search was performed in the PubMed, Web of Science and Scopus scientific databases in order to identify articles published in English between January 1st 2014 and December 31st 2019. The following Medical Subject Headings (MeSH) were used to search for relevant investigations: “toxoplasma OR toxoplasm*” and “diagnosis”. Moreover, references of the included studies were checked for additional papers.

2.2 Selection criteria

Potential studies were selected by screening the title and abstract by three researchers. Full texts of eligible articles were read by these same researchers to decide upon final inclusion. Data were cross-checked and disagreements were solved by a senior researcher.

Before starting the detailed analysis of the content of each selected publication, the following inclusion criteria were defined:

1. Studies that developed one or more serological tests for the diagnosis of *T. gondii* infection.
2. Studies performed in humans and/or animals (one or more host species).
3. Studies with a sample size ≥5.
4. Full text available in English.
5. Review articles and seroprevalence studies that did not include the development of a serological test were discarded, and duplicates were removed.

2.3 Quality assessment (QUADAS 2)

Studies that met the above inclusion criteria were blindly analysed in order to assess their quality following the QUADAS 2 protocol (Whiting, 2011) (Table 1). Articles were included for subsequent data extraction when the following requirements were fulfilled:

6. Articles that answer the review question "Does this study evaluate one or more serological techniques for detection of anti-*T. gondii* antibodies in animals and/or humans?" and the consequent signalling questions (Table 1). When the answers to these questions were not clearly specified in the text of the article, the answer was considered "unclear" and the article was not selected for the subsequent meta-analysis.
7. Articles with low or medium risk of bias, being those with less than two high or unclear risks of bias or concerns regarding applicability. Articles with high risk of bias in "Index test" and "flow and timing" domains, which were considered as qualifying questions, were not eligible for the meta-analysis.

2.4 Data extraction

Data were extracted using a standardized Excel sheet (Microsoft Excel®, Version 2016) including the information related to variables classified into the following main categories detailed in Table 2:

- Bibliometrics: research area, quartile in which the article was published according to the Journal Citation Reports (JCR) list, authors' affiliation, uni- or interdisciplinary team, type of interdisciplinarity based on the co-authors' areas of expertise, continent and country of the origin of the research team (based on the information provided by the last author's affiliation) and country where the study was conducted (according to the study area from which samples were taken).
- Study aims: study category (related to the serological technique employed and the purpose for which it was used) and the One Health quotation (the mention of this concept in the article).
- Methodology: applied to human or animal samples, host category, natural or experimental infection, sample selection criteria (considering whether samples were retrospectively and/or prospectively selected), evaluated technique, number of evaluated techniques, reference technique and number of reference techniques.

2.5 Data analysis

The frequencies of the different factor labels within the variables were graphically studied by pie charts and bar charts. Differences between qualitative variables were analysed by Pearson's Chi-Square test or Fisher's exact test, as appropriate. Inference analyses for the quantitative variables regarding the qualitative variables were performed by Student's T test when parametric conditions for normality and homoscedasticity were met, checked by Shapiro-Wilk and Fligner-Killen tests, respectively; while Mann-Whitney's U or Kruskal-Wallis tests were carried out when these conditions were not met. Bonferroni corrected *post hoc* test was performed when significant differences were found.

For the clustering of the selected articles according to their authors' affiliation, the One Health quotation and the human or animal host, a Multiple Correspondence Analysis (MCA) (Kassambara, 2017) was carried out with the variables "Authors' affiliation", "Interdisciplinary team", "Study category", "One Health quotation", "Humans or animals", "Sample selection criteria", "Evaluated technique" and "Reference technique". Then, MCA was graphically represented for "Authors' affiliation", "One Health quotation" and "Humans or animals" variables, respectively.

Graphical representation of quality assessment was done by Microsoft Excel Software (Microsoft Excel®, Version 2016). World map was created by Mapchart on-line tool (https://mapchart.net/world.html). Meta-analysis and graphical representations of data extracted from the finally included articles, as well as MCA analysis, was performed by R software (R Core Team 2020). Statistically significant differences were considered when p value < 0.05.

3. Results

3.1 Diagram of articles selection

The flow diagram followed is shown in Figure 1. After applying the selection criteria and performing quality assessment, 73 out of 191 articles initially chosen for quality assessment were selected for data extraction and meta-analysis.

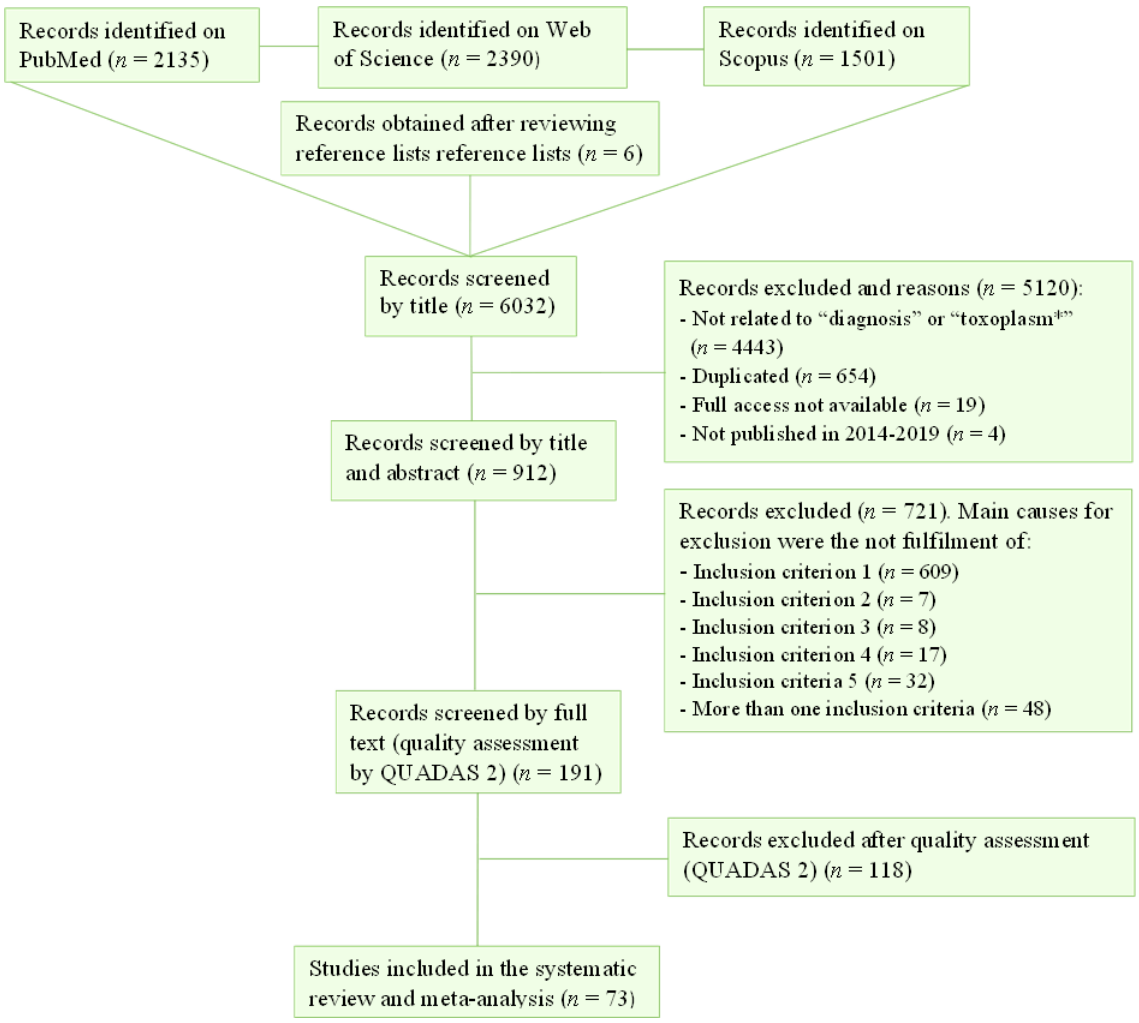


Figure 3. Flow diagram for the selection of articles in the systematic review and meta-analysis.

3.2 Quality assessment (QUADAS 2)

From the 191 articles assessed by the QUADAS 2 protocol, 126 were performed in humans, 63 in animals and only two in both humans and animals. The number of included and excluded articles is summarized in Table 1. According to the quality assessment, only 37 out of 191 (19.4%) and 36 out of 191 (18.8%) had low and medium risk of bias, respectively (Table 1 and Supplementary File 1). Discarded studies (118/191; 61.8%) had a negative or unclear answer to the review question and/or to the consequent signalling questions, high or unclear risk of bias in the qualifying questions and/or had two or more high risk of bias concerning the different domains (selection of study subjects, index test, reference standard and flow and timing). A total of 58/191 (30.4%) articles had a negative or unclear answer to the review question, whereas 38/191 (19.9%) had a negative answer to one or more signalling questions. With regards

to the qualifying questions, 38/191 (19.9%) articles did not pre-specified the threshold, whilst this information was unclear in 15/191 (7.8%) articles; the reference standard was not or was unclearly applied to all the study subjects in 5/191 (2.6%) and 4/191 (2.1%) articles, respectively; the study subjects did not use the same reference standard technique or it was unclear in 18/191 (9.4%) and 8/191 (4.2%) articles, respectively; and not all the study subjects were included in the statistical analysis or it was unclear, respectively, in 23/191 (12%) and 5/191 (2.6%). On the other hand, there were articles that had high or unclear risk concerning the selection of the study subjects (100/191; 52.4%), the index test (49/191; 25.6%) and the flow and timing domain (89/191; 46.6%); no articles had high or unclear risk regarding the reference standard. Focusing on the host species, 66.1% (78/118) of the excluded studies were conducted exclusively in humans, 33.1% (39/118) in animals and 1.7% (2/118) on both humans and animals. After applying the selection criteria, the remaining 73 articles (38.2%) were selected for further data-extraction and statistical analysis.

3.3 Systematic review and meta-analysis

The 73 articles finally included for the systematic review and meta-analysis are summarized in Supplementary File 2, and all data regarding bibliometrics, study aims and methodology of these articles are shown in Supplementary File 3. Significant associations between the different variables and their statistical values are included in Supplementary File 4. The most relevant findings are described below.

3.1.1 Bibliometrics

Frequencies of the factor labels within each qualitative “Bibliometrics” variable are represented in Figure 2. Eight out of the 73 selected articles (Q10, Q12, Q15, Q26, Q28, Q33, Q48, Q61) (see Supplementary Files 2 and 3) were published in journals not indexed in JCR but, as they met the inclusion criteria and the quality assessment, they

were included for the systematic review and meta-analysis.

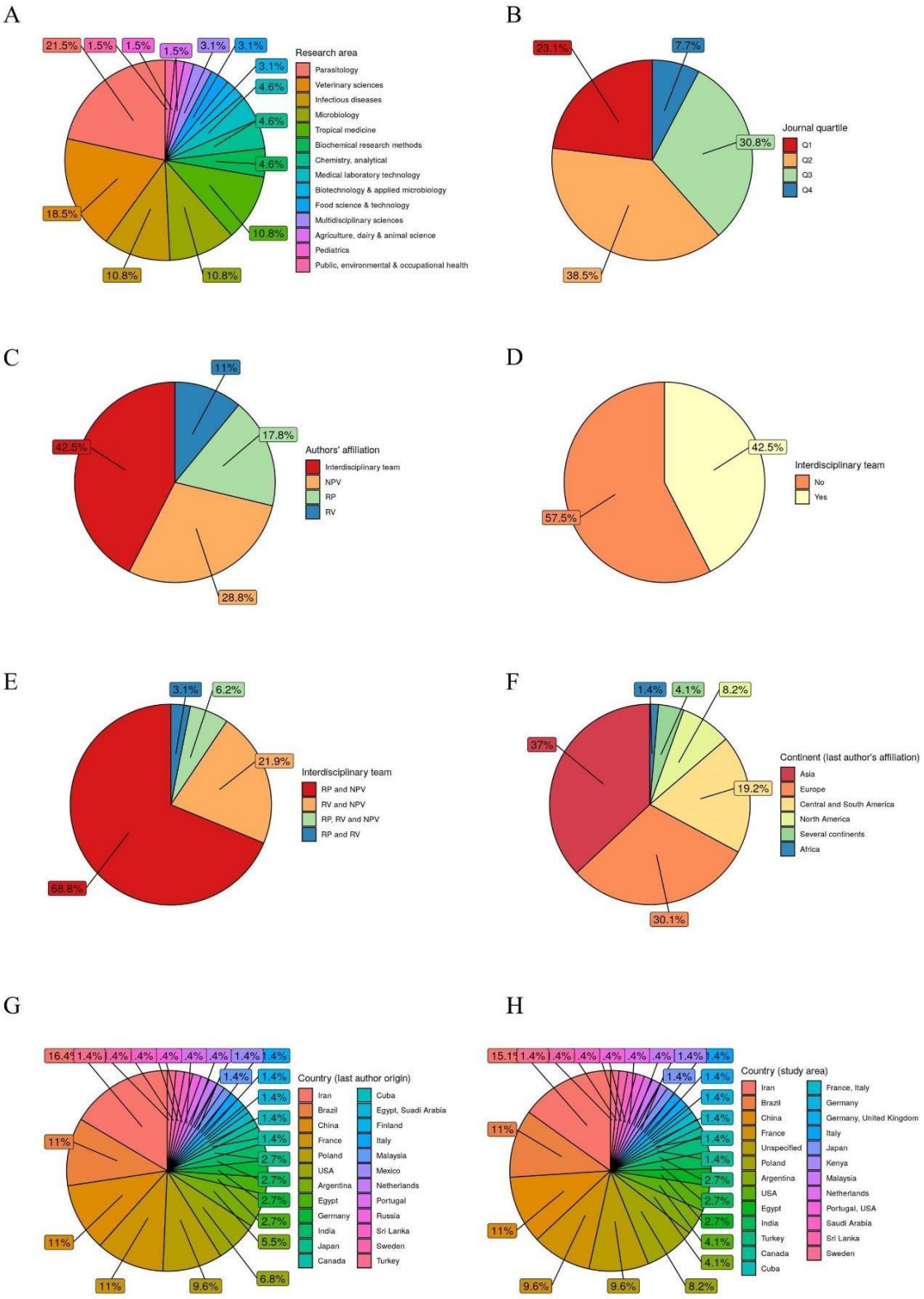


Figure 4. Pie charts representing the frequency of studies in each factor label from the "Bibliometrics" variables: "Research area" (A), "Journal quartile in JCR" (B), "Authors' affiliation" (C), "Interdisciplinary team" (D), "Type of interdisciplinarity" (E), "Continent (last author's affiliation)" (F), "Country (last author's affiliation)" (G) and "Country (study area)" (H). Q = Journal quartile; RP = Research physicians; RV = Research veterinarians; NPV = neither physicians nor veterinarians.

Focusing on the 65 articles published in journals indexed in JCR, all research areas were included in “Life Science and Biomedicine”, one of the five broad categories in Web of Science. Research areas were related to the authors’ affiliation (p = 0.043) (Table 3). Specifically, most of the studies indexed in JCR and carried out by research physicians (RP) were published in journals related to “Microbiology” (5/9; 55.6%); research veterinarians (RV) published more frequently in “Parasitology” (3/8; 37.5%) and “Veterinary Sciences" (3/8; 37.5%) journals; and researchers who were neither physicians nor veterinarians (NPV) published in “Veterinary Sciences" (5/20; 25%) and “Parasitology” (4/20; 20%) research areas. Interdisciplinary teams had preference for publishing in the research areas “Infectious disease” (5/28; 17.9%) and “Parasitology” (5/28; 17.9%), followed by “Veterinary Sciences” (4/28; 14.3%) and “Tropical medicine” (4/28; 14.3%). There was a relationship between research areas and the “One Health quotation” variable (p = 0.044) (Table 3), being a 50% (7/14) of the articles that implicitly mentioned the One Health approach published in the “Veterinary sciences” research area, and a 28.6% (4/14) in “Parasitology”. Research area also showed a relationship according to whether the study was carried out in human or animal hosts (p < 0.001) (Table 4). Specifically, studies performed in humans were more frequently published in “Parasitology” (8/42; 19%) and “Microbiology” (7/42; 16.7%), while those carried out in animal hosts were more frequently published in “Veterinary Sciences” (12/23; 52.2%). Frequencies of studies published in the different journal quartiles in JCR regarding the human or animal host are represented in Figure 1 of the Supplementary File 5.

Regarding “Authors’ affiliation” variable, it was significantly related to the “One Health quotation” variable (p < 0.001) (Table 3), being this approach more frequently implicitly mentioned in studies conducted by RV (6/15; 40%) than by NPV (5/15; 33%) and interdisciplinary research teams (4/15; 26.7%), the latter composed by RV and other researchers in all cases. None of the studies exclusively performed by RP implicitly referred to the fundamental principles on which the One Health approach is based. In addition, authors’ affiliation showed a relationship with the host studied, humans or animals (p < 0.001) (Table 4). Most of articles in humans were carried out by interdisciplinary teams (23/49; 46.9%), followed by RP (13/49; 26.5%) and NPV (13/49; 26.5%). From the 23 articles carried out in humans by interdisciplinary teams, 21/23

(91.3%) were performed by RP and NPV, whereas only 2/23 (8.7%) were conducted by RP, RV and NPV. Studies focused on animal hosts were equally performed by RV (8/24; 33.3%), NPV (8/24; 33.3%) and interdisciplinary teams (8/24; 33.3%; 7 of these 8 articles were conducted by RV and NPV, and 1/8 by RP and RV). None of the articles in humans and animals were exclusively carried out by RV and RP, respectively. Authors' affiliation was also related to the reference technique (p = 0.018) (Table 4). Specifically, RP teams more frequently used ELISA techniques as reference technique (7/13 of the articles performed by RP; 58,3%), RV mostly based their research on IFAT (3/8 of the articles performed by RV; 37.5%) and agglutination techniques (2/8 of the articles performed by RV; 25%), and NPV and interdisciplinary teams more often used several techniques as reference (11/21 of the articles performed by NPV, 52.4%; and 12/31 of the articles performed by interdisciplinary teams, 38.7%).

The worldwide distribution of the 73 articles according to the last author's country of origin is shown in Figure 3. When analysing the country of origin of the samples, it coincided with the last author's country of origin in 76.7% (56/73) of articles, or with other co-authors' country of origin in 13.7% (10/73). In the remaining 9.6% (7/73) of cases, country of origin of the samples was not specified. Iran (11/49; 22.4%), France (8/49; 16.3%), Brazil (6/49; 12.2%) and USA (5/49; 10.2%) showed the higher number of articles performed on humans, whereas China (7/24; 29.2%), Poland (4/24; 16.7%), Brazil (2/24; 8.3%) and India (2/24; 8.3%) had the higher number of articles carried out on animals (Figure 3).

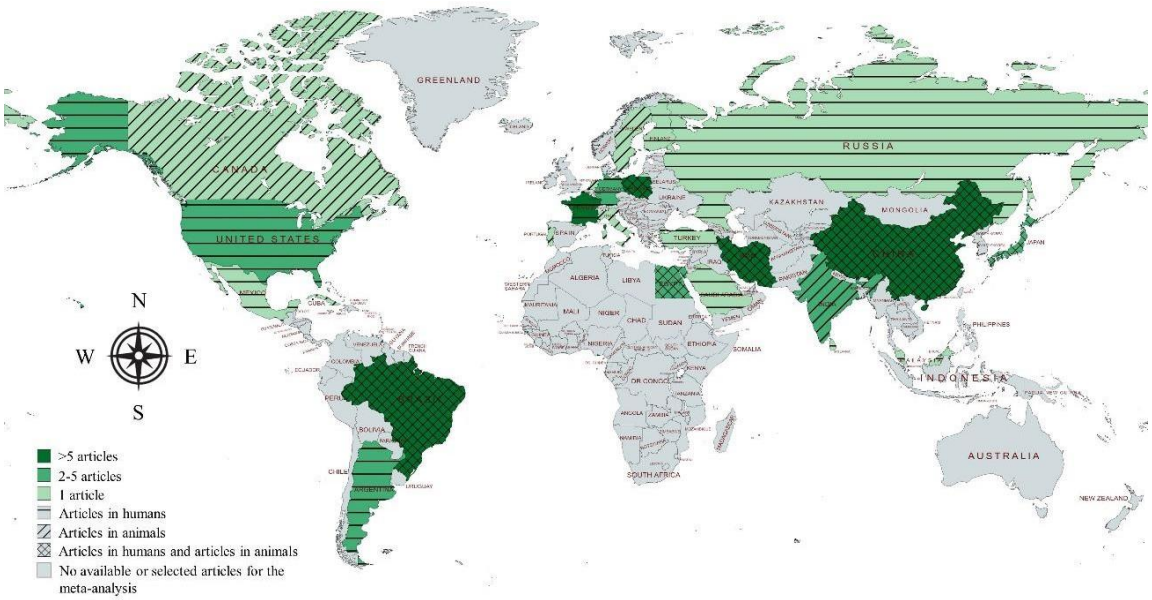


Figure 5. Global distribution of the 73 finally selected articles according to the last author's country of origin.

3.1.2 Study aims

Data regarding the "Study aims" variables are presented in Figure 4. Study category showed a significant relationship with the human or animal host (p = 0.012) (Table 4). Articles focusing on humans had a wider variety of study categories, although they did not develop any technique for several host species (category six). In contrast, 12.5% (3/24) of the studies performed on animals developed techniques applicable to several host species. The rest of articles belonged to categories "1) Design and/or validation of a new technique" (11/24; 45.8%) and "2) Modification of a previously described technique" (10/24; 41.7%).

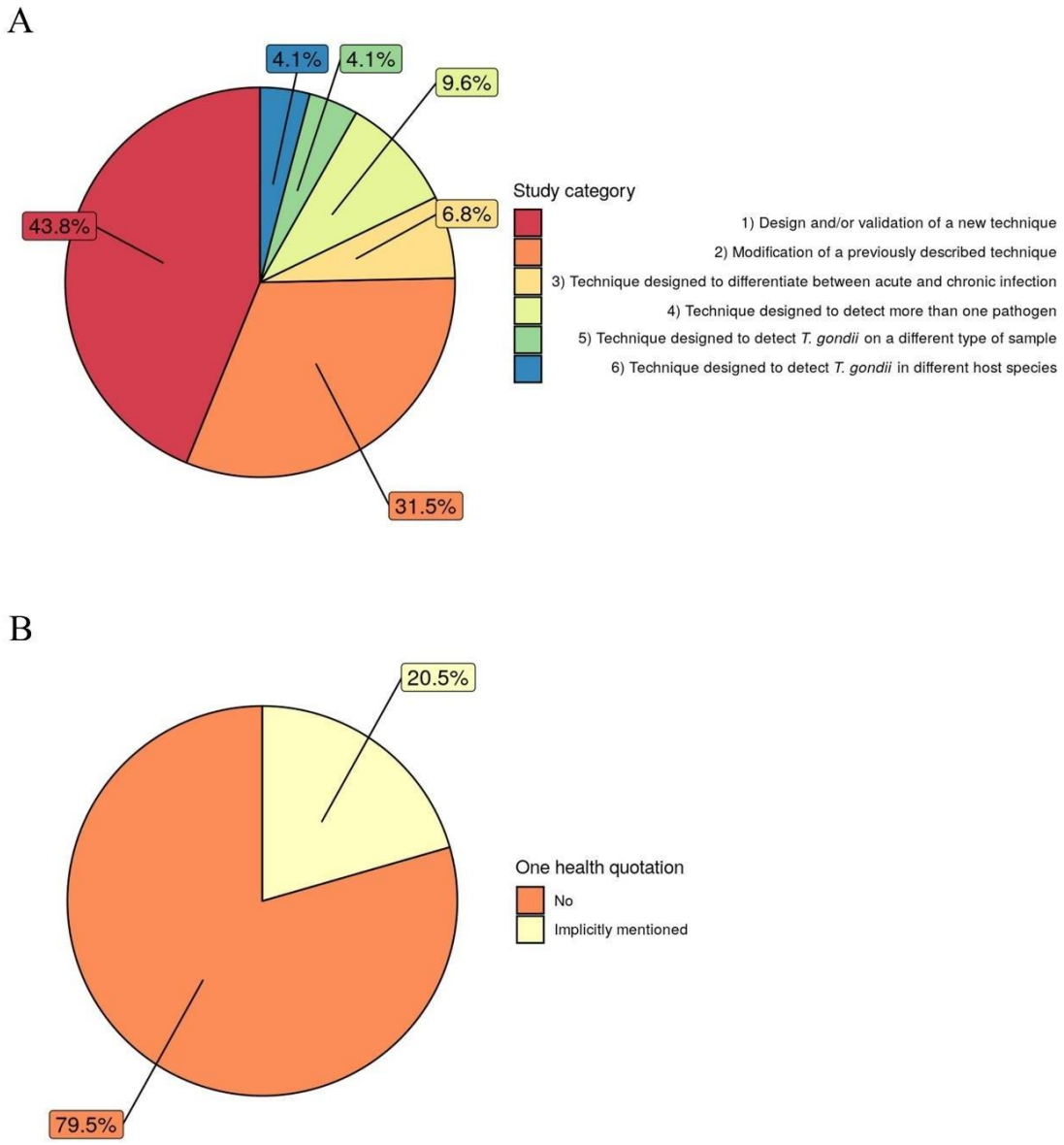


Figure 6. Pie charts representing the frequency of studies in each factor label from the "Study aims" variables: "Study category" (A) and "One Health quotation" (B).

Although none of the selected articles explicitly cited the words "One Health", 20.5% (15/73) of them implicitly mentioned the fundamental principles underlying the One Health approach to the study of diseases; specifically, these articles (Q02, Q05, Q09, Q22, Q23, Q30, Q53, Q54, Q56, Q58, Q61, Q63, Q66, Q68 and Q69) (see Supplementary Files 2 and 3) mentioned the need for joint efforts by different disciplines in order to control toxoplasmosis. There was a relationship between this implicit mention and the type of studied host/s, both with humans or animals ($p < 0.001$) or with the particular host category ($p < 0.001$) (Table 4). The One Health approach was not implicitly

mentioned in any study performed in human host, while it was referred in 15 out of 24 (62.5%) of the studies in animal hosts. From these 15 articles, six were carried out in ruminants (40%), three in cats (20%), two in pigs (13.3%), and four in other animal species (26.7%) (Figure 5).

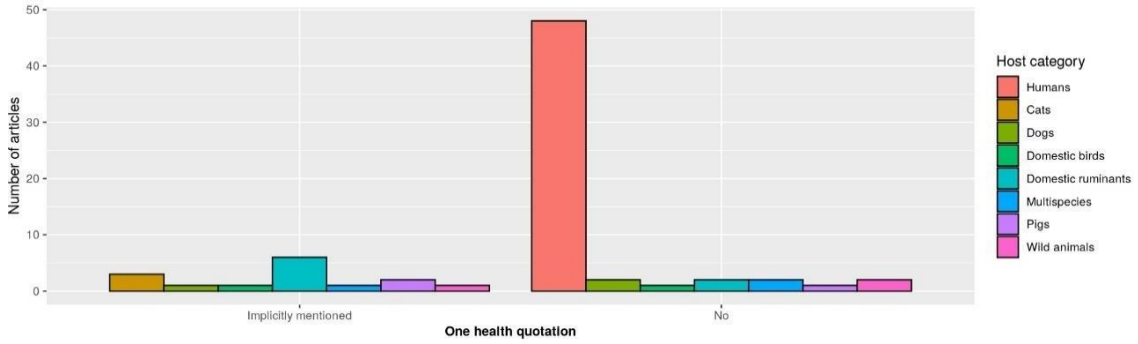


Figure 7. Graphical representation of the variable "One Health quotation" regarding the host category.

On the other hand, from the 73 articles, there was only one article (Q73) that applied the One Health approach for developing a multispecies technique for domestic (the human activity component – in this case, pigs, cats and mice) and wild animals (the ecological component – in this case, seals), according to Calistri *et al.* (2013).

3.1.3 Methodology

Figure 6 represents the frequency of each factor label within "Methodology" variables. Articles focusing on "wild animals" were specifically carried out in the following host species: red fox (*Vulpes vulpes*), raccoon dog (*Nyctereutes procyonoides*), badger (*Meles meles*), marten (*Martes martes*), mink (*Neovison vison*) and polecat (*Mustela putorius*) (Q39); mink (*Neovison vison*) (Q50); and boar (*Sus scrofa*) (Q63). From the total of three studies carried out in multiple species, one of them was performed in humans and mice (Q06), one in horses, sheep and pigs (Q54), and the last one in pigs, cats, mice and grey seals (*Halichoerus grypus*) (Q73).

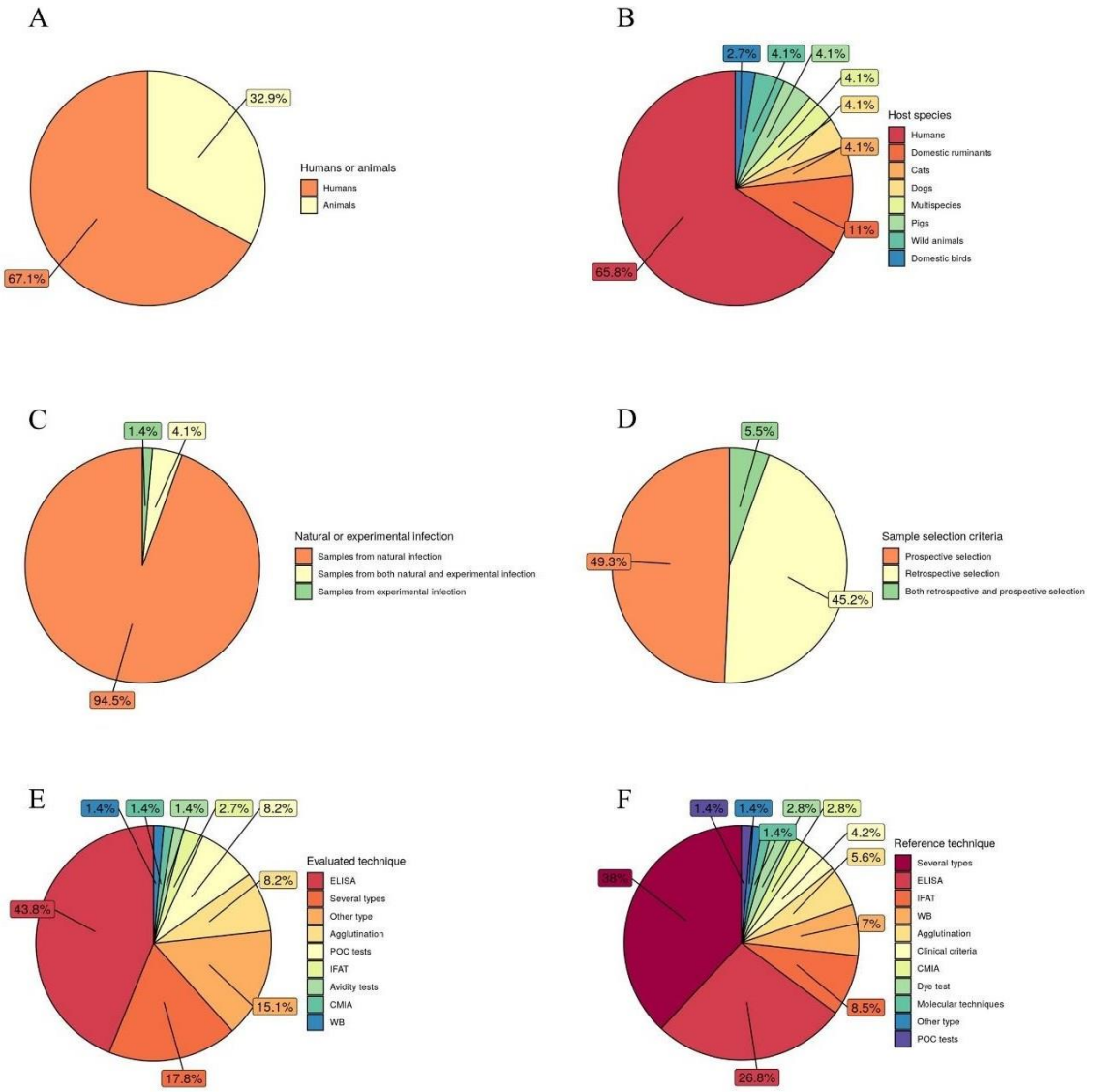


Figure 8. Pie charts representing the frequency of studies in each factor label from the "Methodology" qualitative variables: "Humans or animals"1(A), "Host category" (B), "Natural or experimental infection" (C), "Sample selection criteria" (D), "Evaluated technique" (E) and "Reference technique" (F). 1Although study Q70 did not specify the host species, it was considered as human host because it talks about "individuals" and also because of the "Authors' affiliation" (interdisciplinary, mostly physicians). ELISA = Enzyme-linked immunosorbent assay; IFAT = Indirect fluorescent antibody tests; WB = Western blot; POC = Point-of-care; CMIA = Chemiluminescent microparticle immunoassay.

The studied host was statistically related with the sample selection criteria, both generally regarding humans or animals (p = 0.003) and specifically regarding the host category (p < 0.001) (Table 5). Most of the studies performed on humans made a retrospective selection of the samples (29/49; 59.2%), while 36.7% (18/49) prospectively selected the samples. In animals, most of studies made a prospective selection of the samples (18/24; 75%), whereas only 16.7% (4/24) were retrospective studies. The frequencies of each type of sample selection regarding the specific host category is graphically represented in Figure 2 of the Supplementary File 5. Additionally,

comparison of human or animal host with the type of reference technique showed a relationship (p = 0.002) (Table 5). Studies performed in human host more often used several techniques (20/49; 42.6%) and ELISA (16/49; 34%) as the reference technique, whilst those carried out in animals more frequently used several techniques (7/24; 29.2%), IFAT (5/24; 20.8%), agglutination (4/24; 16.7%), ELISA (3/24; 12.5%) and WB (3/24; 12.5%) as reference techniques. Moreover, when analysing the host category with regard to the type of the reference technique, a relationship was also found (p < 0.001) (Table 5). In detail, agglutination was the most used reference technique for cats (2/3 of the studies in this species; 66.7%); several techniques, IFAT and WB were equally used when studies analysed dog samples (each technique represents 1/3 of the studies; 33.3%); ELISA, WB and agglutination were equally used for wild animals (each technique represent 1/3 of the studies; 33.3%); several techniques and IFAT were most used for domestic ruminants (each technique represents 3/8 of the studies; 37.5%); ELISA, IFAT and another type of technique were equally used for pigs (each technique represent 1/3 of the studies; 33.3%); WB and molecular techniques for domestic birds (each technique represent 1/2 of the studies; 50%); and several techniques when multispecies samples were tested (3/3 of the studies; 100%).

The number of evaluated techniques was significantly related to the study category (p = 0.003) (Table 6), and Bonferroni corrected *post hoc* test showed a significant difference between categories 1 (Design and/or validation of a new technique) and 2 (Modification of a previously described technique). The number of reference techniques also showed a significant relationship with the study category (p = 0.016) (Table 6), but no differences were detected in Bonferroni corrected *post hoc* test. In addition, the number of reference techniques was related to the specific host species (p = 0.049) (Table 6), although Bonferroni corrected *post hoc* test did not detect any difference, probably due to the low number of studies from each animal species.

3.1.4 Multiple Correspondence Analysis (MCA)

The MCA (Figure 7) showed that for dimension 1 (10.9% variability), higher contribution (above 10%) concerned the factor labels “Humans or animals - Animals”, “One Health quotation – Implicitly mentioned” and “Authors’ affiliation - RV”. For dimension 2 (7.4% variability), the most contributory (above 10%) factor labels were

"Authors' affiliation - RP", "Authors' affiliation – Interdisciplinary team", and "Interdisciplinary team - Yes". The percentage of contribution of all the factor labels which contribute to dimensions 1 and 2 are graphically represented in Figure 8.

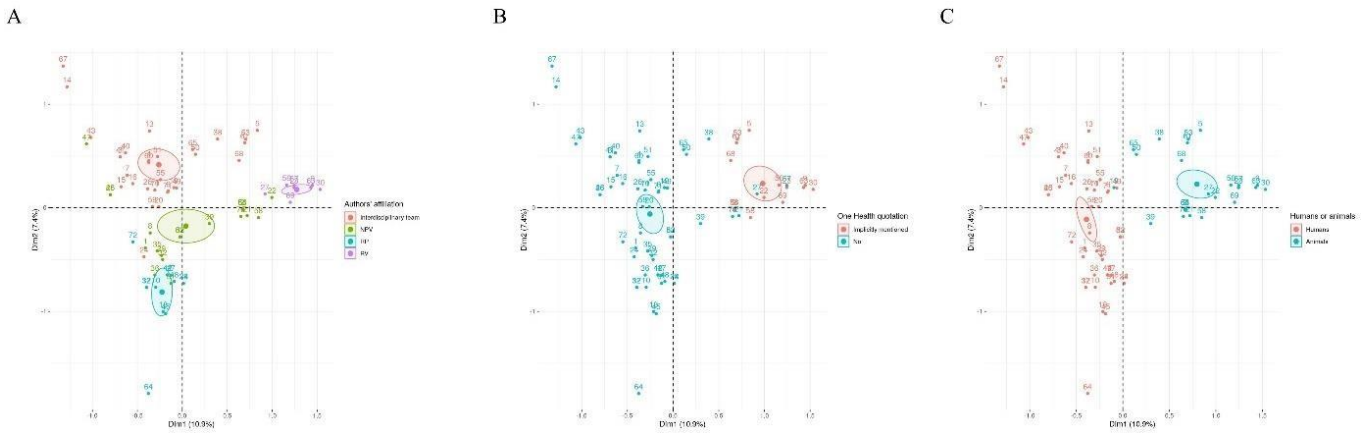


Figure 9. Graphical depicting of individual distribution of the studies within dimension 1 and dimension 2 in Multiple Correspondence Analyses (MCA) for "Authors' affiliation" (A), for "One Health quotation" (B) and for "Humans or animals" (C), being Dim-1 = dimension 1 (factor labels with higher contribution: "Humans or animals- Animals", "One Health quotation – Implicitly mentioned" and "Authors' affiliation - RV") and Dim-2 = dimension 2 (factor labels with higher contribution: "Authors' affiliation - RP", "Authors' affiliation – Interdisciplinary team", and "Interdisciplinary team - Yes").

Regarding the graphical representation of MCA performed with "Authors' affiliation" variable (Figure 7A), the distribution of the 73 articles included in this MCA showed a clear separation between the four different authors' affiliations. Studies carried out by RV had a strongly positive influence by the factor labels from dimension 1, and a relatively less strong positive influence by labels from dimension 2, contrary to what happened with the studies performed by RP, which were strongly negatively influenced by the factor labels from dimension 2 and slightly negatively influenced by labels from dimension 1. Moreover, interdisciplinary teams had a slightly negative influence by the labels from dimensions 1 and a strongly positive influence by the labels from dimension 2. Finally, studies carried out by NPV showed relatively slight positive influence of the factor labels from dimension 1 and slight negative influence by the labels from dimension 2. In the light of these results, and taking into account all the contributory factor labels (Figure 8), RV carried out studies on animals, implicitly mentioned One Health, evaluated other type of techniques, used techniques designed to detect more than one pathogen and used agglutination as reference technique more

frequently compared to the remaining authors' affiliations (regarding the most positive influence of labels from dimension 1). As far as RP, they studied techniques to differentiate between acute and chronic infections or designed to detect more than one pathogen, and evaluated avidity tests and other type of techniques less frequently than other authors' affiliations (regarding the most negative influence of labels from dimension 2), being exactly the opposite to interdisciplinary teams (regarding the most positive influence of labels from dimension 2).

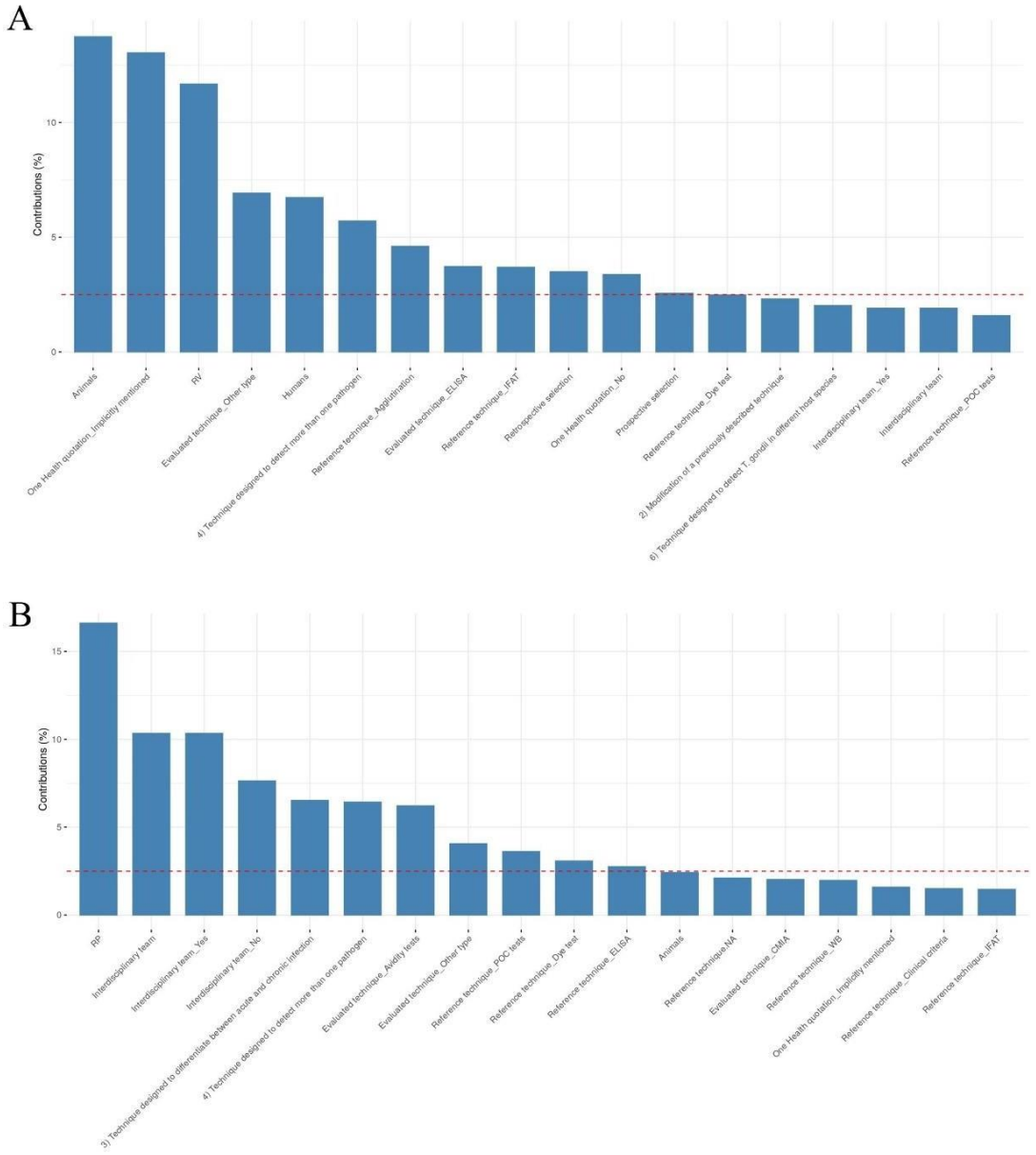


Figure 8. Degree of contribution of factor labels in dimensions 1 (A) and 2 (B) for Multiple Correspondence Analyses (MCA).

There was also a clear separation of the 73 articles included in this MCA regarding the variable "One Health quotation" (Figure 7B). Specifically, studies that implicitly mentioned this approach were positively influenced by the factor labels from dimension 1 and dimension 2, whereas studies that did not mentioned the One Health approach were negatively influenced by the factor labels from both dimensions. Therefore, these results reinforce the idea that the implicit mention of One Health appears more commonly in studies carried out on animals and by RV. The positive influence of dimension 2 in this implicit mention of One Health approach could be due to other labels different from "Authors' affiliation - RP", such as "Authors' affiliation – Interdisciplinary team", and "Interdisciplinary team - Yes", as none of the studies performed exclusively by RP implicitly mentioned this approach.

Finally, MCA indicated a clear separation between articles depending on their performance in humans or animals (Figure 7C). Specifically, studies conducted in humans showed a negative influence by the factor labels from dimension 1 and 2, contrary to what was detected in studies performed in animals, which were positively influenced by the labels of both dimensions. These similar results between MCAs for "Humans and animals" and "One Health quotation" showed a resemblance between the characteristics of the studies performed in animals and those which implicitly mentioned the One Health approach, and also, between the characteristics of the studies carried out in humans and those that did not mentioned the One Health perspective.

4. Discussion

A systematic review and meta-analysis on the serological tests developed for the detection of *T. gondii* infection in humans and animals are the necessary baseline to obtain a global, critical and comparative overview of the diagnostic methods used. The achieved results, therefore, provide the possibility to identify potential knowledge gaps and research lines that should be improved and, ultimately, to guide the way forward to address the prevention of toxoplasmosis from the One Health perspective (Ferreira González et al., 2011; Schmiege et al., 2020; Tiwari et al., 2020).

Prior to the systematic review, our study has evidenced that QUADAS-2 tool, specifically designed to assess the quality of studies that evaluate diagnostic accuracy (Whiting, 2011), is essential for the standardization of the quality assessment of diagnostic accuracy studies as done in previous studies (Laurenti et al., 2016; Nagai et al., 2016; Chaves et al., 2020; Lisboa Bastos et al., 2020; Zhang et al., 2021). In fact, the quality evaluation is often applied for systematic reviews and meta-analysis in order to limit the introduction of bias, which is a requirement of PRISMA statement (Page et al., 2021). In this sense, our results prove that the quality of studies on the development and validation of new serological methods for *T. gondii* diagnosis should be improved. Only 38.2% of assessed articles were considered to have low/medium risk of bias or concerns regarding applicability by QUADAS-2 tool and, consequently, were included in the meta-analysis. Therefore, even though the studies initially selected werepublished in scientific journals and found in specialized databases, our results highlight the need of performing a quality assessment of the studies before conducting any statistical analysis, in order to minimize the introduction of errors and biases into the analyses, especially regarding the selection of the study subjects, the specification of theevaluated test's threshold and the flow of study subjects.

Remarkably, this review emphasises the considerable variability and lack of implementation of the One Health approach. The importance of this interdisciplinary perspective has been highlighted in numerous studies regarding zoonotic diseases such as toxoplasmosis (Thompson, 2013; Dantas-Torres and Otranto, 2014; Krecek et al., 2020). However, none of the 73 studies selected in this review mentioned the One Health concept, and only one applied this approach by developing a multispecies *T. gondii* diagnostic technique for domestic animals (species closely related to humans) and wild animals (species that have constant contact with the environment and, therefore, can serve as a proxy for the sylvatic cycle of *T. gondii*) (Calistri et al., 2013).

With regards to the authors' affiliation, the scarcity of research teams integrated by physicians and veterinarians is evidenced that the study of toxoplasmosis has not been approached from a One Health perspective, despite being one of the most widespread multi-host parasitic zoonoses in the world (Dubey, 2016). From the few articles that made an implicit reference to the One Health concept, none of them were performed

by physicians. On the other hand, when comparing *T. gondii* serological studies performed in humans (mainly by physicians) with those conducted in animals (by veterinarians, non-physician researchers and/or interdisciplinary teams), it was found that the latter mentioned the need to undertake an interdisciplinary focus in the study of this zoonosis. This result suggests that veterinarians are more aware than physicians of the necessity to address the study of toxoplasmosis from a One Health perspective. Moreover, according to MCA analysis, there is a clear separation in the characteristics that define the studies according to the research profile of the authors. This shows that there is little collaboration between researchers belonging to different knowledge areas and, therefore, a scarcity of interdisciplinary teams that can integrate a One Health approach in their research. The encouragement of interdisciplinary teams that, in a holistic way, can deal with the study of toxoplasmosis would provide a more comprehensive and accurate knowledge of *T. gondii* infection in the environment, humans and animals, including domestic and wild host species (Humboldt-Dachroeden et al., 2020).

Another main evidence that we detected is that there is a considerable difference in the characteristics of the studies regarding whether One Health approach is quoted (or rather implicitly quoted), or if they are performed on humans or animals, according to MCA analyses. Moreover, there is a considerable difference in the number of published studies on the serological diagnosis of *T. gondii* depending on whether the study was carried out on humans or animals. Specifically, from the 191 articles initially selected, the number of those performed on humans (n = 126) doubled the number of studies conducted on animal hosts (n = 63). Only two articles studied serological techniques applied, at the same time, for the diagnosis of *T. gondii* in humans and animals. After quality assessment, this difference was also noticeable (49 on humans *vs* 24 on animals). This result suggests that the scientific community is more concerned about the development of new serological methods for the diagnosis of toxoplasmosis in humans. This finding contrasts with the traditional way to control zoonotic diseases in domestic and wild animals, frequently focused on the testing and mass vaccination and/or culling, which is not considered a One Health approach (Narrod et al., 2012). In fact, a shared research investment between physicians and veterinarians, as well as

other disciplines such as epidemiology, ecology and social sciences, has proven to be necessary to increase the cost-effectiveness of the zoonoses control (Narrod et al., 2012; Babo Martins et al., 2016).

On the other hand, from the 24 articles conducted on animal hosts, only three of them were carried out on wild species. Difficulty in obtaining species-specific conjugates and/or positive and negative control samples may be the reason why serological techniques are less developed in wild hosts (Jacobson, 1998; Wyrosdick and Schaefer, 2015), despite their importance in the sylvatic cycle of *T. gondii* (Dubey, 2016) and, consequently, in the implementation of the One Health approach (Aguirre et al., 2019). Moreover, the differences in the sample selection criteria found between studies focusing on human or animal hosts should be highlighted. In this regard, the availability of wide stocks of human samples in biobanks from hospitals and research institutions, such as the biobank at Palo Alto Medical Foundation Toxoplasma Serology Laboratory (PAMF-TSL) (Gomez et al., 2018; Li et al., 2019), facilitates the use of samples for retrospective studies of *T. gondii* infection in humans. In fact, the existence of these biobanks makes easier to obtain well-characterized and standardized samples that can be used as controls for the development and validation of new serological techniques and other research aimed at the study of human toxoplasmosis (Jacobson, 1998; Godard etal., 2003), as it has been shown in this study. In contrast, the scarcity of such biobanks for animal species, especially in the case of wild hosts, and even the difficulty to find positive and negative controls from some host species (Jacobson, 1998), make it difficult to carry out retrospective studies in domestic and wild animals and, consequently, explains why studies on the serodiagnosis of *T. gondii* infection in animals are mainly prospective. These results therefore emphasise that international collaboration and investment would benecessary in order to create banks of multispecies sera, available for performing standardized research.

Concerning the evaluated technique, our results reveal that the studies included in this systematic review and meta-analysis did not show any tendency to evaluate one type of serological technique or another according to the profile of the research team, nor to the target host species. However, there were differences in the choice of the technique used as reference depending on the profile of the research team and the host

category analysed; in particular, studies made by physicians for the diagnosis of *T. gondii* in humans commonly used ELISA, while those conducted by veterinarians for animal species select more frequently other types of techniques as a reference, such as IFAT and agglutination tests, which are commonly performed as *in house* techniques and whose results could be more subjective to interpret (Frössling et al., 2003; Wyrosdick and Schaefer, 2015). However, agglutination methods, which do not require species-specific reagents such as secondary antibodies, have the advantage of being suitable for use in a wide range of host species (de Almeida et al., 2016), and this could be the reason why these techniques are often used for *T. gondii* serodiagnosis in animals. The easiness and quickness of use, in combination with the high availability of commercial tests and specific reagents, and its normally high sensitivity and specificity (Ybañez et al., 2020b), could make ELISAs the best choice as reference technique for humans, but also would be ideal for animals, where the use of subjective techniques should be replaced by automatized and easy-to-implement techniques, to make results comparable between different laboratories. In addition, it is noteworthy that, despite Sabin-Feldmandye test and mice bioassay are considered the gold standards for the detection of *T. gondii* infection in humans and animals, respectively (Liu et al., 2015), only a few of the73 articles included in the present review employed dye test, and none used mice bioassay as reference technique. Furthermore, although several studies have previouslyreviewed the existing techniques for diagnosis of *T. gondii* infection in both humans andanimals (Liu et al., 2015; Wyrosdick and Schaefer, 2015), this information should be kept continually updated and publicly reported to help in the control of toxoplasmosis. International guidelines for the diagnosis of this zoonotic disease in humans and animals including the different available serological techniques would be advisable, like those established by the WHO for other zoonoses, such as echinococcosis (Ammann et al., 2002) or trichinellosis (Boireau et al., 2007).

The overall results of our meta-analysis show a high heterogeneity in the characteristics of the studies developing serological techniques for the diagnosis of *T. gondii* in humans and animals. Moreover, this study highlights the incomplete application of the One Health perspective, especially concerning human medicine. This lack of a holistic approach that characterizes this type of research is particularly evident

when analysing the results obtained in relation to bibliometrics, study aims, and methodology. Finally, in the light of these results, it would be necessary to implement a general protocol for the diagnosis of *T. gondii* infection for humans and animals. Furthermore, (1) the increase of interdisciplinary research teams working on the topic; (2) the establishment of an international group of experts, composed by specialists from different disciplines, who compile, share and report significant data on different aspects of *T. gondii* infection (epidemiology, pathogenesis, diagnosis, treatment, etc); (3) the creation of international biobanks of multispecies sera; (4) altogether with the reporting of a global free access and updated list of available and validated serological techniques for each host species, would be helpful for the control of this zoonosis.

5. Conclusions

The present study highlights the wide heterogeneity found in the studies on the development of serological techniques for the detection of *T. gondii* infection, mainly between those performed by physicians and veterinarians, or focused on humans or domestic and wild animals. The One Health approach should be implemented in future research on the serological diagnosis of *T. gondii* infection to achieve the complete control of this zoonosis.

6. Acknowledgements

The authors would like to express their gratitude to Statistical Support Section (SAE), Scientific and Research Area (ACTI), University of Murcia (www.um.es/web/acti). In addition, the authors would like to acknowledge the financial supporters: Ana Huertas López was supported by a pre-doctoral grant from University of Murcia (R-1207/2017); Gema Álvarez García is part of the TOXOSOURCES consortium, supported by funding from the European Union's Horizon 2020 Research and Innovation Programme under grant agreement No. 773830: One Health European Joint Programme; Ana Cantos Barreda was supported by a contract co-financed 91.89% by the European Social Fund and the "Iniciativa de Empleo Juvenil (POEJ)" through the Seneca Foundation of Murcia Region, Spain (21327/PDGI/19); Roberto Sánchez was supported by the Community of Madrid, Spain (PLATESA2, S2018/BAA-4370); finally, this work was supported by the Seneca Foundation of Murcia Region, Spain (project 19894/GERM/15).

TABLES

Table 1. Evaluation of 191 potentially eligible reports by QUADAS2 tool. Article Q06 only met the quality assessment for the IgG ELISA, and not for IgM ELISA and IgG-Avidity assay, so the statistical analysis was only performed on the IgG ELISA data. * Qualifying question (if the answer is "No" or "Unclear" = high risk of bias and not eligible study)

Stage	Methodology		Results		
	Area/Domain	Evaluation question	Answer	Eligibility	Number of articles (%)
Stage 1	Review question	Does the study analyse a serological technique for the detection of *T. gondii* infection in animals or humans?	Yes	Yes	133/191 (69.6%)
			No	No	54/191 (28.3%)
			Unclear	Unclear, continue quality assessment	4/191 (2.1%)
	Signalling questions	Is the study performed in humans or animals?	Yes	Yes	189/191 (99%)
			No	No	2/191 (1%)
			Unclear	Unclear, continue quality assessment	0/191 (0%)
		Is the analysed technique a serological technique?	Yes	Yes	187/191 (97.9%)
			No	No	4/191 (2.1%)
			Unclear	Unclear, continue quality assessment	0/191 0%)

			Does the study use a reference technique?	Yes	Yes	159/191 (83.2%)
				No	No	32/191 (16.8%)
				Unclear	Unclear, continue quality assessment	0/191 (0%)
			Is *T. gondii* the pathogen or one of the investigated pathogens?	Yes	Yes	191/191 (100%)
				No	No	0/191 (0%)
				Unclear	Unclear, continue quality assessment	0/191 (0%)
Stage 2	Flowchart		Hand drawn diagram (to facilitate evaluation in Stage 3)			
Stage 3	Domain 1: Selection of study subjects	Risk of bias	Was a consecutive or random sample of study subjects enrolled?	Yes	Low risk of bias	133/191 (69.6%)
				No	High risk of bias	21/191 (11%)
				Unclear	Unclear risk	37/191 (19.4%)
			Was a case-control design avoided?	Yes	Low risk of bias	129/191 (67.5%)
				No	High risk of bias	53/191 (27.8%)
				Unclear	Unclear risk	9/191 (4.7%)
			Did the study avoid inappropriate exclusions?	Yes	Low risk of bias	154/191 (80.6%)
				No	High risk of bias	32/191 (16.8%)
				Unclear	Unclear risk	5/191 (2.6%)
			Could the selection of study subjects have introduced bias?	No	Low risk of bias	91/191 (47.6%)
				Yes	High risk of bias	58/191 (30.4%)

			Unclear	Unclear risk	42/191 (22%)
	Applicability	Are there concerns that the included study subjects and setting do not match the review question?	No	Low concerns regarding applicability	186/191 (97.4%)
			Yes	High concerns regarding applicability	5/191 (2.6%)
			Unclear	Unclear concerns regarding applicability	0/191 (0%)
Domain 2: Index test	Risk of bias	Were the index test results interpreted without knowledge of the results of the reference standard?	Not applicable	No reference standard, not eligible study	33/191 (17.3%)
			Yes	Low risk of bias	19/191 (9.9%)
			No	High risk of bias	4/191 (2.1%)
			Unclear	Unclear risk	135/191 (70.7%)
		If a threshold was used, was it pre-specified?*	Not applicable	Index test without threshold (e.g. POC tests)	11/191 (5.8%)
			Yes	Low risk of bias	127/191 (66.5%)
			No	High risk of bias, not eligible study	38/191 (19.9%)
			Unclear	Unclear risk	15/191 (7.8%)
		Could the conduct or interpretation of the index test have introduced bias?	No	Low risk of bias	142/191 (74.3%)
			Yes	High risk of bias, not eligible study	43/191 (22.5%)

			Unclear	Unclear risk, not eligible study	6/191 (3.1%)
	Applicability	Are there concerns that the index test, its conduct, or interpretation differ from the review question?	No	Low concerns regarding applicability	138/191 (72.3%)
			Yes	High concerns regarding applicability	52/191 (27.2%)
			Unclear	Unclear concerns regarding applicability	1/191 (0.5%)
Domain 3: Reference standard	Risk of bias	Is the reference standard likely to correctly classify the target condition?	Not applicable	No reference standard, not eligible study	33/191 (17.3%)
			Yes	Low risk of bias	38/191 (19.9%)
			No	High risk of bias	4/191 (2.1%)
			Unclear	Unclear risk	116/191 (60.7%)
		Were the reference standard results interpreted without knowledge of the results of the index test?	Not applicable	No reference standard, not eligible study	33/191 (17.3%)
			Yes	Low risk of bias	105/191 (55%)
			No	High risk of bias	0/191 (0%)
			Unclear	Unclear risk	53/191 (27.7%)
		Could the reference standard, its conduct, or its interpretation have introduced bias?	Not applicable	No reference standard, not eligible study	33/191 (17.3%)
			No	Low risk of bias	158/191 (82.7%)
			Yes	High risk of bias	0/191 (0%)

			Unclear	Unclear risk	0/191 (0%)
	Applicability	Are there concerns that the target condition as defined by the reference standard does not match the question?	Not applicable	No reference standard, not eligible study	33/191 (17.3%)
			No	Low concerns regarding applicability	151/191 (79.1%)
			Yes	High concerns regarding applicability	4/191 (2.1%)
			Unclear	Unclear concerns regarding applicability	3/191 (1.6%)
Domain 4: Flow and timing	Risk of bias	Was there an appropriate interval between index test and reference standard?	Not applicable	No reference standard, not eligible study	33/191 (17.3%)
			Yes	Low risk of bias	148/191 (77.5%)
			No	High risk of bias	4/191 (2.1%)
			Unclear	Unclear risk	6/191 (3.1%)
		Did all study subjects receive the reference standard?*	Not applicable	No reference standard, not eligible study	33/191 (17.3%)
			Yes	Low risk of bias	149/191 (78%)
			No	High risk of bias, not eligible study	5/191 (2.6%)
			Unclear	Unclear risk, not eligible study	4/191 (2.1%)

	Did all study subjects receive the same reference standard?*	Not applicable	No reference standard, not eligible study	33/191 (17.3%)
		Yes	Low risk of bias	132/191 (69.1%)
		No	High risk of bias, not eligible study	18/191 (9.4%)
		Unclear	Unclear risk, not eligible study	8/191 (4.2%)
	Were all study subjects included in the statistical analysis?*	Not applicable	No statistical analysis, not eligible study	20/191 (10.5%)
		Yes	Low risk of bias	143/191 (74.9%)
		No	High risk of bias, not eligible study	23/191 (12%)
		Unclear	Unclear risk, not eligible study	5/191 (2.6%)
	Could the flow of study subjects have introduced bias?	No	Low risk of bias	102/191 (53.4%)
		Yes	High risk of bias, not eligible study	51/191 (26.7%)
		Unclear	Unclear risk, not eligible study	38/191 (19.9%)
Summatory of bias and concerns regarding applicability	No bias		Low risk, eligible study	37/191 (19.4%)
	One high or unclear bias (not qualifying question)		Medium risk, eligible study	36/191 (18.8%)
	One high or unclear bias (qualifying question) or two or more high or unclear bias		High risk, not eligible study	118/191 (61.8%)

Table 2. Categories and variables studied in the 73 selected articles. JCR = Journal Citation Report; RP = Research physicians; RV = Research veterinarians; NPV = Neither physicians nor veterinarians; OIE = World Organization For Animal Health; ELISA = Enzyme-linked immunosorbent assay; IFAT = Indirect fluorescentantibody tests; WB = Western blot; POC = Point-of-care; CMIA = Chemiluminescent microparticle immunoassay.

Category	Variable	Factor label	Type of variable
Bibliometrics	Research area	Infectious diseases; Parasitology; Veterinary sciences; Tropical medicine; Chemistry, analytical; Microbiology; Medical laboratory technology; Biochemical research methods; Food science & technology; Pediatrics; Biotechnology & applied microbiology; Public, environmental & occupational health; Agriculture, dairy & animal science; Multidisciplinary sciences	Qualitative
	Journal quartile in JCR	Q1; Q2; Q3; Q4	Qualitative
	Authors' affiliation	RP; RV; NPV; Interdisciplinary team	Qualitative
	Interdisciplinary team	Yes/No	Qualitative
	Type of interdisciplinarity	RP and NPV; RV and NPV; RP and RV; RP, RV and NPV	Qualitative
	Continent (last author's affiliation)		Qualitative
	Country (last author's affiliation)		Qualitative
	Country (study area)		Qualitative
Study aims	Study category	1) Design and/or validation of a new technique; 2) Modification of a previously described technique (with new antigens or secondary antibodies); 3) Technique designed to differentiate between acute and chronic infection; 4) Technique designed to detect more than one pathogen; 5) Technique designed to detect *T. gondii* on a different type of sample (e.g., blood serum, saliva, urine); and 6) Technique designed to detect *T. gondii* in different host species.	Qualitative
	One Health quotation	Yes; No; One Health is not quoted but it is implicitly mentioned	Qualitative
Methodology	Humans or animals	Humans; Animals; Both humans and animals	Qualitative

	Host category	Humans; Cats; Dogs; Domestic ruminants; Pigs; Domestic birds; Wild animals (one or several species); Multispecies	Qualitative
	Natural or experimental infection	Samples from natural infection; Samples from experimental infection; Samples from both natural and experimental infection	Qualitative
	Sample selection criteria	Retrospective selection (samples from Banks of samples or known sero-status in samples analyzed before the study); Prospective selection (unknown sero-status in samples from natural infection analyzed in the study or samples from experimental infection performed in the study); Both retrospective and prospective selection	Qualitative
	Evaluated technique	ELISA; IFAT; WB; Agglutination; POC tests; Avidity tests; CMIA; Other type; Several types	Qualitative
	Number of evaluated techniques		Quantitative
	Reference technique	ELISA; IFAT, WB; Agglutination; Dye test; POC tests; CMIA; Clinical criteria; Molecular techniques; Other type; Several types	Qualitative
	Number of reference techniques		Quantitative

Table 3. Results of Pearson's Chi-Square test *p*-values for comparison of "Bibliometrics" and "Study aims" variables. JCR = Journal Citation Reports; (F) = Fisher's exact test; Bold = Statistically significant differences.

		Bibliometrics					Study aims	
		Research area	Journal quartile in JCR	Authors' affiliation	Interdisciplinary team	Type of interdisciplinarity	Study category	One Health quotation
Bibliometrics	Research area		**0.019**	**0.043**	0.683	**0.018**	**0.001**	**0.044**
	Journal quartile in JCR	**0.019**		0.294	0.685	0.634	0.691	0.619
	Authors' affiliation	**0.043**	0.294		**0.000**	0.926	0.682	**0.000**
	Interdisciplinary team	0.683	0.685	**0.000**		0.926	0.657	0.165/0.243(F)
	Type of interdisciplinarity	**0.018**	0.634	0.926	0.926		**0.002**	**0.001**
Study aims	Study category	**0.001**	0.691	0.682	0.657	**0.002**		0.090
	One Health quotation	**0.044**	0.619	**0.000**	0.165/0.243 (F)	**0.001**	0.090	

Table 4. Results of Pearson's Chi-Square test *p*-values for comparison of "Bibliometrics" and "Study aims" parameters with "Methodology" variables. JCR = Journal Citation Reports; (F) = Fisher's exact test; Bold = Statistically significant differences.

		Bibliometrics					Study aims	
		Research area	Journal quartile in JCR	Authors' affiliation	Interdisciplinary team	Type of interdisciplinarity	Study category	One Health quotation
Methodology	Humans or animals	**0.000**	0.390	**0.000**	0.269/0.320 (F)	**0.000**	**0.012**	**0.000/0.000 (F)**
	Host category	0.395	0.676	**0.021**	0.728	**0.001**	0.307	**0.000**
	Natural or experimental infection	0.878	0.555	0.693	0.481	0.782	**0.006**	0.117
	Sample selection criteria	0.290	0.789	**0.044**	0.737	0.051	0.056	0.243
	Evaluated technique	**0.001**	0.736	0.624	0.555	**0.047**	**0.000**	0.248
	Reference technique	0.654	0.587	**0.018**	0.609	0.889	0.427	**0.040**

Table 5. Results of Pearson's Chi-Square test *p*-values for comparison of "Methodology" variables. Bold = Statistically significant differences.

		Methodology					
		Humans or animals	Host category	Natural or experimental infection	Sample selection criteria	Evaluated technique	Reference technique
Methodology	Humans or animals		**0.000**	0.151	**0.002**	0.069	**0.002**
	Host category	**0.000**		**0.000**	**0.000**	0.999	**0.000**
	Natural or experimental infection	0.151	**0.000**		**0.000**	0.995	0.973
	Sample selection criteria	**0.002**	**0.000**	**0.000**		0.564	0.228
	Evaluated technique	0.069	0.999	0.995	0.564		0.616
	Reference technique	**0.002**	**0.000**	0.973	0.228	0.616	

Table 6. Association between quantitative and qualitative variables by Student's T test or ANOVA for parametric conditions or by Mann-Whitney's U test or Kruskal-Wallis test for non-parametric conditions. Bold = Statistically significant differences; * = Significant differences in Bonferroni corrected *post hoc* test.

			Quantitative variables			
			Number of evaluate techniques		Number of reference techniques	
			Statistical analysis	*p*-value	Statistical analysis	*p*-value
Qualitative variables	Bibliometrics	Research area	Kruskal-Wallis test	0.373	Kruskal-Wallis test	0.222
		Journal quartile in JCR	Kruskal-Wallis test	0.736	Kruskal-Wallis test	0.255
		Authors' affiliation	Kruskal-Wallis test	0.989	Kruskal-Wallis test	0.344
		Interdisciplinary team	Student's T test	0.427	Mann-Whitney's U test	0.850
		Type of interdisciplinarity	Kruskal-Wallis test	0.414	Kruskal-Wallis test	0.094
		Continent (last author's affiliation)	Kruskal-Wallis test	0.548	Kruskal-Wallis test	0.052
	Study aims	Study category	Kruskal-Wallis test	**0.003***	Kruskal-Wallis test	**0.016**
		One Health quotation	Mann-Whitney's U test	0.374	Mann-Whitney's U test	0.248
	Methodology	Humans or animals	Mann-Whitney's U test	0.893	Mann-Whitney's U test	0.055
		Host category	Kruskal-Wallis test	0.733	Kruskal-Wallis test	**0.049**

	Natural or experimental infection	Kruskal-Wallis test	0.548	Kruskal-Wallis test	0.206
	Sample selection criteria	Kruskal-Wallis test	0.644	Kruskal-Wallis test	0.238

Supplementary material not included from Huertas-López, A., Sánchez-Sánchez, R., Cantos-Barreda, A., Álvarez-García, G., Ibáñez-López, F. J., Martínez-Subiela, S., Cerón, J. J., and Martínez-Carrasco, C. The systematic review and meta-analysis on the serological diagnosis of *Toxoplasma gondii* infection highlight the lack of a One Health approach.

- **Supplementary File 1:**

  https://docs.google.com/document/d/1vIKqx2ExTqOaOwz2rLLH3OE0IsvZABhl/edit?usp=sharing&ouid=117908795181800371023&rtpof=true&sd=true

  - Table S1. QUADAS-2 assessment of 192 potentially eligible papers.
  - Figure 1 S1. Proportion of studies with low, medium or high summatory of risk.
  - Figure 2 S1. Proportion of studies with low, high or unclear risk of bias (A) or concerns regarding applicability (B) from the 192 reports selected for quality assessment by QUADAS 2 protocol.
- **Supplementary File 2:** articles included for systematic review and meta-analysis (n = 73).

  https://docs.google.com/document/d/183sxjgbPFESDtLsR9ePZYxBEsR7BUpkq/edit?usp=sharing&ouid=117908795181800371023&rtpof=true&sd=true
- **Supplementary File 3:** data extraction from the 73 selected articles.

  https://docs.google.com/spreadsheets/d/1MCQ51aslb0WphV3C3nRxPVVoQBtesK2S/edit?usp=sharing&ouid=117908795181800371023&rtpof=true&sd=true
- **Supplementary File 4:**

  https://docs.google.com/document/d/1hgIrdqzHcIWdYhOiXgvcGDcbCY09RnWM/edit?usp=sharing&ouid=117908795181800371023&rtpof=true&sd=true

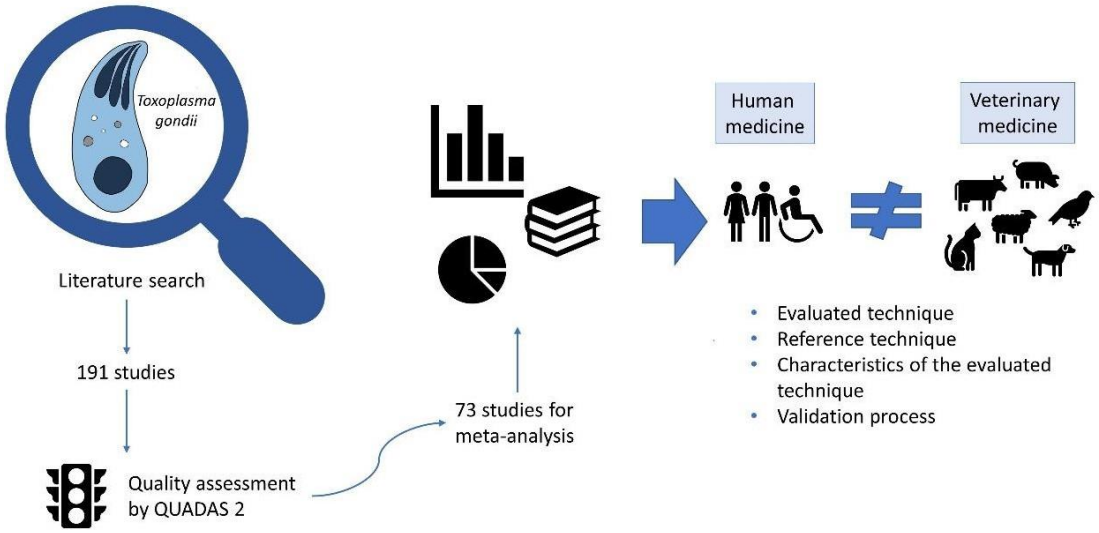
  - Table 1 S4: significant associations between qualitative variables by Pearson's chi-square or Fisher's exact tests.
  - Table 2 S4: Significant associations between quantitative and qualitative variables by Kruskal-Wallis test (non-parametric conditions) and subsequent analysis by Bonferroni corrected *post hoc* test.
- **Supplementary File 5:**

  https://docs.google.com/document/d/124hdjVTsbaSgQ8TtLbRljp68VztF4yrb/e

dit?usp=sharing&ouid=117908795181800371023&rtpof=true&sd=true

- Figure 1 S5: frequencies of studies published in journals classified in quartiles 1 to 4, regarding their performance on human or animal host.
- Figure 2 S5: frequencies of studies that use each type of sample selection criteria, regarding their performance on each host category.

Chapter 1b: A systematic review and meta- analysis on the validation of serological methods for detection of anti-*Toxoplasma gondii* antibodies in humans and animals



Huertas-López, A.[a], Cantos-Barreda, A.[b,c], Sánchez-Sánchez[d], R., Martínez-Carrasco, C.[c], Ibáñez-López, F. J.[e], Martínez-Subiela, S.[a], Cerón, J. J.[a], and Álvarez-García, G[d]. A systematic review and meta-analysis on the validation of serological methods for detection of anti-*Toxoplasma gondii* antibodies in humans and animals.

[a] Interdisciplinary Laboratory of Clinical Analysis, Interlab-UMU, University of Murcia – Regional Campus of International Excellence "Campus Mare Nostrum", 30100 Espinardo, Murcia, Spain

[b] Department of Biochemistry and Molecular Biology-A, University of Murcia - Regional Campus of International Excellence "Campus Mare Nostrum", 30100 Espinardo, Murcia, Spain

[c] Animal Health Department, University of Murcia - Regional Campus of International Excellence "Campus Mare Nostrum", 30100 Espinardo, Murcia, Spain

[d] Animal Health Department, Complutense University of Madrid, Ciudad Universitaria s/n, 28040 Madrid, Spain

[e] Statistical Support Section (SAE), Scientific and Research Area (ACTI), University of Murcia - Regional Campus of International Excellence "Campus Mare Nostrum", 30100 Espinardo, Murcia, Spain

Abstract

Toxoplasmosis is a zoonotic disease subjected to a high concern from the economical and public health points of view. For this reason, numerous serological techniques to detect anti-*T. gondii* antibodies in humans and animals have been developed. The present systematic-review and meta-analysis aims to evaluate the main characteristics of the recently developed serological techniques, as well as the validation process they had, from a One Health's perspective. For this purpose, data regarding techniques, characteristics of the techniques and analytical and diagnostic validation parameters were extracted from 73 studies published from 2014 to 2019 which had been previously selected and quality assessed. Statistical analysis was made focusing in the comparison between human and veterinary medicine. Commercial techniques were most commonly used as reference by physicians, non-physicians and non-veterinarians professionals and interdisciplinary teams, and also in studies carried out on humans; whereas non-commercial techniques were more frequently used as reference by veterinarians and in those studies conducted on animals. The "Gold standard" concept could have been misinterpreted, specially by physicians and non-medical and non-veterinarian professionals. Several areas of improvement were regarding the characteristics of the evaluated techniques, mainly related to the absence of some important data in a considerable proportion of the studies, which could limit their reproducibility. On the other hand, a high heterogeneity was found in their validation process, specially between human and animal hosts. Few studies performed analytical validation of the techniques, while most of them determined the diagnostic sensitivity and specificity. A higher proportion of studies performed in animals chose the determination of agreement in order to compare the analyzed technique with a reference test, while studies performed in human host more frequently used the correlation coefficient instead. This study highlights the need of the development of an internationally consensual protocol for validation of diagnostic techniques to detect *T. gondii* infection in both human and animal species.

Keywords: *Toxoplasma gondii*, validation, serological techniques, One Health, meta-analysis

1. Introduction

Toxoplasmosis is a zoonotic disease caused by the Apicomplexan parasite *Toxoplasma gondii* (Dubey, 2016). The importance of this disease in terms of economic impact and public health has been largely discussed in the scientific literature (Hoffmann et al., 2012; EFSA and ECDC, 2018; Koutsoumanis et al., 2018). In humans, *T. gondii* is frequently asymptomatic, but it may cause reproductive failures in pregnant women, and other health problems, such as ophthalmological and neurological disorders, in congenitally infected children and immunocompromised patients (Koutsoumanis et al., 2018). People can get infected by the consumption of raw meat containing *T. gondii* cysts, and also by the oocysts excreted in cats' faeces which contaminate water, vegetables and soil (Cook et al., 2000; Koutsoumanis et al., 2018; Aguirre et al., 2019; Attias et al., 2020;). On the other hand, a wide range of mammals and birds can be hosts of *T. gondii* and, consequently, suffer from different disorders. In some domestic species, such as small ruminants, *T. gondii* infection is particularly relevant due to the high economic impact associated to the reproductive problems caused by this parasite (Dubey, 2016).

Diagnosis of *T. gondii* infection is made by molecular and serological techniques that have been developed, in many cases, during the last decade (Liu et al., 2015; Wyrosdick and Schaefer, 2015; Ybañez et al., 2020b; Uddin et al., 2021). According to the World Organisation for Animal Health (OIE) (2019), all diagnostic techniques should be validated before use on the aimed species. For this purpose, analytical and diagnostic performance characteristics need to be assessed, and then the validation status should be continuously checked. For analytical validation, parameters such as assay repeatability or inter-assay precision, reproducibility or intra-assay precision, analytical sensitivity (Se) (limit of detection) and analytical specificity (Sp) (evaluation of potential cross-reactivities) are analysed. Diagnostic performance is normally assessed through determining the optimal cut-off, diagnostic Se, diagnostic Sp and the area under the curve (AUC) by a receiver operating characteristics (ROC) analysis or by Bayesian analysis, as well as estimating the agreement (by calculating *kappa* value) and correlation with a reference technique. These analysesought to be carried out comparing with a previously validated technique or, ideally, witha gold standard (Jacobson, 1998;

Thrusfield et al., 2018; OIE, 2019a). In this regard, Sabin-Feldman dye test and mice bioassay are considered as gold standard to diagnose toxoplasmosis in human and veterinary medicine, respectively (Liu et al., 2015; Wyrosdick and Schaefer, 2015; Ybañez et al., 2020b; Uddin et al., 2021).

Several problems can be found when validating new diagnostic techniques. For example, some of the techniques used for the diagnosis of toxoplasmosis, especially in veterinary medicine, are not validated comparing with an international standard, and Se and Sp values are not always high enough (Gardner et al., 2010; Robert-Gangneux and Dardé, 2012; Wyrosdick and Schaefer, 2015; Zhang et al., 2016). Moreover, the lack of reference sera from naturally and experimentallyinfected animals is a limitation for an accurate cut-off estimation (Robert-Gangneux and Dardé, 2012). Finally, the absence of species-specific conjugates for some animal species also may difficult the use of serological techniques in these species (Wyrosdick and Schaefer, 2015).

The present study aimed to evaluate the validation process followed with the recently developed serological assays for human and animal hosts and identify potential areas of improvement. With this purpose, this study consisted of: i) data extraction from articles published between January 1st 2014 and December 31st 2019 on the development of serological techniques for diagnosis of *T. gondii* infection in both humans and animals; and ii) meta-analysis of the data in order to identify the differences in the characteristics and validation method of new serological techniques between humans and animals regarding the guidance for methods validation proposedby the OIE (2019).

2. Material and methods

2.1 Selection of articles and data extraction

The study carried out in Chapter 1a has been the basis of the present systematic review and meta-analysis. Studies on the development of serological techniques for humans and animals were reviewed with the aim of determining whether they followed a One Health approach in Chapter 1a, so variables regarding bibliometrics, study aims and type of evaluated and reference techniques were included. However, for the

present review, we focused on the characteristics of the evaluated techniques and the validation process, so the following 47 variables were analysed:

1. Authors' affiliation.
2. Study population: humans or animals and host category , sample size.
3. Techniques: do they evaluate different techniques?, evaluated technique, number of evaluated techniques, commercial/non-commercial (referred to the evaluated technique/s), type of Enzyme immunoassay (EIA) (evaluated technique), type of agglutination test (evaluated technique), type of Point-of-care (POC) test (evaluated technique), type of Immunoglobulin G (IgG) avidity test (evaluated technique), other assays (evaluated technique), reference technique, number of reference techniques, commercial/non-commercial (reference technique), type of EIA (reference technique), type of agglutination test (reference technique), "gold standard" quotation, methodology quoted as gold standard, and is the reference technique the same quoted as gold standard?
4. Characteristics of the evaluated technique/s: antigen employed, native antigens, recombinant antigens, single/mixed use (recombinant antigens), parasite specific stage antigen, other antigens, type of secondary antibody, antibody isotype detected, positive control, negative control, samples tested in duplicate, do the specify the cut-off?, discrimination between acute and chronic phases and novelty of the method.
5. Type of validation: analytical, diagnostic validation or both of them.
6. Parameters studied for analytical validation: intra-assay precision determination, inter-assay precision determination, limit of detection determination, cross-reactivity determination and pathogen/s considered for studying cross-reactivity.
7. Analysis performed and parameters studied for diagnostic validation: ROC analysis, AUC determination, Se determination, Sp determination, agreement determination and correlation coefficient (Pearson/Spearman) determination.

All studied variables and their factor labels are detailed in Table 1. Whenever possible, the number of true positive (TP), true negative (TN), false positive (FP) and false negative (FN) obtained by the evaluated technique with regards to the reference

technique were also collected from the selected articles.

The present systematic review and meta-analysis was performed according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement (Page et al., 2021). The search strategy, the selection criteria and the quality assessment were described in Chapter 1a. In brief, search was performed using "toxoplasma OR toxoplasm*" and "diagnosis" Medical Subject Headings (MeSH) terms, between January1st 2014 and December 31st 2019, in the PubMed, Web of Science and Scopus databases.Then, articles were screened by title and abstract, and inclusion criteria (articles that analysed one or more serological techniques, were performed in humans and/or animals, had sample size > 5, had an available full text in English and were not review articles) were applied. 192 articles were preliminary selected. Quality assessment was carried out by QUADAS 2 tool, and 73 out of the 192 studies met the quality criteria, with low or medium risk of bias. Selection of studies and quality assessment were madeby three independent researchers. From the 73 finally selected articles, data were blindly extracted by the three researchers using a standardized excel form. Extracted data regarding the 47 analysed variables are described in Supplementary File 1.

2.2 Data analysis

The frequency of each variable was determined graphically. Statistical relationship between "Authors' affiliation", "Humans and animals" and "Hostcategory" variables and the remaining qualitative variables were analysed by Pearson'sChi-Square test, when assumptions were met, or Fisher's exact test, when they were not. The type of evaluated and reference techniques had been previously studied in Chapter 1a. However, some modifications of the variables "Evaluated technique" and "Reference technique" were made for this study, so qualitative inference analyses wererepeated for both variables. Inference analyses were also carried out for the quantitative variables "Sample size", "Number of evaluated techniques", "Number of reference techniques" and "Number of studied pathogens (including *T. gondii*)" by one-way ANOVA when parametric conditions for normality and homoscedasticity were met,or by Kruskal-Wallis test when these conditions were not met. In order to cluster the selected articles according to the authors' affiliation, and the humans or animal host, two Multiple

Correspondence Analysis (MCA) (Kassambara, 2017) were carried out withthe following qualitative variables: evaluated technique, reference technique, methodology quoted as gold standard, antigen employed, antibody isotype detected, discrimination between acute and chronic phases, novelty of the method, analytical or diagnostic validation, cross-reactivity determination, ROC analysis, Se determination, Sp determination, agreement determination, correlation coefficient (Pearson/Spearman) determination. The two MCA were graphically represented for "Authors' affiliation" and "Humans or animals" variables, respectively. Finally, a diagnostic test accuracy (DTA) meta-analysis was performed. In this meta-analysis, the pooled Se, Sp and diagnostic odds ratio (DOR) of the evaluated techniques was estimated by a random effects model(Cota et al., 2012) that included the studies in which true positives (TP), true negatives (TN), false positives (FP), false negatives (FN) data were available. To perform the meta-analysis, each of the techniques evaluated in the studies was considered separately and, therefore, included independently in this analysis. Evaluated techniquesthat showed undetermined results regarding the reference technique/s were also excluded from this analysis.

Statistical analysis of data was performed by R software (R Core Team 2020). Significant relationship between variables was considered when p value < 0.05. The article Q20 was excluded for statistical analysis of variables concerning antigens employed and antibody isotype detected. The article Q06 only met quality assessment for the analysisof IgG enzyme-linked immunosorbent assay (ELISA), and not for IgM ELISA and Avidity assay, so statistical analysis was performed on IgG ELISA data (Chapter 1a).

3. Results

The 73 articles included in the present systematic review and meta-analysis are shown in Supplementary File 1 of Chapter 1a. All the results obtained in the chi-square and Fisher's exact tests between "Authors' affiliation", "Humans and animals" and "Host category" variables and the other qualitative variablesare included in Supplementary File 2. All the results obtained in the inference analyses for the quantitative variables "Sample size", "Number of evaluated techniques", "Number of reference techniques" and "Number of studied pathogens (including *T. gondii*)" variables and the other

qualitative variables are included in Supplementary File 3. From these results, the most relevant findings are described hereunder.

3.1 Study population

The significant relationships of the quantitative variable "Sample size" with the qualitative variables are shown in Table 2 and Figure 1.

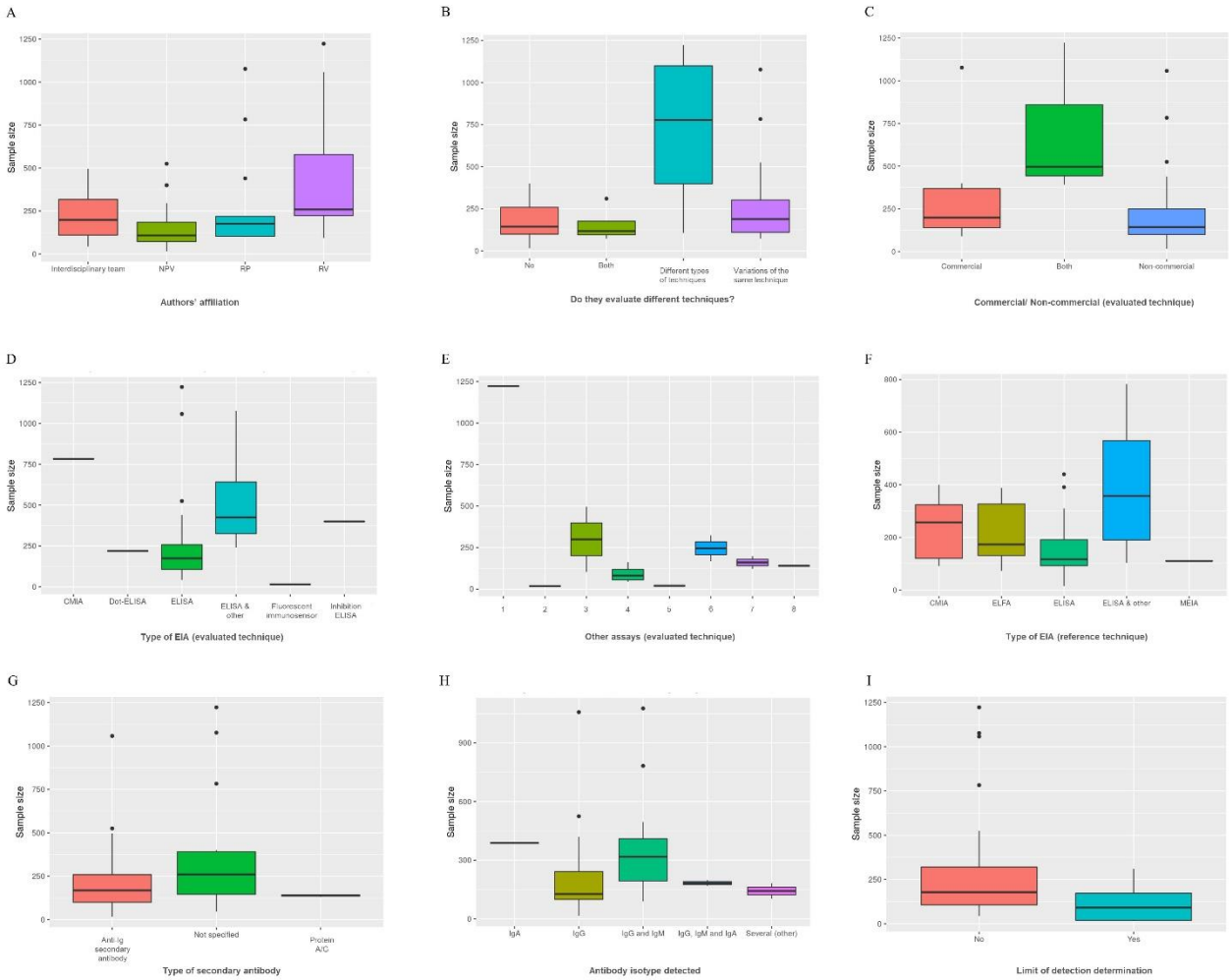


Figure 1. Boxplots of "Sample size" regarding the qualitative variables with statistically significant relationships. NPV = Neither physicians nor veterinarians; RP = Research physicians; RV = Research veterinarians; EIA = Enzyme immunoassay; CMIA = Chemiluminescent microparticle immunoassay; ELISA = Enzyme-linked immunosorbent assay; 1 = Bead-based assay (only for T. gondii detection); 2 = Electrochemical sensing platform; 3 = Flow cytometry; 4 = Multiplex bead assay; 5 = Multiplex dot-immunoassay based; 6 = Peptide microarray; 7 = Several; 8 = Suspension immunoassay (SIA); ELFA = Enzyme-linked fluorescence assay; MEIA = Microparticle enzyme immunoassay; ; Ig = Immunoglobulin.

3.2 Techniques

The frequency of variables from "Techniques" category is shown in Figures 2 (variables regarding the evaluated technique) and 3 (variables regarding the reference

technique). Figures 4 and 5 represent the boxplots for “Number of evaluated techniques” and “Number of reference techniques”, respectively, regarding the qualitive variables with which they had significant relationships (Table 2).

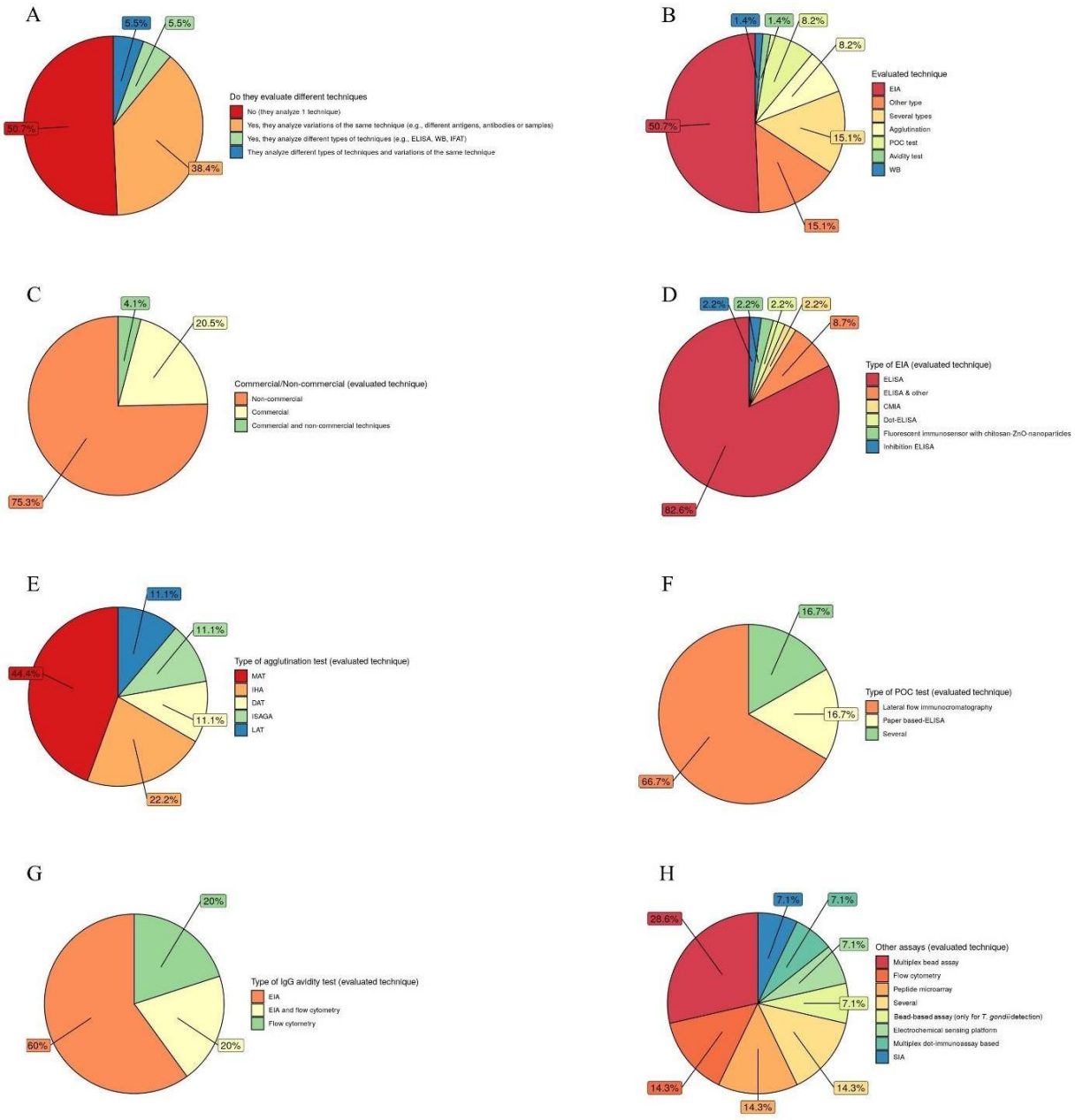


Figure 2. Frequencies of variables regarding the evaluated technique/s. EIA = Enzyme immunoassay; POC = Point-of-care; WB = Western Blot; ELISA = Enzyme-linked immunosorbent assay; CMIA = Chemiluminescent microparticle immunoassay; MAT = Modified agglutination assay; IHA = Indirect hemagglutination test; DAT = Direct agglutination test; ISAGA = Immunosorbent agglutination assay; LAT = Latex agglutination test; SIA = Suspension immunoassay.

There was a significant relationship between the evaluated technique and the human or animal host (p = 0.035) (Table 3). From the total of 49 studies performed on

humans, the most frequently evaluated technique was EIA (44.9%; 22/49), whereas avidity test and western blot (WB) were each evaluated in only 2% (1/49) of studies. In contrast, from the 24 studies carried outon animals, 62.5% (15/24) evaluated EIA, and POC test was the less frequently evaluated technique (4.2%; 1/24). Agglutination methods were more often evaluated on animals (16.7%; 4/24) in comparison with humans (4.1%; 2/49). On the contrary, POC tests were more evaluated on humans (10.2%; 5/49) in comparison with animals (4.2%; 1/24).

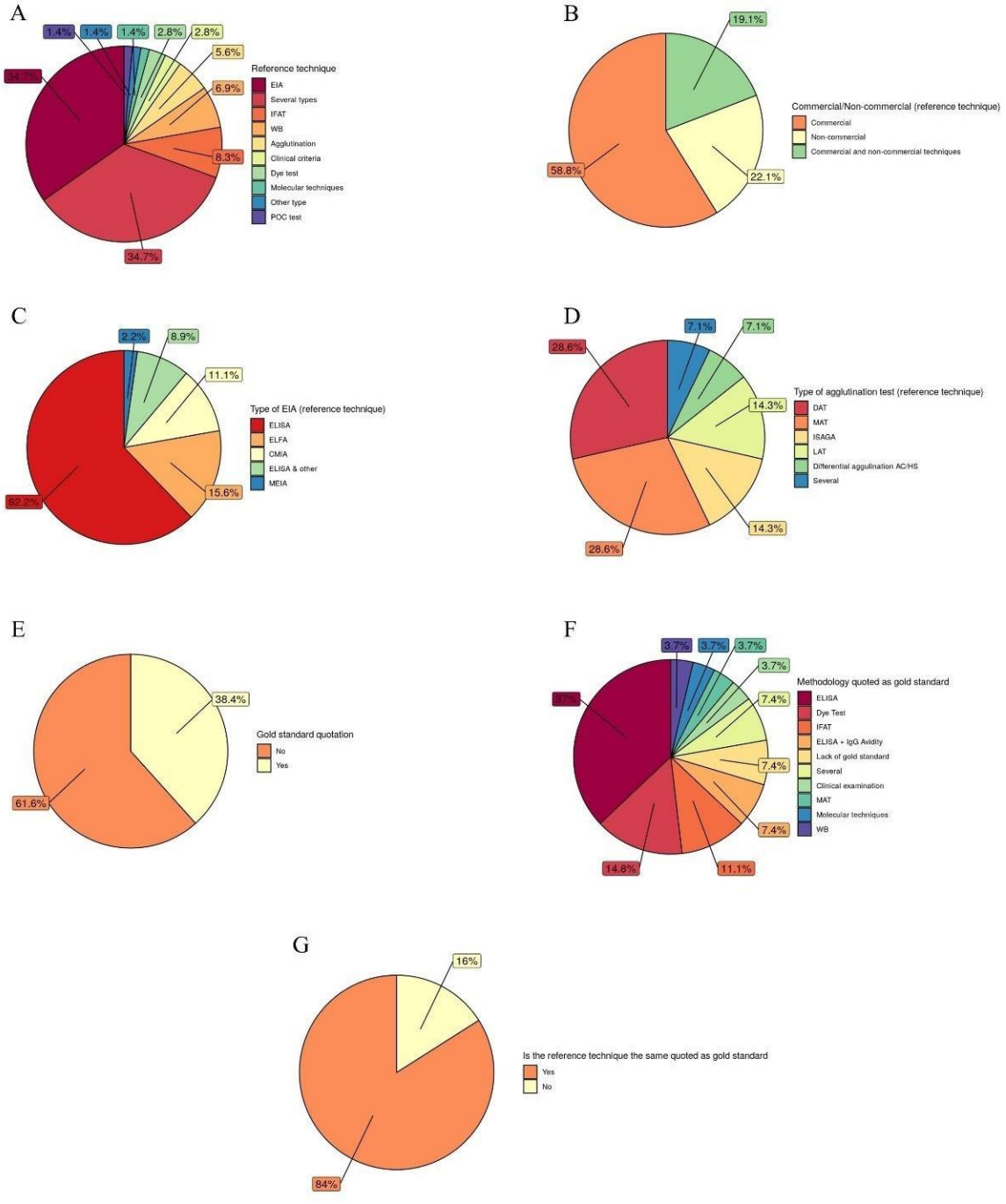


Figure 3. Frequencies of variables regarding the reference technique/s. EIA = Enzyme immunoassay; IFAT = Indirect fluorescent antibody tests; WB = Western Blot; POC = Point-of-care; ELISA = Enzyme-linked immunosorbent assay; ELFA = Enzyme-linked fluorescence assay; CMIA = Chemiluminescent microparticle immunoassay; MEIA = Microparticle enzyme immunoassay; DAT = Direct agglutination test; MAT = Modified agglutination assay; ISAGA = Immunosorbent agglutination assay; LAT = Latex agglutination test.

The reference technique also showed a significant relationship with the variables "Authors' affiliation" ($p = 0.037$), "Humans or animals" ($p < 0.001$) and "Host category" ($p < 0.001$) (Table 3). Most of studies conducted by an interdisciplinary team used EIA (32.3%; 10/31) or several types of technique (32.3%; 10/31) as the reference technique, similarly to those performed by NPV and research physicians (RP), which also used EIA (38.1%; 8/21 and 58.3%; 7/12, respectively) and several types of techniques (47.6%; 10/21 and 33.3%; 4/12, respectively) as the reference technique more frequently. However, studies conducted by RV more commonly used indirect fluorescent antibody tests (IFAT) (37.5%; 3/8) and agglutination tests (25%; 2/8). Regarding the host studied, 44.9% (22/49) studies performed on humans used EIA as reference technique, and 36.7% (18/49) used severaltechniques. In contrast, studies conducted on animals preferred to use several techniques (29.2%; 7/24), IFAT (20.8%; 5/24) and agglutination tests (16.7%; 4/24) as the reference tests. The most used reference technique was different regarding the specific host category. For example, it is noteworthy that IFAT was frequently used on dogs (33.3%; 1/3), domestic ruminants (37.5%; 3/8) and pigs (33.3%; 1/3) and agglutination methods on cats (66.6%; 2/3) and wild animals (33.3%; 1/3).

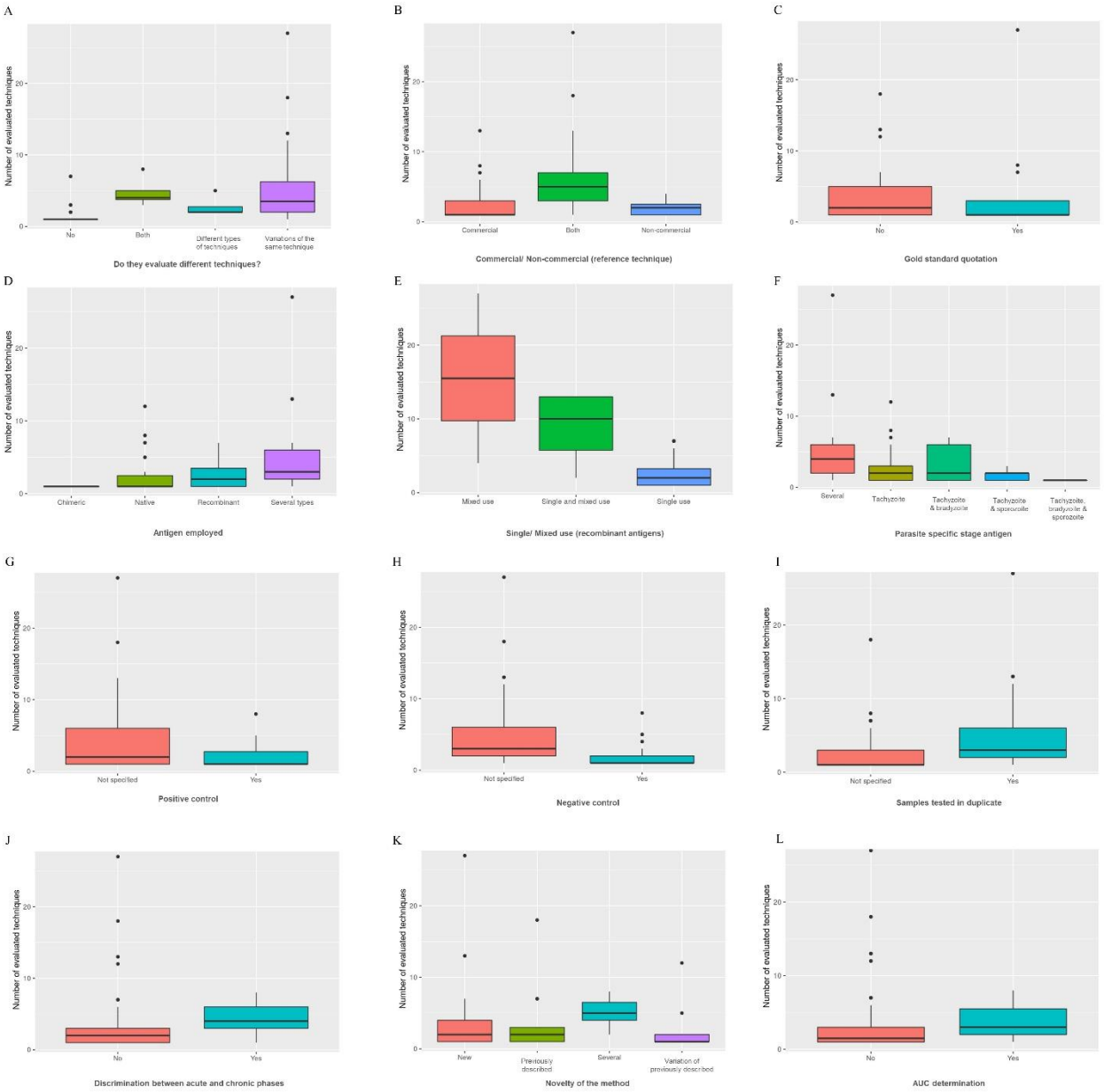


Figure 4. Boxplots of "Number of evaluated techniques" regarding the qualitative variables with statistically significant relationships.

The use of commercial or non-commercial techniques as reference tests was also significantly related to the authors' affiliation (p = 0.019), the studied host (human or animal) (p < 0.001) and the specific host category (p = 0.001) (Table 3). Most of studies conducted by interdisciplinary teams (55.2%; 16/29), NPV (61.9%; 13/21) and RP (81.8%; 9/11) used commercial techniques as reference, while most of studies performed by RV (71.4%; 5/7) used non-commercial techniques. With regards to the study host, 65.3% (32/49) of studies conducted on humans used commercial techniques as reference, only 8.2% (4/49) applied non-commercial techniques, and the remaining 18.4% (9/49) used both commercial and non-commercial techniques. In contrast, most of the studies

conducted on animals chose non-commercial techniques (45.8%: 11/24) as reference, whereas 33.3% (8/24) used commercial techniques and 16.7% (4/24) both commercial and non-commercial techniques.

The type of agglutination method used as reference technique had a significant relationship with the human or animal host (p = 0.005) (Table 3). From the four articles that used agglutination techniques as reference in humans, 50% (2/4) used immunosorbent agglutination assay (ISAGA), 25% (1/4) differential agglutination AC/HS and 25% (1/4) several agglutination tests. In animals, 40% (4/10) of studies used direct agglutination test (DAT), 40% (4/10) modified agglutination test (MAT) and 20% (2/10) latex agglutination test (LAT) as reference technique.

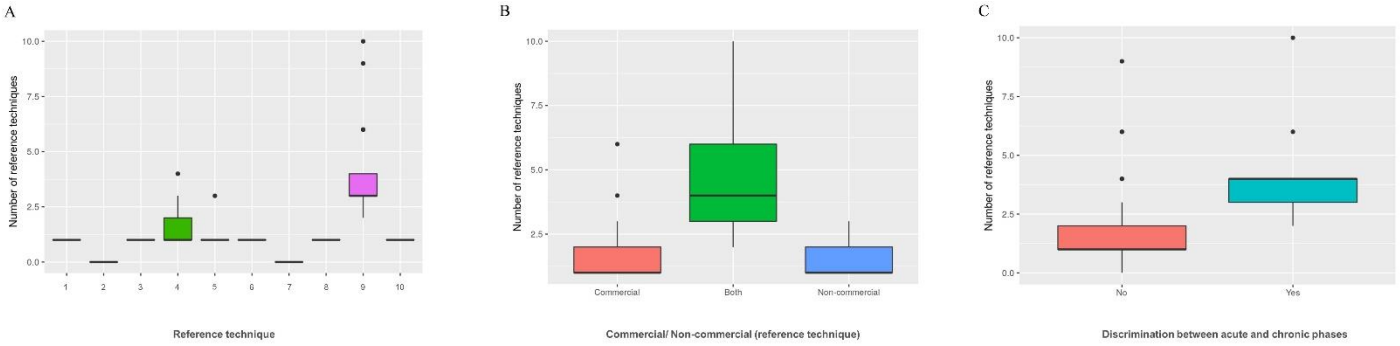


Figure 5. Boxplots of "Number of reference techniques" regarding the qualitative variables with statistically significant relationships. 1 = Agglutination; 2 = Clinical criteria; 3 = Dye test; 4 = EIA (Enzyme immunoassay); 5 = IFAT (Indirect fluorescent antibody test); 6 = Molecular technique; 7 = Other type; 8 = POC (Point-of- care) test; 9 = Several types; 10 = WB (Western Blot).

From the 28 out of 73 (38.4%) articles who mentioned "gold standard", there was no consensus regarding the considered gold standard technique (Figure 3F).Moreover, from the 25 articles that mentioned a specific method as gold standard, 21 (84%) used that reference technique. On the other hand, the "gold standard" concept quotation, the techniques mentioned as gold standard, and the use as reference technique the same technique quoted as gold standard, were not related to the authors' affiliation (p = 0.083, p = 0.285 and p = 0.405, respectively) and to the human or animal subject of study (p = 0.718, p = 0.079 and p = 1.000, respectively) (Supplementary File 2). However, the "Gold standard quotation" variable was significantly related to "Host category" (p = 0.037) (Table 3), as it was quoted in 100% (3/3) of the studies conducted on pigs and 66.6% (2/3) studies performed on multispecies, while it was not quoted in

all or most of the studies conducted in other host categories: humans (58.3%; 28/48); cats (66.6%; 2/3); dogs (66.6%; 2/3); domestic birds (100%; 2/2), domestic ruminants (100%; 8/8) and wild animals (66.6%; 2/3).

3.3 Characteristics of the evaluated techniques

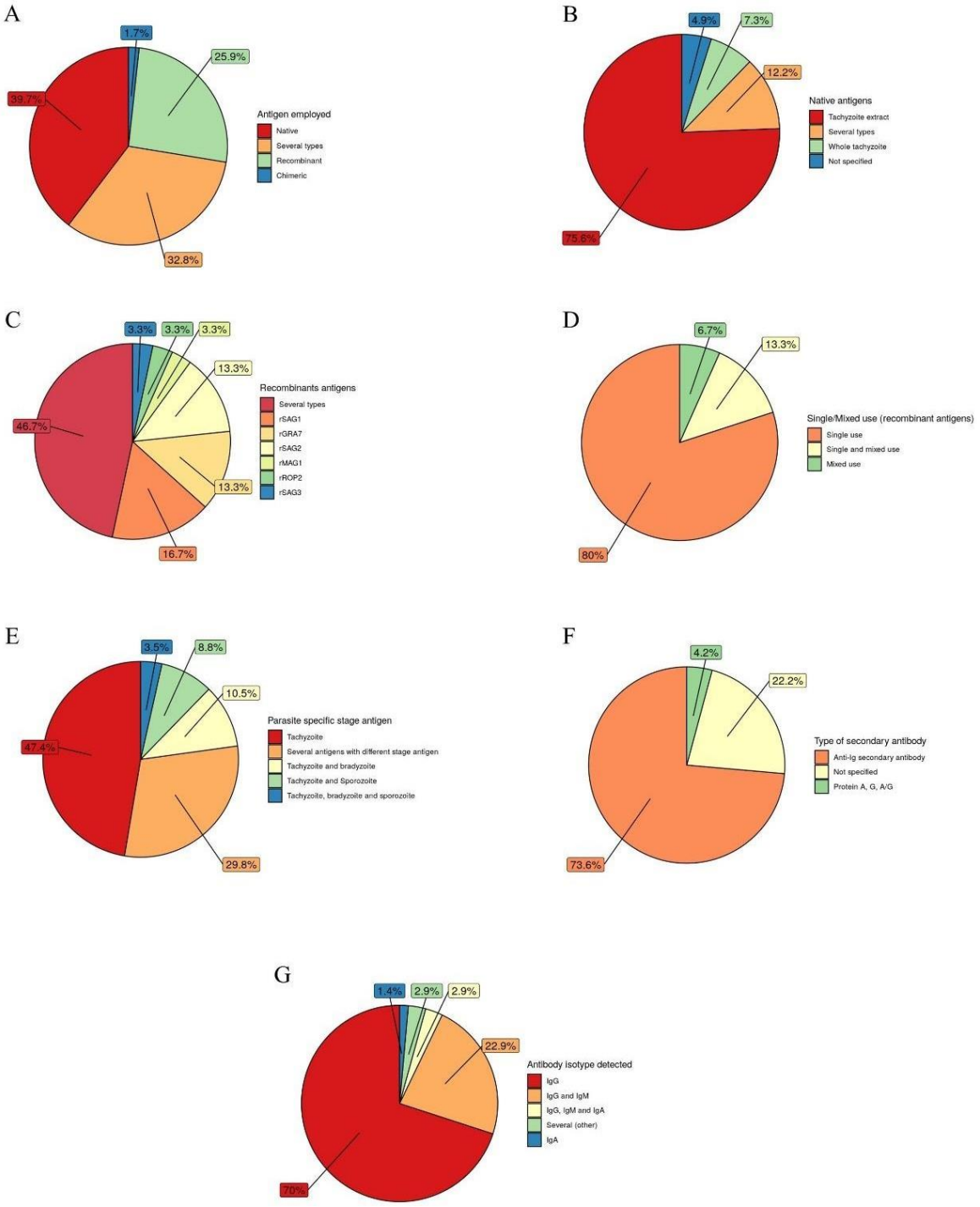


Figure 6. Frequencies of variables regarding the characteristics of the evaluated technique/s related to antigens and secondary antibodies employed. SAG = Surface antigen; GRA = Dense granular antigen; MAG = Matrix antigen; ROP = Rhoptry antigen; Ig = Immunoglobulin.

Some fundamental data were not appropriately described in all articles. Concretely, the antigen employed and the antibody isotype detected were not specified in 19.2% (14/73) and 2.7% (2/73) of the articles, respectively. Regarding the variables included in "Characteristics of the evaluated techniques" category from those articles that included these data, their frequencies are graphically represented in Figures 6 (including variables related to antigens and secondary antibodies employed) and 7 (variables analysing othercharacteristics of the evaluated techniques). Figure 8 shows the frequency of antigens employed regarding "Authors' affiliation" (Figure 8A), "Humans or animals" (Figure 8B)and "Host category" (excluding humans) (Figure 8C) variables. In Figure 9, the frequencies of antibody isotype detected were represented also regarding the authors'affiliation (Figure 9A), the human or animal host (Figure 9B) and the animal host category (excluding humans) (Figure 9C). Finally, Figure 10 shows the boxplots for the variable "Number of studied pathogens (including *T. gondii*)" regarding the qualitative variables to which it was significantly related (Table 2).

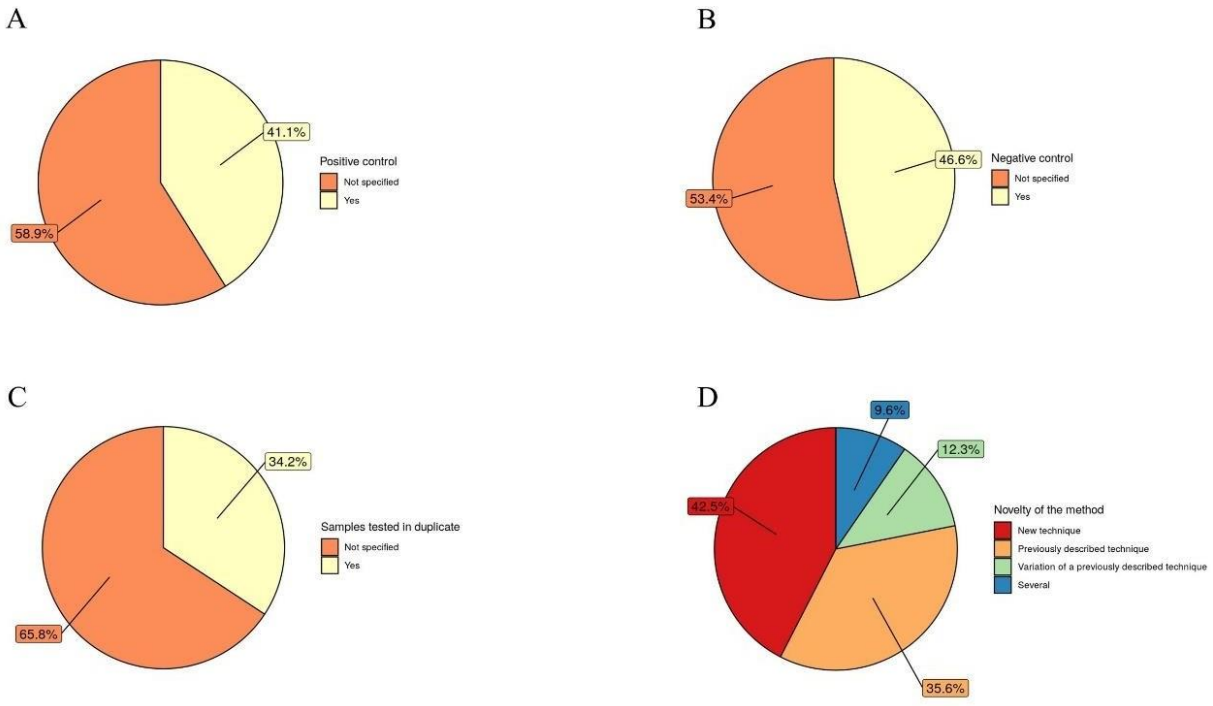


Figure 7. Frequencies of variables regarding other characteristics of the evaluated technique/s.

While 72 out of 73 articles described serological techniques that detected antibodies, only one report (Q20) evaluated a technique for detection of *T. gondii* antigens. The only article that evaluated a non-protein antigen was Q03, which used a synthetic Glycosylphosphatidylinositol (GPI) glycans on a bead-based multiplex assay.

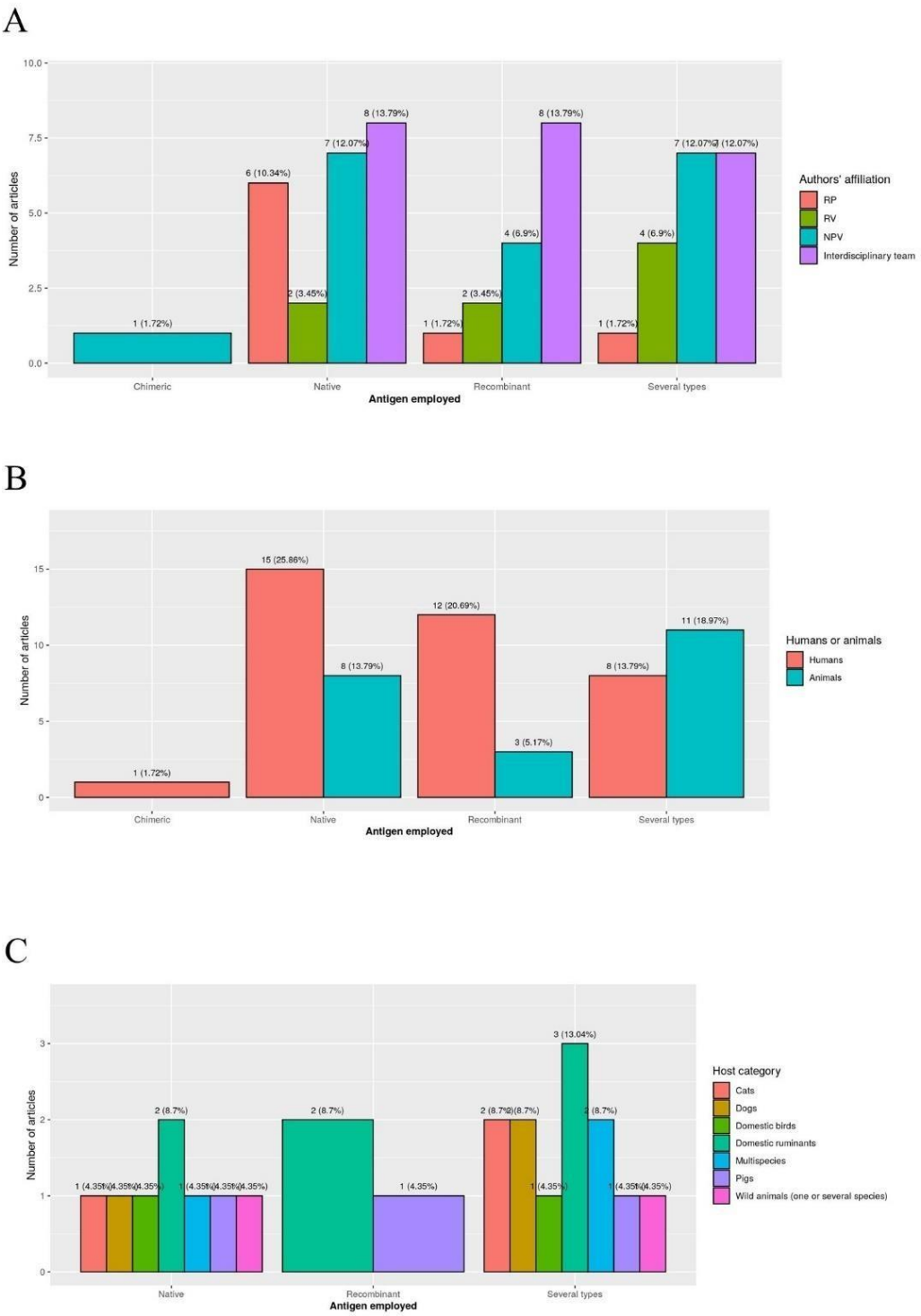


Figure 8. Frequencies of antigens employed regarding "Authors' affiliation" (A), "Humans or animals" (B) and "Host category" (C) variables.

The antibody isotype detected was significantly related to the authors' affiliation (p = 0.013) (Table 3). Most of studies performed by interdisciplinary teams and NPV evaluated technique for the detection of IgG (61.3%; 19/31 and 90.5%; 19/21, respectively). All studies conducted by RV detected IgG. In contrast, 50% (6/12) of studies performed by RP evaluated technique to detect both IgG and IgM, and 41.7% (5/12) to detect only IgG.

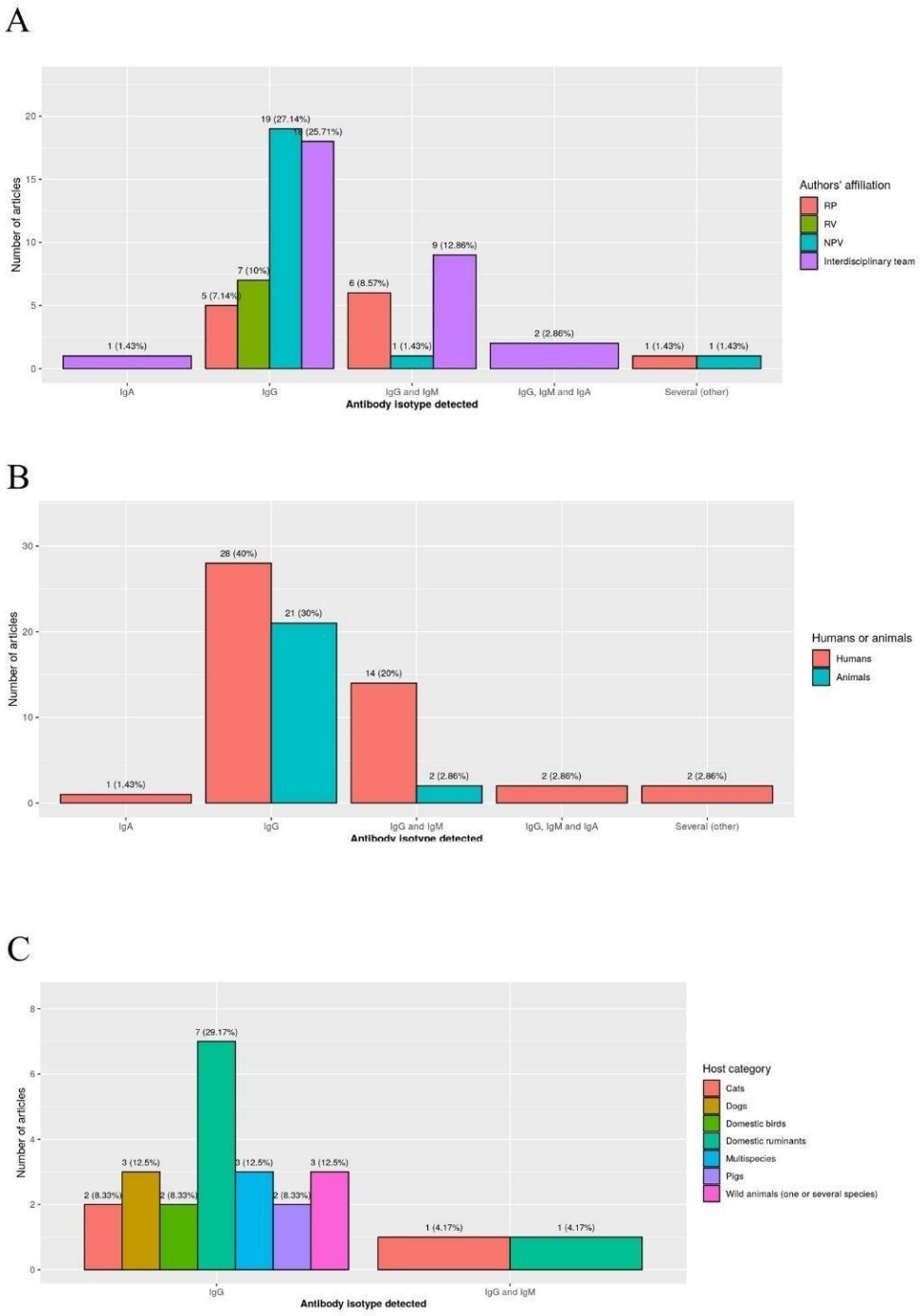


Figure 9. Frequencies of antibody isotypes detected regarding "Authors' affiliation" (A), "Humans or animals" (B) and "Host category" (C) variables.

The description of the use of duplicate samples was significantly higher inanimals (58.3%; 14/24) than in humans (22.4%; 11/49) (p = 0.006) (Table 3).

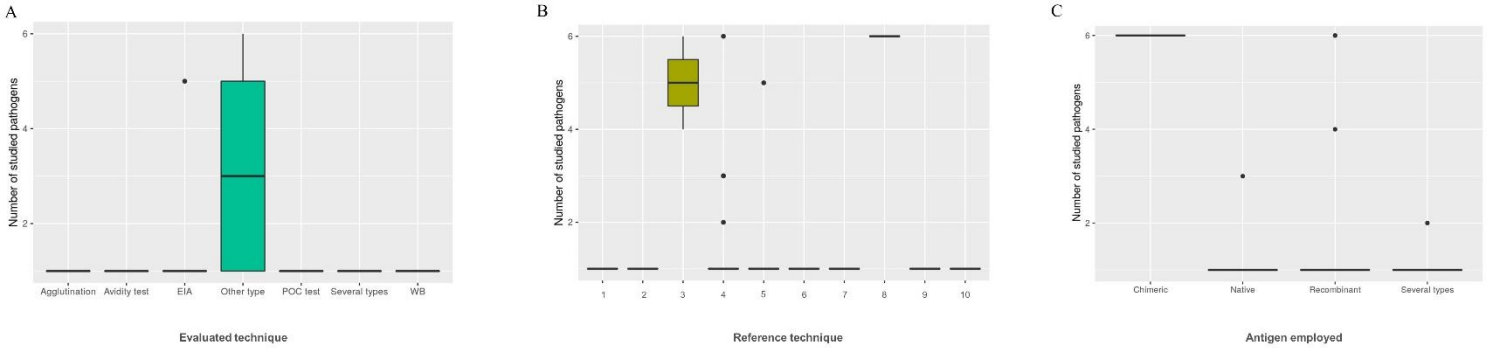


Figure 10. Boxplots of "Number of studied pathogens (including T. gondii)" regarding the qualitative variables with statistically significant relationships. EIA = Enzyme immunoassay; POC = Point-of-care; WB = Western Blot; 1 = Agglutination; 2 = Clinical criteria; 3 = Dye test; 4 = EIA; 5 = IFAT (Indirect fluorescent antibody test); 6 = Molecular technique; 7 = Other type; 8 = POC test; 9 = Several types; 10 = WB.

The exact cut-off was specified in 98.5% of the 67 studies where applicable (excluding POC tests). Only one study did not mention the exact value of the established cut-off, but described how to calculate it.

From the total of 73 articles, only 12.3% discriminated between acute and chronic phases of *T. gondii* infection. Discrimination between acute and chronic phases was only analysed in studies performed in human host and not in animal hosts (p = 0.026) (Table 3).

3.4 Validation

Most of articles exclusively performed a diagnostic validation (89%), whereas 2.7% of articles only carried out an analytical validation and 8.2% performed both analytical and diagnostic validations. Figure 11 shows the frequencies of the type of validation represented regarding the authors' affiliation (Figure 11A) and the human or animal host (Figure 11B).

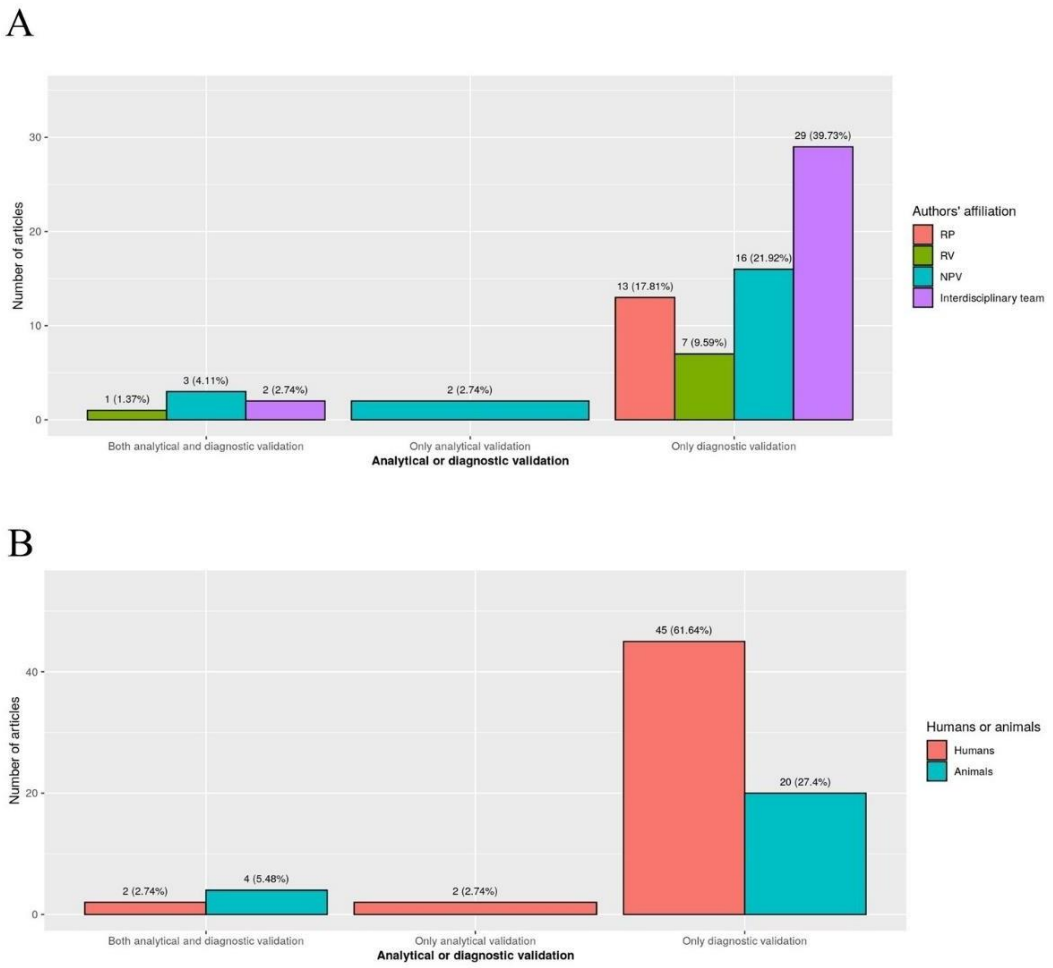


Figure 11. Frequencies of analytical or diagnostic validation regarding "Authors' affiliation" (A) and "Humans or animals" (B) variables.

Focusing on the parameters evaluated for analytical validation, 9.6% of articles determined the intra- and inter-assay precision and the limit of detection. Cross-reactivity was estimated in 14 out of 73 articles (19.2%), from which six studied cross-reactivity with other parasites, one with virus, and seven with several pathogens (caused by virus, bacteria, parasites and other non-infectious agents). From the six articles studying cross-reactivity with other parasites, five out of them (Q02, Q54,Q65, Q66 and Q73) analysed cross-reactivity with *N. caninum*. In addition, the study Q73 also analysed it with *Besnoitia besnoiti*, *Besnoitia tarandi* and *Trichinella spiralis*. Although Q05 did not specifically describe the study of cross-reactivity, it was considered as such because the serum samples utilized as negative controls were positive to *Hammondia hammondi*. The pathogen considered for studying cross- reactivity has a significant relationship with the variables "Humans or animals" (p = 0.012) and "Host category" (p = 0.014) (Table 3). In humans, several pathogens were studied in 83.3% (5/6) of the articles analysing the

cross-reactivity, while 16.7% (1/6) focused on virus. From the 8 studies analysing cross reactivity in animals, 75% (6/8) studied other parasites (different from *T. gondii*) and 33.3% (2/6) studied severalpathogens. Regarding the specific host category, 100% (1/1) of studies on cats, 100% (1/1) on domestic birds, 100% (2/2) on domestic ruminants and 100% (2/2) on multispecies studied other parasites (different from *T. gondii*), while 100% (1/1) of studies on dogs and 100% (1/1) on pigs studied several pathogens.

The graphical representation of frequencies of the validation variables from "Analysis performed and parameters studied for diagnostic validation" category is shown in Figure 12. From a total of 35 studies that determined the agreement between the evaluated and reference techniques, 77.1% (27/35) used the Cohen's *kappa* value and 22.9% (8/35) an agreement percentage. A statistical relationship was found between determination of agreement and authors' affiliation ($p = 0.038$), the human or animal host ($p < 0.001$) and the specific host category ($p = 0.06$) (Table 3). This parameter was estimated in most of the studies performed by interdisciplinary teams (51.6%; 15/16), by RP (61.5%; 8/13) and by RV (75%; 6/8). However, it was not determined in the majority of the studies conducted by NPV (76.2%; 16/21). In addition, from the articles performed on humans, 32.7% (16/49) estimated the agreement, in comparison with 79.2% (19/24) of studies on animals that studied it. Within the 19 articles performed inanimals that determined agreement, 31.6% (6/19) were carried out in domestic ruminants, 15.8% (3/19) in cats, 15.8% (3/19) in dogs, 10.5% (2/19) in domestic birds,10.5% (2/19) in pigs, 10.5% (2/19) in wild animals and 5.3% (1/19) in several species at the same time.

Determination of the correlation coefficient (Pearson/Spearman) showed a statistical relationship with the human or animal host ($p = 0.014$) (Table 3), being 94.1% (16/17) of the articles which estimated this parameter performed on humans. From the 16 articles that calculated the correlation coefficient in humans, 12.5% (2/16) did not provide the exact result.

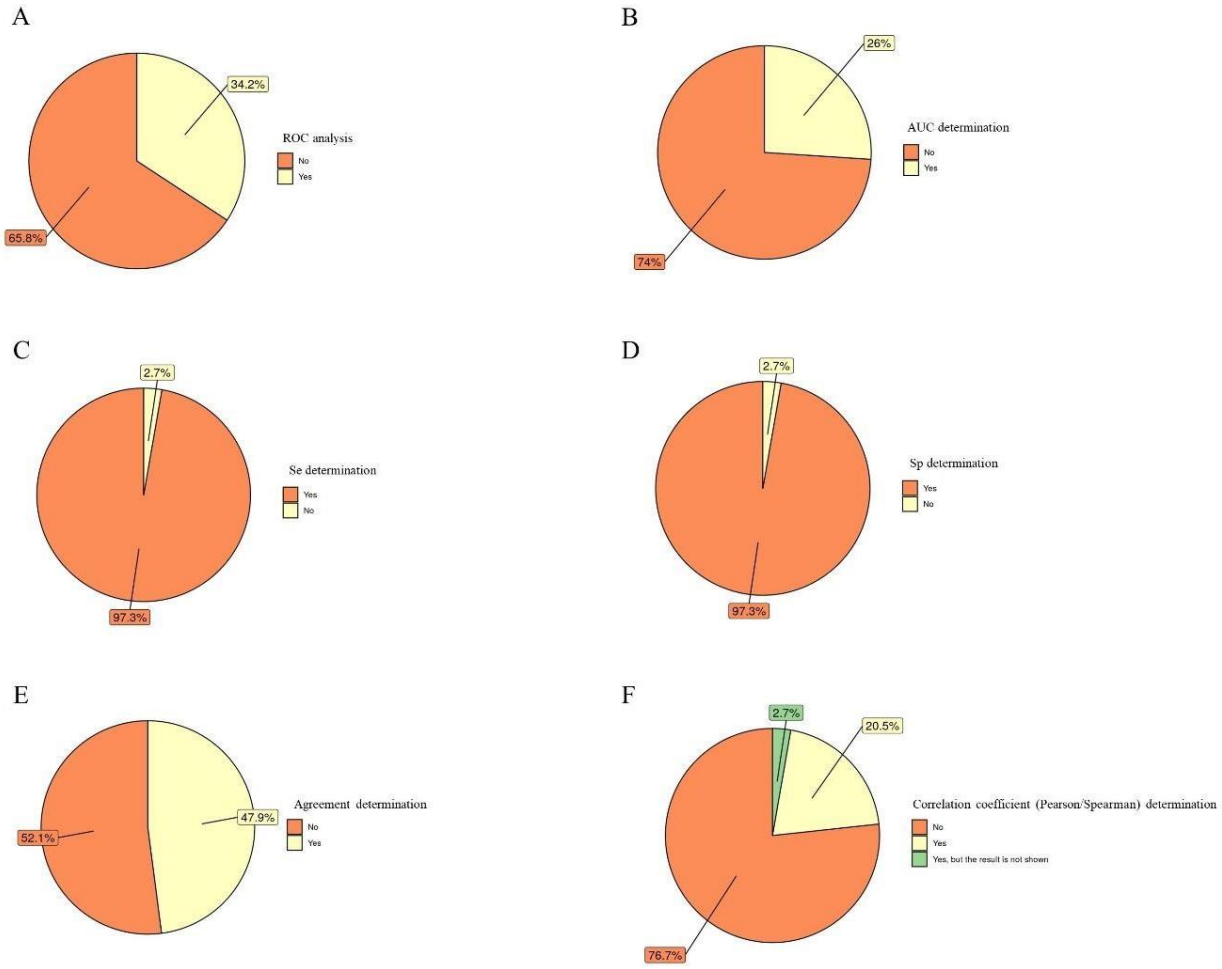


Figure 12. Frequencies of variables regarding the analysis performed and parameters studied for diagnostic validation. ROC = Receiver operating characteristic; AUC = Area under de curve; Se = Sensitivity; Sp = Specificity.

3.5 Multiple Correspondence Analysis (MCA)

Factor labels with higher contribution for dimension 1 (6.7% variability) were "Analytical validation" (from the variable "Analytical or diagnostic validation"), "No" (from "Se determination"), "No" (from "Sp determination") and "Yes" (from "Correlation coefficient (Pearson/Spearman) determination") (Figure 13A). On the other hand, for dimension 2 (7.7% variability) "Animals" (from the variable "Humans or animals", "RV" (from "Authors' affiliation"), "RP" (from "Authors' affiliation") and "Native" (from "Antigen employed") were the most contributory factor labels (Figure 13B).

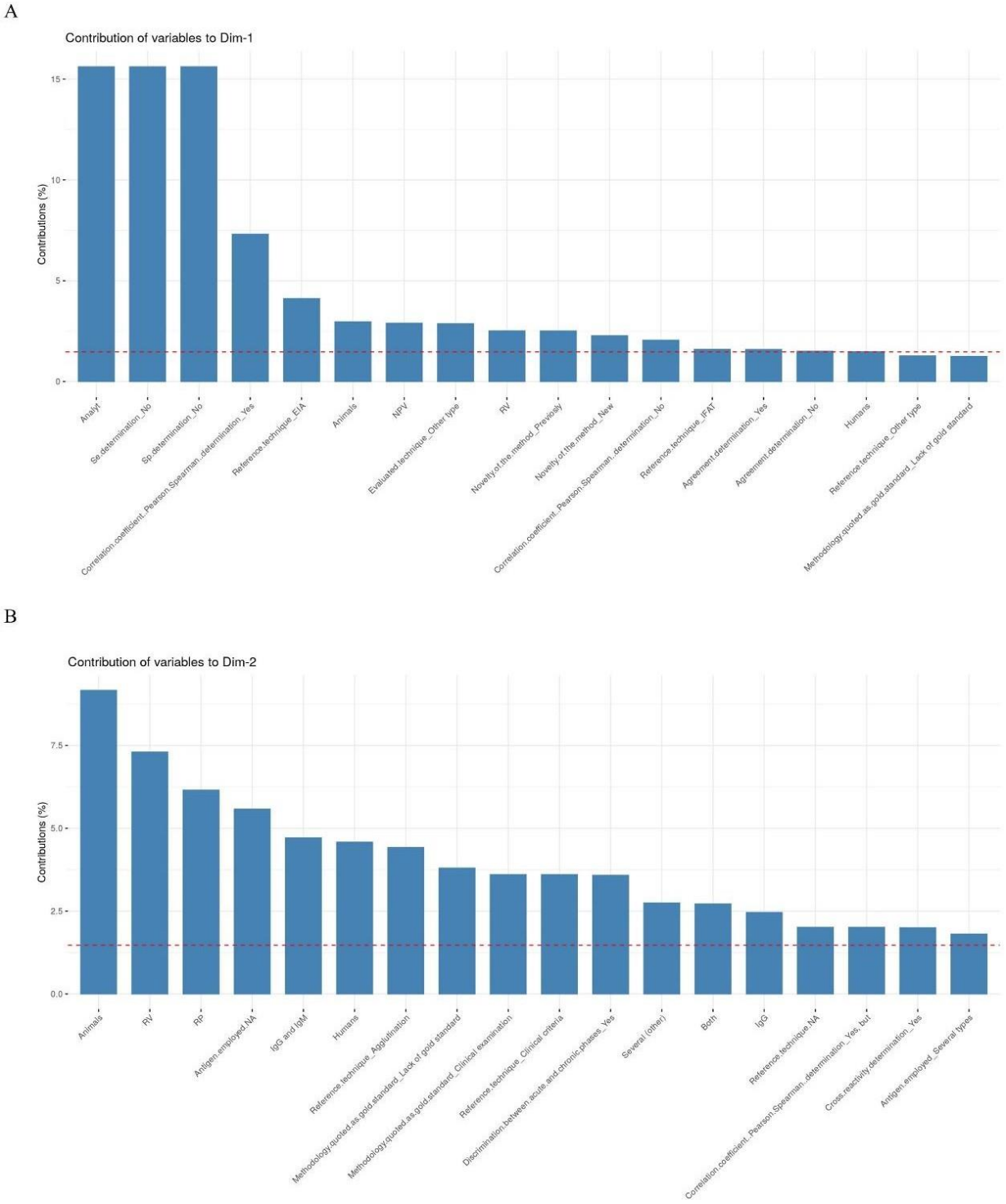


Figure 13. Degree of contribution of factor labels in dimensions 1 (A) and 2 (B) for Multiple Correspondence Analyses (MCA).

The distribution of the 72 articles included in the MCA for "Authors' affiliation" is depicted in Figure 14A, showing a clear separation in those articles performed by RV

and by RP with regards to the other authors' affiliation. Studies carried out by RV were negatively influenced by variables from dimensions 1 and 2, while studies performed by MP were only positively influenced by variables from dimension 2. Moreover, studies carried out by NPV were positively influenced by variables from dimension 1 and negatively by dimension 2 variables. Conversely, interdisciplinary teams showed a relatively slight negative influence by variables from dimension 1 and positive from dimension 2.

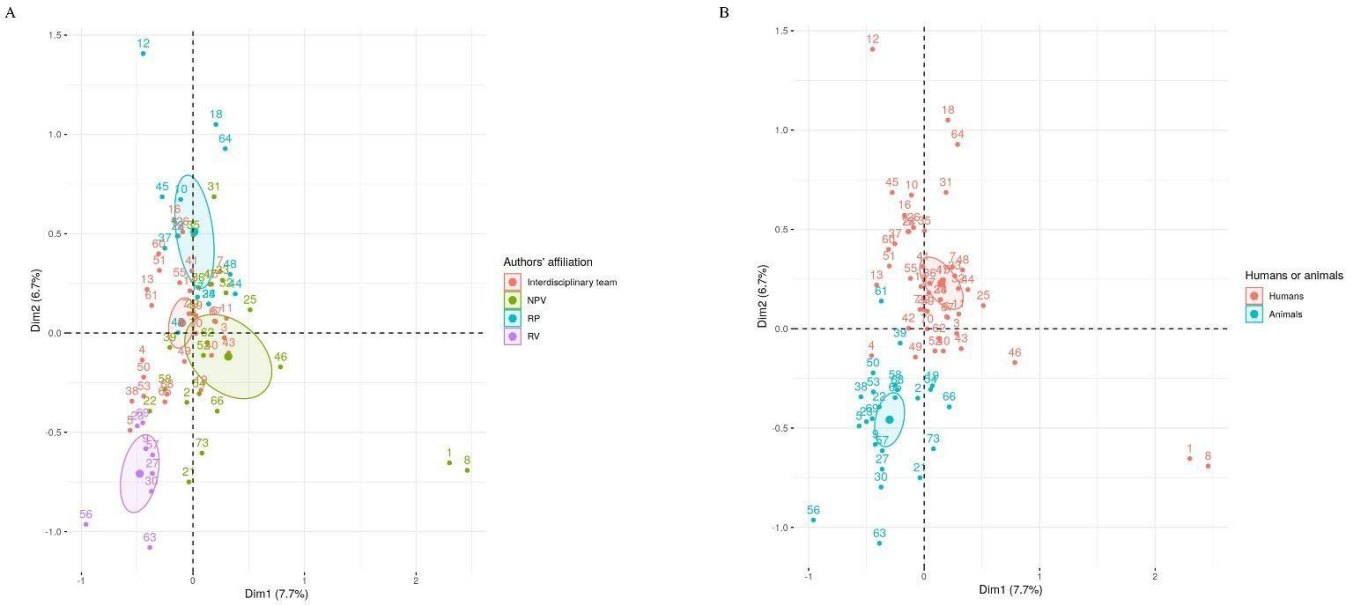


Figure 14. Graphical depicting of individual distribution of the studies within dimension 1 and dimension 2 in Multiple Correspondence Analyses (MCA) for "Authors' affiliation" (A) and for "Humans or animals" (B), being Dim-1 = dimension 1 (factor labels with higher contribution: "Analytical or diagnostic validation- Analytical validation", "Se determination – No", "Sp determination – No" and "Correlation coefficient (Pearson/Spearman) determination - Yes") and Dim-2 = dimension 2 (factor labels with higher contribution: "Humans or animals – Animals", "Authors' affiliation - RV", "Authors' affiliation – RP", and "Antigen employed - Native").

Figure 14B represents the MCA for "Humans or animals" variable, and it showed a clear separation of the articles depending on their performance in human or animal hosts. Particularly, studies performed in human host showed a positive influence by variables from dimensions 1 and 2, in contrast with those carried out in animal hosts, which are negatively influenced by variables from both dimensions.

3.6 Diagnostic test accuracy meta-analysis

Data of TP, TN, FP and FN were available in 53.4% (39/73) articles. From them, 11 techniques showed undetermined results and were not included in the analysis. Results of DTA meta-analysis are summarized in Table 4.

4. Discussion

The aim of this systematic review and meta-analysis was to evaluate the characteristics and validation procedure of the recently developed serological assays for *T. gondii* diagnosis in both human and veterinary medicine. A total of 73 records were analysed. The overall results of this meta-analysis confirm the elevated grade of heterogeneity in the way serological methods are currently developed and validated for both human and animal hosts. Though the World Health Organization (WHO) does not have any official protocol for the evaluation of serological assays to diagnose *T. gondii* infection in humans, it has established several protocols for evaluation of diagnostic assays for other diseases, such as Malaria or HIV (Banerjee et al., 2022; WHO, 2016), which could be used as a model. In a similar way, a protocol for validation of serological assays in animals was established by the OIE, consisting in four stages with different levels of evaluation: analytical characteristics (Stage 1), diagnostic characteristics (Stage 2), reproducibility (Stage 3) and implementation (Stage 4) (OIE, 2019a).

"Gold standard" can be defined as the technique that could lead to a perfect diagnosis of an infection status (Gardner et al., 2010). Sabin-Feldman dye test has been historically considered as gold standard for the diagnosis of *T. gondii* infection in humans (Liu et al., 2015; Wyrosdick and Schaefer, 2015; Ybañez et al., 2020b). In animals, mice bioassay has been reported as gold standard (Liu et al., 2015), although the high costs and complexity of this assay are not comparable with the dye test. Maybe for this reason some authors consider that there is a lack of a reliable gold standard in veterinary medicine (Wyrosdickand Schaefer, 2015). However, we found that several studies considered different techniques, such as ELISA (in studies performed by RP and NPV), as gold standard. A hypothesis to explain this finding is that there could be a misinterpretation of "Gold Standard" concept, considering it as a highly sensitive and specific technique. This could agree with some authors who think that there is no

technique that meets these high analytical requirements, so they propose to change the "Gold standard" term by the "Best available technique" (Finbarr Duggan, 1992; Claassen, 2005). On the other hand, only five articles performed in humans used Sabin-Feldman dye test as referencetechnique, while none of the articles in animals used mice bioassay as reference. This could be due to the disadvantages associated to these techniques: dye test requires theuse of live tachyzoites, with a considerable biohazard (Wyrosdick and Schaefer, 2015; Ybañez et al., 2020b), and mice bioassay is high costly and has a very long runtime (Liu et al., 2015).

In the present review, the differences found between human and veterinary medicine regarding the description of the evaluated techniques' protocol and reagents and the validation process are considerable. These differences are foreseeable due to the variety of species included in this review, and also, because of the lack of specific reagents, reference sera and commercially available techniques for anti-*T. gondii* antibodies detection in animals, which can influence the variety of reference techniques used (Wyrosdick and Schaefer, 2015).

Focusing on the evaluated and reference techniques, the differences found between the articles according to the authors' affiliations and the human or animal host have been previously discussed in Chapter 1a. However, in the present study, we must highlight that most of the articles performed on humans by RP, NPV and interdisciplinary teams used a commercial technique as reference. On the contrary, the majority of studies conducted on animals or by RV used non-commercial techniques as reference. The lower availability of commercial tests for serological studies of *T. gondii* in animals may be due to the difficulty of standardization of serological assays, caused by the lack of available reference sera or species-specific conjugates (Wyrosdick and Schaefer, 2015). Moreover, the reference techniques more commonly used on animals (IFAT, agglutination tests) are normally based on native antigens that are more difficult to standardize (Liu et al., 2015; Rostami et al., 2019; Uddin et al., 2021). The only exception were pigs and wild animals, in which commercial techniques were used as reference in all the studies. In pigs, it could be probably due to the increasing economic importance of this species in human food (Xie et al., 2022) and the significant importance of pork consumption as a risk factor for *T. gondii* infection in humans (Thebault et al., 2021).

From the two studies using commercial techniques on wild animals, one was performed on wild boars (*Sus scrofa*) by a commercial technique developed for humans (Toxo-Screen DA, bioMérieux SA, France) but previously used in other species, as it does not include any species-specific reagent (Galat et al., 2019). The other study was conducted on wild carnivores, and they used the multispecies ID Screen Toxoplasmosis Indirect kit (IDvet, France), which employs an anti-multispecies IgG-HRP conjugate. This kit was developed for detection of anti-T. *gondii* antibodies in ruminant, swine, dog and cat samples, although it is not validated for use in wild animals (Liyanage et al., 2021).Regarding the characteristics of the analysed techniques, this review highlights the considerable proportion of studies that did not specify important data for the complete description of the analysed techniques, such as the type of antigen or secondary antibody employed, which could limit the reproducibility of the study (Begley and Ioannidis, 2015). Moreover, they did not often mention other characteristics during the testing procedure, such as the use of reliable positive and negative controls or duplicate samples, which could also have a negative influence on the quality and reproducibility of the experiment (Begley, 2013; Begley and Ioannidis, 2015). On the other hand, comparing between human and animal hosts, there were not significant differences in the type of antigen employed, secondary antibody or antibody isotype detected, although they were found in the specification of the use of some quality controls, such as the use of duplicate samples. The more often detection of IgG and IgM in studies conducted by RP, and also the exclusive discrimination between acute and chronic phases in studies performed on humans, could be associated with the importance of an early diagnosis of acute toxoplasmosis in humans, and especially in pregnant women, for whom numerous serological methods for detection of IgM and/or IgG avidity are available (Emelia et al., 2014; Smets et al., 2016; Trotta et al., 2016; Laboudi and Sadak, 2017).

The present study showed that, regardless the studied host or the authors' affiliation, most of articles on development and validation of serological techniques evaluated their diagnostic performance, normally through the calculation of their diagnostic Se and Sp. However, few of them performed an analytical validation, with particular attention in those articles that developed a new technique, which should

analyze at least the intra- and inter-assay precision, the analytical sensitivity or the accuracy of the technique (Jacobson, 1998; Andreasson et al., 2015). Moreover, few studies performed an analysis of the possible cross-reactivities with other pathogens, especially with those phylogenetically similar, such as Apicomplexan parasites (*Neospora caninum*, *Sarcocystis* spp.) (Gondim et al., 2017). This fundamentalaspect should be improved in future studies developing new serological techniques for the diagnosis of *T. gondii* infection.

Correlation coefficient and agreement were most frequently employed for the comparison of the evaluated technique with a reference standard in humans and animals, respectively. Pearson's correlation coefficient can be used for comparison of two diagnostic tests with a dichotomous result (positive/negative) (Hazra and Gogtay, 2017; Schober and Schwarte, 2018), while Spearman correlation coefficient can evaluate the monotonic relationship between the exact results of two techniques (Schober and Schwarte, 2018). In contrast, agreement is considered a measure for studying the inter-rater agreement of two diagnostic tests on categorical values (Cohen, 1960; McHugh, 2012; Hazra and Gogtay, 2017), and it is stronger when it is estimated via Cohen's *kappa* value than through the calculation of the concordance or agreement percentage (Hazra and Gogtay, 2017). In the future, it would be appropriate to harmonise the comparison between the evaluated and the reference techniques by the same statistical analyses in humans and animals.

Concerning the MCA for "Humans or animals" and "Authors' affiliation" variables, the clear separation between studies that had a human or an animal host, or that were performed by RP, RV, NPV or interdisciplinary teams, showed that they met different criteria when developing and validating a new serological technique. The high variability found between *T. gondii* serological studies performed in human and veterinary medicine differs from theOne Health perspective, which consider that a collaborative effort should be made in order to control this zoonosis (Thompson, 2013; Suijkerbuijk et al., 2018; Krecek et al., 2020).

OIE protocol (OIE, 2019a) should be followed by future studies on the validation of serological assays for the detection of anti-*T. gondii* antibodies in human or animal hosts. But ideally, a new and unified protocol should be developed for validation of

serological techniques for detection of anti-*T. gondii* antibodies in both human and animal hosts. For this new protocol, there are some key issues that should be taken into account: i) new serological techniques should be described in detail in order to assure the reproducibility of the results; ii) a panel of *T. gondii* seropositive and seronegative reference sera, whose analytical Se and Sp had been appropriately assessed, is necessary for evaluating serological assays in each species; and iii) analytical validation should be assessed for all new developed serological techniques.

5. Conclusions

The present review highlights the areas for improvement in the studies focused on the development and validation of serological techniques for the diagnosis of *T. gondii* infection, and confirms the wide heterogeneity recently evidenced between those studies performed in human or veterinary medicine. This could be the starting point to define a more accurate procedure for the validation of serological techniques to standardize, in future studies, the diagnosis of *T. gondii* in human and animal hosts.

6. Acknowledgements

The authors would like to express their gratitude to Statistical Support Section (SAE), Scientific and Research Area (ACTI), University of Murcia (www.um.es/web/acti). In addition, the authors would like to acknowledge the financial supporters: Ana Huertas López was supported by a pre-doctoral grant from University of Murcia (R-1207/2017); Ana Cantos Barreda was supported by the Seneca Foundation of Murcia Region, Spain and the European Social Fund (21327/PDGI/19); Gema Álvarez García is part of the TOXOSOURCES consortium, supported by funding from the European Union's Horizon 2020 Research and Innovation Programme under grant agreement No. 773830: One Health European Joint Programme; and this work was supported by the Seneca Foundation of Murcia Region, Spain (project 19894/GERM/15).

TABLES

Table 1. Categories and variables studied from the 73 selected papers. RP = Research physicians; RV = Research veterinarians; NPV = Neither physicians nor veterinarians; EIA = Enzyme immunoassay; IFAT = Indirect fluorescent antibody test; WB = Western Blot; POC = Point-of-care; ELISA = Enzyme-linked immunosorbent assay; CMIA = Chemiluminescent microparticle immunoassay; LAT = Latex agglutination test; MAT = Modified agglutination test; IHA = Indirect hemagglutination test; ISAGA = Immunosorbent agglutination assay; DAT = Direct agglutination test; SIA = Suspension immunoassay; ELFA = Enzyme-linked fluorescence assay; MEIA = Microparticle enzyme immunoassay; SAG = Surface antigen; GRA = Dense granular antigen; MAG = Matrix antigen; ROP = Rhoptry antigen; Ig = Immunoglobulin; ROC = Receiver operating characteristic; AUC = Area under de curve; Se = Sensitivity; Sp = Specificity.

Category	Variable	Factor label	Type of variable
Bibliometrics	Authors' affiliation	RP; RV; NPV; Interdisciplinary team	Qualitative
Study population	Humans or animals	Humans; Animals; Both humans and animals	Qualitative
	Host category	Humans; Cats; Dogs; Domestic ruminants; Pigs; Domestic birds; Wild animals (one or several species); Multispecies	Qualitative
	Sample size		Quantitative
	Do they evaluate different techniques?	No (they evaluate 1 technique); Yes, they evaluate different types of techniques (e.g., EIA, IFAT, etc.); Yes, they evaluate variations of the same technique (e.g., different antigens, antibodies or samples); Yes, they evaluate different type of techniques and variations of the same technique	Qualitative
	Evaluated technique	EIA; IFAT, WB; Agglutination; POC test; Avidity test; Other type; Several types	Qualitative
	Commercial/ Non-commercial (evaluated technique)	Commercial; Non-commercial; Commercial and not commercial techniques	Qualitative
	Number of evaluated techniques		Quantitative

Techniques	Type of EIA (evaluated technique)	ELISA; Inhibition ELISA; Fluorescent immunosensor with chitosan-ZnO-nanoparticles; Dot-ELISA; CMIA; ELISA & Other	Qualitative
	Type of agglutination test (evaluated technique)	LAT; MAT; IHA; ISAGA; DAT	Qualitative
	Type of POC test (evaluated technique)	Lateral flow immunocromatography; paper-based ELISA	Qualitative
	Type of IgG avidity test (evaluated technique)	EIA; Flow cytometry; EIA and flow cytometry	Qualitative
	Other assays (evaluated technique)	Multiplex bead assay; Bead-based assay (only for *T. gondii* detection); Electrochemical sensing platform; Flow cytometry; Multiplex dot-immunoassay based; SIA; Peptide microarray; Several	Qualitative
	Reference technique	EIA; IFAT, WB; Agglutination; Dye test; POC test; Clinical criteria; Molecular techniques; Other type; Several types	Qualitative
	Commercial/ Non-commercial (reference technique)	Commercial; Non-commercial; Commercial and not commercial techniques	Qualitative
	Number of reference techniques		Quantitative
	Type of EIA (reference technique)	ELISA; ELFA; MEIA; CMIA; ELISA & Other	Qualitative
	Type of agglutination test (reference technique)	LAT; MAT; ISAGA; DAT; Differential agglutination AC/HS; Several	Qualitative
	"Gold standard" quotation	Yes; No	Qualitative
	Methodology quoted as gold standard	ELISA; ELISA & IgG Avidity; IFAT; WB; MAT; Dye test; Molecular techniques; Clinical examination; Authors highlight the lack of gold standard; Several	Qualitative
	Is the reference technique the same quoted as gold standard?	Yes; No	Qualitative
	Number of studied pathogens (including *T. gondii*)		Quantitative
	Antigen employed	Native; Recombinant; Chimeric; Several types	Qualitative
	Native antigens	Not specified; Whole tachyzoite; Tachyzoite extract; Several types	Qualitative
	Recombinant antigens	rSAG1; rSAG2; rSAG3; rGRA7; rMAG1; rROP2; Several types	Qualitative

Characteristics of the evaluated technique/s	Single/ Mixed use (recombinant antigens)	Single use; Mixed use; Single and mixed use	Qualitative
	Parasite specific stage antigen	Tachyzoite; Tachyzoite and bradyzoite; Tachyzoite and sporozoite; Tachyzoite, bradyzoite and sporozoite; Several antigens with different stage antigen	Qualitative
	Type of secondary antibody	Not specified; Anti-Ig secondary antibody; Protein A, G, A/G	Qualitative
	Antibody isotype detected	IgG; IgA; IgG and IgM; IgG, IgM and IgA; Several (other)	Qualitative
	Positive control	Yes; Not specified	Qualitative
	Negative control	Yes; Not specified	Qualitative
	Samples tested in duplicate	Yes; Not specified	Qualitative
	Do they specify the cut-off?	Yes; No; Not applicable	Qualitative
	Discrimination between acute and chronic phases	Yes; No	Qualitative
	Novelty of the method	New technique; Previously described technique; Variation of a previously described technique (e.g., new antigen); Several	Qualitative
Type of validation	Analytical or diagnostic validation	Analytical validation; Diagnostic validation; Both analytical and diagnostic validation	Qualitative
Parameters studied for analytical validation	Intra-assay precision determination	Yes; No	Qualitative
	Inter-assay precision determination	Yes; No	Qualitative
	Limit of detection determination	Yes; No	Qualitative
	Cross-reactivity determination	Yes; No	Qualitative
	Pathogen/s considered for studying cross-reactivity	Other parasites (different from *T. gondii*); Virus; Bacteria; Non-infectious diseases; Several	Qualitative
Analysis performed and parameters studied for diagnostic validation	ROC analysis	Yes; No	Qualitative
	AUC determination	Yes; No	Qualitative
	Se determination	Yes; No	Qualitative
	Sp determination	Yes; No	Qualitative
	Agreement determination	Yes; No	Qualitative
	Correlation coefficient (Pearson/Spearman) determination	Yes; Yes, but the result is not shown; No	Qualitative

Table 2. Significant associations between quantitative and qualitative variables by one-way ANOVA when parametric conditions for normality and homoscedasticity were met, or by Welch-James Approximate Degrees of Freedom or Kruskal-Wallis tests when these conditions were not met. (df) = Degree of freedom.

Quantitative variable	Qualitative variable	Statistical analysis	Statistical value (df)	*p*-value
Sample size	Authors' affiliation	Kruskal-Wallis test	$K = 9.37$	< 0.05
	Do they evaluate different techniques?	One-way ANOVA	$F(3, 69) = 9.2$	< 0.05
	Commercial/ Non-commercial (evaluated technique)	Kruskal-Wallis test	$K = 9.53$	< 0.05
	Type of EIA (evaluated technique)	Kruskal-Wallis test	$K = 11.25$	< 0.05
	Other assays (evaluated technique)	One-way ANOVA	$F(7, 6) = 10.04$	< 0.05
	Type of EIA (reference technique)	One-way ANOVA	$F(4, 40) = 3.14$	< 0.05
	Type of secondary antibody[a]	One-way ANOVA	$F(2, 69) = 3.75$	< 0.05
	Antibody isotype detected[a]	Kruskal-Wallis test	$K = 14.28$	< 0.05
	Limit of detection determination	Kruskal-Wallis test	$K = 4.81$	< 0.05
Number of evaluated techniques	Do they evaluate different techniques?	One-way ANOVA	$F(3, 69) = 6.76$	< 0.05
	Commercial/ Non-commercial (reference technique)	One-way ANOVA	$F(2, 65) = 8.28$	< 0.05
	"Gold standard" quotation	Kruskal-Wallis test	$K = 4.86$	< 0.05
	Antigen employed[a]	Kruskal-Wallis test	$K = 11.77$	< 0.05
	Single/ Mixed use (recombinant antigens)[a]	One-way ANOVA	$F(2, 27) = 11.95$	< 0.05
	Parasite specific stage antigen[a]	Kruskal-Wallis test	$K = 10.49$	< 0.05

	Positive control	One-way ANOVA	F (1, 71) = 5.63	< 0.05
	Negative control	One-way ANOVA	F (1, 71) = 9.09	< 0.05
	Samples tested in duplicate	One-way ANOVA	F (1, 71) = 6.79	< 0.05
	Discrimination between acute and chronic phases	Kruskal-Wallis test	K = 5.09	< 0.05
	Novelty of the method	Kruskal-Wallis test	K = 9.01	< 0.05
	AUC determination	Kruskal-Wallis test	K = 6.49	< 0.05
Number of reference techniques	Reference technique	Kruskal-Wallis test	K = 51.05	< 0.05
	Commercial/ Non-commercial (reference technique)	One-way ANOVA	F (2, 65) = 23.09	< 0.05
	Discrimination between acute and chronic phases	Kruskal-Wallis test	K = 16.28	< 0.05
Number of studied pathogens (including *T. gondii*)	Evaluated technique	One-way ANOVA	F (6, 66) = 0.51	< 0.05
	Reference technique	One-way ANOVA	F (9, 62) = 8.14	< 0.05
	Antigen employed[a]	Kruskal-Wallis test	K = 12.71	< 0.05

Table 3. Significant relationships of "Authors' affiliation", "Humans or animals" and "Host category" variables with other qualitative variables by Pearson's chi-square or Fisher's exact tests. (df) = Degree of freedom.

		Statistical analysis	Statistical value (df)	*p*-value
Authors' affiliation	Reference technique	Fisher's exact test	$X^2(27) = 41.00$	0.037
	Commercial/ non-commercial (reference technique)	Fisher's exact test	$X^2(6) = 16.61$	0.019
	Antibody isotype detected	Fisher's exact test	$X^2(12) = 19.48$	0.013
	Agreement determination	Fisher's exact test	$X^2(3) = 8.38$	0.038
Humans or animals	Evaluated technique	Fisher's exact test	$X^2(6) = 11.23$	0.035
	Reference technique	Fisher's exact test	$X^2(9) = 28.29$	<0.001
	Commercial/ non-commercial (reference technique)	Fisher's exact test	$X^2(2) = 13.93$	<0.001
	Type of agglutination (reference technique)	Fisher's exact test	$X^2(5) = 14.00$	0.005
	Samples tested in duplicate	Pearson's chi-square test	$X^2(1) = 7.69$	0.006
	Discrimination between acute and chronic phases	Fisher's exact test	$X^2(1) = 5.03$	0.026
	Pathogen/s considered for studying cross-reactivity	Fisher's exact test	$X^2(2) = 8.17$	0.012
	Agreement determination	Pearson's chi-square test	$X^2(1) = 12.16$	<0.001
	Correlation coefficient (Pearson/Spearman) determination	Fisher's exact test	$X^2(2) = 7.35$	0.014
Host category	Reference technique	Fisher's exact test	$X^2(63) = 126.81$	<0.001
	Commercial/ non-commercial (reference technique)	Fisher's exact test	$X^2(14) = 34.10$	0.001
	Gold standard quotation	Fisher's exact test	$X^2(7) = 12.38$	0.037
	Samples tested in duplicate	Fisher's exact test	$X^2(7) = 20.64$	<0.001
	Pathogen/s considered for studying cross-reactivity	Fisher's exact test	$X^2(14) = NC$	0.014
	Agreement determination	Fisher's exact test	$X^2(7) = 16.24$	0.006

Table 4. Pooled Se, Sp and DOR of the serological techniques evaluated in humans and animals. * From the 3 potentially evaluable techniques, 2/3 had undetermined results.

Host category	Evaluated technique	Antibody isotype detected	Number of evaluated techniques included in the analysis	Pooled Se (95% CI)	Pooled Sp (95% CI)	Pooled DOR (95% CI)
Humans	ELISA	IgG	22	91.02% (84.42-95.94%)	96.48% (90.72-99.54%)	273.67 (76.20-982.85)
	CMIA	IgG	3	90% (46.95-100%)	98.57% (94.65-100%)	1293.87 (10.54-158785.88)
	ECLIA	IgG	1*	100% (99.54-100%)	100% (99.52-100%)	166383 (3285.64-8425545.98)
	ICT	IgG	3	100% (99.76-100%)	96.87% (93.93-98.86%)	6024.03 (1018.15-35642)
	ICT	IgM	2	44.81% (14.45%-77.52%)	93.82% (82.06-99.58%)	12.78 (7.36-22.18)
	ICT	IgG and IgM	2	87.37% (55.30-100%)	92.35% (82.38-98.39%)	97.24 (3.02-3128.14)
	BBA	IgG	3	90.30% (82.70-95.88%)	90.28% (84.84-94.61%)	66.18 (28.68-152.68)
	Multiplex plasmonic gold assay	IgG	2	100% (99.33-100%)	100% (99.45-100%)	24501.41 (1518.85-395245.30)
	Multiplex plasmonic gold assay	IgM	2	96.70% (90.62-99.71%)	97.30% (94.98-98.92%)	555.58 (142.02-2173.41)
	Singleplex microarray immunoassay	IgG	2	100% (99.48-100%)	100% (98.41-100%)	11285 (695.31-183157.54)
Cats	ELISA	IgG	2	93.65% (87.21-97.95%)	97.95% (96.01-99.25%)	504.87 (164.08-1553.48)
Cattle	ELISA	IgG	4	87.97% (76.45%-95.98%)	95.46% (88.33-99.35%)	97.38 (25-379.24)
Chickens	ELISA	IgG	4	95.09% (85.95-99.61%)	96.72% (93.67-98.79%)	208.46 (63.90-680.01)

Dogs	ELISA	IgG	5	86. 44% (80.60-91.37%)	97.30% (96.03-98.33%)	164.83 (88.91-305.56)
Goats	ELISA	IgG	4	92.62% (84.32-97.96%)	97.60% (89.23-100%)	354.91 (26.42-4766.97)
Horses	ELISA	IgG	9	84.61% (70.47-94.73%)	100% (99.79-100%)	469.85 (169.66-1301.20)
Pigs	ELISA	IgG	11	80.06% (60.60-94.01%)	99.95% (99.63-100%)	654.03 (173.78-2461.50)
Sheep	ELISA	IgG	25	96.61% (92.38-99.18%)	99.57% (98.72-99.97%)	1145.09 (377.29-3475.38)
Wild carnivorous	ELISA	IgG	3	77.52% (65.88-87.31%)	97.27% (94.49-99.10%)	80.99 (34.42-190.54)

Supplementary material not included from Huertas-López, A., Cantos-Barreda, A., Sánchez-Sánchez, R., Martínez-Carrasco, C., Ibáñez-López, F. J., Martínez-Subiela, S., Cerón, J. J., and Álvarez-García, G. A systematic review and meta-analysis on the validation of serological methods for detection of anti-*Toxoplasma gondii* antibodies in humans and animals.

- **Supplementary File 1**: data extraction from the 73 selected articles. https://docs.google.com/spreadsheets/d/11qVdrzEJfliggmGBjPEfrSTG0y6CSXoc/edit?usp=sharing&ouid=117908795181800371023&rtpof=true&sd=true
- **Supplementary File 2**: significant associations between qualitative variables by Pearson's chi-square or Fisher's exact tests. https://docs.google.com/spreadsheets/d/1Gy2xPiyjMBkNpIh9FJTP1U0tDtE2UUcA/edit?usp=sharing&ouid=117908795181800371023&rtpof=true&sd=true
- **Supplementary File 3**: significant associations between quantitative and qualitative variables by one-way ANOVA (parametric conditions) or by Kruskal- Wallis test (non-parametric conditions). https://docs.google.com/spreadsheets/d/1Dm6vXTOL9oiHm6YPMq9MXYGb5dHJOW_z/edit?usp=sharing&ouid=117908795181800371023&rtpof=true&sd=true

Chapter 2. Seroprevalence of *Toxoplasma gondii* in anthropized areas

Introduction: the importance of detection of *Toxoplasma gondii* in anthropized areas

Felids are definitive hosts of *T. gondii* and the only ones capable of spreading the disease via oocysts shedding through their feces (Dubey, 2016; Attias et al., 2020). In addition, different studies have proven that dogs can be also a mechanical carrier for the transmission of this parasite, because of their coprophagous behavior and the resistance of the sporulated *T. gondii* oocysts through their digestive system, remaining viable when they are excreted with the dog feces (Schares et al., 2005; Cong et al., 2018). Oocysts can also stay in the dogs' fur after rubbing on the ground contaminated by cat feces, and be a source of infection for humans who pet these dogs (Frenkel et al., 2003). The close relationship between these host species and humans could play an important role in the transmission of this zoonotic parasite (Dabritz and Conrad, 2010; Dubey et al., 2020d). Moreover, community dog and cat keeping, which is a practice of feeding stray animals without adopting them, could also facilitate the contact between people and a large number of animals without health control (Savvides, 2013; Toukhsati et al., 2015). This practice is widespread in Thailand, due to the Buddhist principle of feeding those in need (Savvides, 2013).

Epidemiological studies should be carried out in order to determine the status of the parasite in those geographical areas shared by humans and these species, as a measure for the control of this zoonotic disease (Fábrega et al., 2020). In this regard, Bangkok (Thailand) is a synanthropic environment where the number of feral cats and dogs has been increasing over the past few years (OIE, 2019b), and where community pet keeping is a common and deeply-rooted practice among citizens (Savvides, 2013). These characteristics make Bangkok an epidemiological scenario worthy of study for pathogens shared between humans and domestic animals. For this reason, the second chapter of this PhD thesis is focused in the epidemiology of *T. gondii* in outdoors dogs and cats from Bangkok, including the seroprevalence of this parasitic infection and the associated risk factors. Moreover, the plasma samples collected in this study were used

in the study described in Chapter 3a. This activity was made by a research visit of three and a half months to the Parasitology Unit, Department of Pathology (Faculty of Veterinary Sciences, Chulalongkorn University, Bangkok, Thailand),with an ERASMUS + International Mobility grant.

Chapter 2: Seroprevalence of *Toxoplasma gondii* in outdoor dogs and cats in Bangkok, Thailand

Huertas-López, A., Sukhumavasi, W., Álvarez-García, G., Martínez-Subiela, S., Cano-Terriza, D., Almería, S., Dubey, J. P., García-Bocanegra, I., Cerón, J. J., and Martínez-Carrasco, C. (2021). Seroprevalence of *Toxoplasma gondii* in outdoor dogs and cats in Bangkok, Thailand. Parasitology 148, 843–849. https://doi.org/10.1017/S0031182021000421

ARTICLE 1: Seroprevalence of *Toxoplasma gondii* in outdoor dogs and cats in Bangkok, Thailand.

JOURNAL: Parasitology

ABSTRACT: The aim of this study was to estimate the seroprevalence and risk factors associated with *Toxoplasma gondii* exposure in dogs and cats from Bangkok, Thailand. Blood samples from 318 dogs and 321 cats were tested for *T. gondii* antibodies by modified agglutination test (cut-off 1:25). Additionally, 18 dogs and 20 cats were longitudinally sampled for *T. gondii* antibodies during the same study period, between June and July 2019. The overall seroprevalence in dogs and cats was 7.9% (25/318; 95% CI 4.9–10.8%) and 18.7% (95% CI 14.4–23.0%), respectively. For dogs, risk factors identified were being a mixed-breed animal and living totally outdoors, while increasing age was shown to be a risk factor for cats. Seroconversion was not detected and titres from positive animals remained constant over longitudinal study. The present study indicates that there is a prominent presence of *T. gondii* in urban and peri-urban areas of Bangkok, suggesting that outdoor dogs and cats should be considered as a possible risk factor for humans.

URL:
https://www.cambridge.org/core/journals/parasitology/article/abs/seroprevalence-of-toxoplasma-gondii-in-outdoor-dogs-and-cats-in-bangkok-thailand/9AB3C3404F9D5B5726A8E94640FBEA25

Chapter 3. Development of Time-resolved fluorescence immunoassay (TRFIA) for detection of anti-*Toxoplasma gondii* antibodies in cats and goats

Introduction: detection of anti-*Toxoplasma gondii* antibodiesin cats and goats by TRFIA, a highly sensitive technique

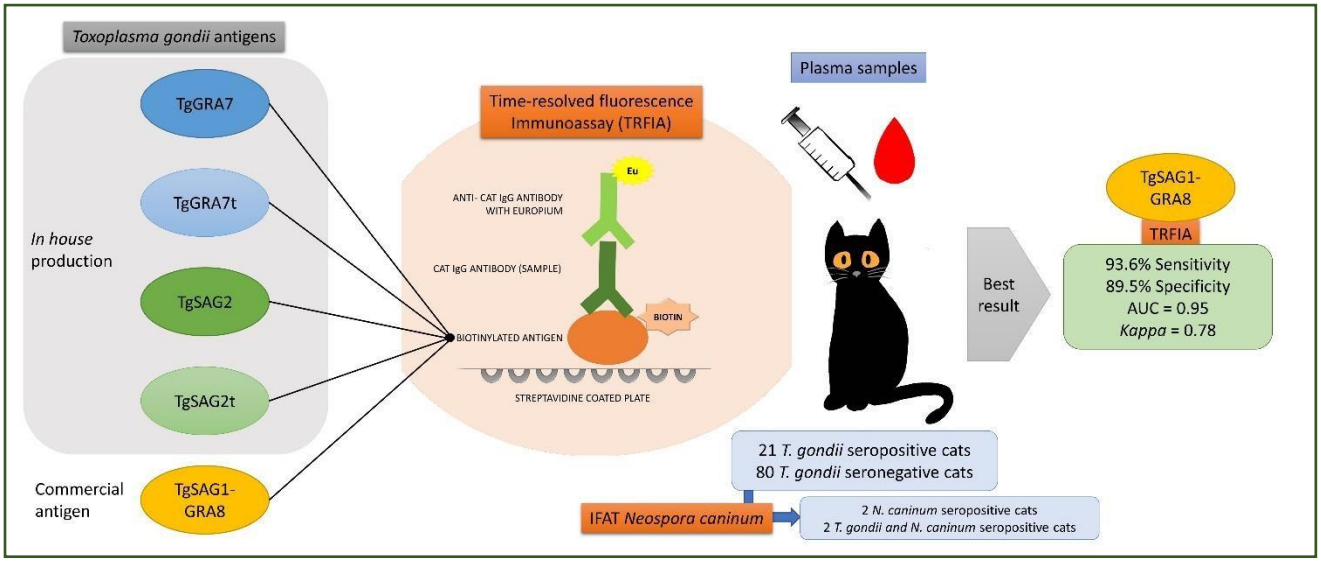
In this chapter, new serological techniques for the detection of anti-*T. gondii* antibodies in animals have been developed and evaluated. In particular: a) five TRFIAs based on four recombinant and a chimeric antigen for the diagnosis of feline toxoplasmosis (Chapter 3a), and b) one TRFIA based on a chimeric antigen for the diagnosis of caprine toxoplasmosis (Chapter 3b).

Cats have a significant importance in the transmission of *T. gondii*, as they are the only definitive host that could spread the disease via oocysts shedding through their feces (Dabritz and Conrad, 2010; Dubey, 2016). Although oocyst shedding normally occurs during primoinfection, and then stops due to the development of an immunological response, several studies have proven that re-shedding is possible, especially when cats are re-infected by new *T. gondii* strains (Dubey et al., 1995; Zulpo et al., 2018). For this reason, knowing the serological status of a cat can help in the control of this zoonotic disease by the identification of potential oocyst shedders that contaminate the environment (Silaghi et al., 2014; Fábrega et al., 2020). In addition, in the case of household cats, special hygienic measures should be taken at home, such as a daily cleaning of litter box (to avoid the potential sporulation of the oocysts in the environment), or wearing gloves when gardening or contacting the sand of the litter box, and washing hands after it (Dabritz and Conrad, 2010). On the other hand, serological diagnosis is important to identify clinical feline toxoplasmosis, as clinical signs could sometimes be unspecific (e.g., fever, depression, diarrhea, anorexia…) (Lappin, 2010). Although there is a wide variety of available serological techniques for the detection of anti-*T. gondii* antibodies, such as the MAT used in the previous chapter of this PhD thesis, most of them present limitations, normally regarding to the costs, the need of maintenance of *T. gondii* cell cultures, the subjectivity or the variable sensitivityof the tests (Liu et al., 2015; Wyrosdick and Schaefer, 2015; Dubey et al., 2020a).

Small ruminants are intermediate hosts of *T. gondii* (Dubey, 2016). This

Apicomplexan parasite causes significant economic losses in sheep and goat farms worldwide associated to reproductive failures, including embryonic or fetal death, with the consequent reabsorption or abortion, respectively, and also stillbirth. Caprine toxoplasmosis is considered more severe than ovine toxoplasmosis (Dubey et al., 2020b; Lindsay and Dubey, 2020). Due to the lack of a vaccine in goats to prevent *T. gondii* infection and the unspecific clinical signs, the control of this zoonotic pathogen usually relies on the diagnosis, which is based on serological, molecular and histopathological techniques (Lindsay and Dubey, 2020). Numerous serological methods have been developed for the diagnosis of caprine toxoplasmosis, but none of them have been validated in naturally infected goats (Dubey et al., 2020b).

Chapter 3a: Comparative performance of five recombinant and chimeric antigens in a time-resolved fluorescence immunoassay for detection of *Toxoplasma gondii* infection in cats.



Huertas-López, A., Contreras Rojo, M., Sukhumavasi, W., Martínez-Subiela, S., Álvarez-García, G., López-Ureña, N. M., Cerón, J. J., and Martínez-Carrasco, C. (2022). Comparative performance of five recombinant and chimeric antigens in a time-resolved fluorescence immunoassay for detection of *Toxoplasma gondii* infection in cats. Vet. Parasitol. 304, 109703. https://doi.org/10.1016/j.vetpar.2022.109703

ARTICLE 2: Comparative performance of five recombinant and chimeric antigens in a time-resolved fluorescence immunoassay for detection of *Toxoplasma gondii* infection in cats.

JOURNAL: Veterinary Parasitology

ABSTRACT: Felids are definitive hosts of *Toxoplasma gondii*, being the only hosts that can spread the infection through oocyst shedding in their feces. The elevated presence of this parasite in the domestic cat (*Felis catus*), and its close contact with humans, make it necessary to obtain reliable diagnostic methods to detect positive animals as a public health measure. For this reason, in this study, the diagnostic performance of five different recombinant antigen-based techniques was assessed to diagnose *T. gondii* infection in cat blood plasma samples. Specifically, four *T. gondii* recombinant antigens (GRA7, truncated GRA7, SAG2, and truncated SAG2) and a chimeric antigen (SAG1-GRA8) were used. A time-resolved fluorescence immunoassay (TRFIA) was developed for each antigen, and the results of each of these techniques were compared with those obtained by a commercial enzyme-linked immunoassay (ELISA) and a modified agglutination test (MAT) as reference techniques. The TRFIA based on SAG1-GRA8 antigen showed better discrimination between seropositive and seronegative cats ($p < 0.001$), as well as a better area under the curve (0.95), sensitivity (93.6%), and specificity (89.5%) values for the optimal cut-off, *versus* the other TRFIAs. In addition, SAG1-GRA8 TRFIA showed substantial agreement (*kappa* value = 0.78) and a moderate significant correlation (Spearman's correlation: $r = 0.62$, $p < 0.001$) compared with the reference techniques. On the other hand, since plasma samples were obtained from 101 cats in Bangkok city and four of them were Neospora caninum seropositive by indirect immunofluorescence assay (IFAT), this is the first time that anti-*N. caninum* antibodies are detected in cats in Thailand. In conclusion, our study highlights that the TRFIA with TgSAG1-GRA8 antigen is an accurate and recommended diagnostic technique for detecting anti-*T. gondii* antibodies in cats.

URL:
https://www.sciencedirect.com/science/article/pii/S0304401722000577?via%3Dihub

Supplementary material not included from Huertas-López, A., Contreras Rojo, M., Sukhumavasi, W., Martínez-Subiela, S., Álvarez-García, G., López-Ureña, N. M., Cerón, J. J., and Martínez-Carrasco, C. (2022). Comparative performance of five recombinant and chimeric antigens in a time-resolved fluorescence immunoassay for detection of Toxoplasma gondii infection in cats. Vet. Parasitol. 304, 109703. https://doi.org/10.1016/j.vetpar.2022.109703.

- **Supplementary File 1**:

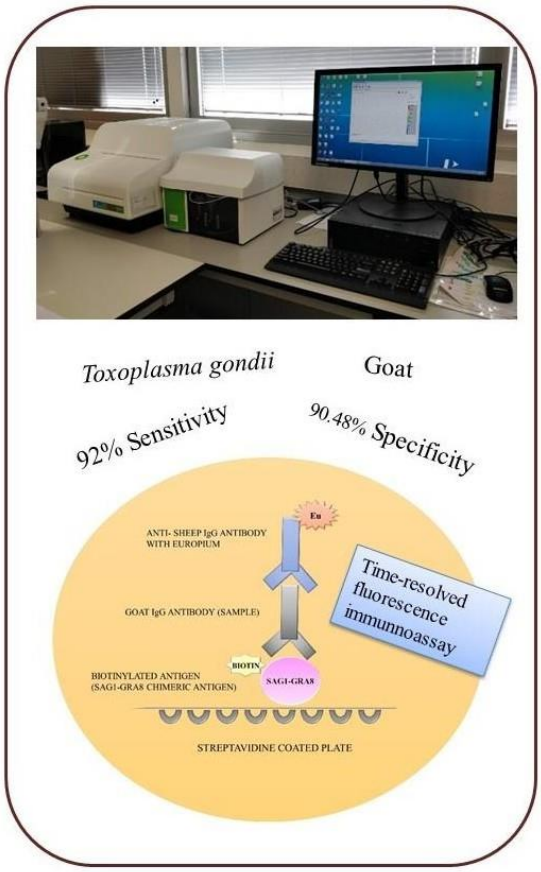
  https://www.sciencedirect.com/science/article/pii/S0304401722000577?via%3Dihub#ec0005

  - Figure 1 S1. Results of recombinant TgGRA7 significant alignements by BLAST tool.
  - Figure 2 S1. Sequences producing significant alignements with TgGRA7 by BLAST tool. A: Sequence alignement of TgGRA7 with uncharacterized protein NCLIV_021640 [*Neospora caninum* Liverpool] (Sequence ID XP_00388408.1); B: Sequence alignement of TgGRA7 with peptide recognized by serum from cattle that aborted due to neosporosis, partial [*Neospora caninum*] (Sequence ID AAC47096.1).
  - Figure 3 S1. Results of recombinant TgSAG2 significant alignements by BLAST tool.

    Figure 4 S1. Sequences producing significant alignements with TgSAG2 by BLAST tool. A: Sequence alignement of TgSAG2 with NcSRS domain-containing protein [*Neospora caninum* Liverpool] (Sequence ID XP_003883827.1); B: Sequence alignement of TgSAG2 with NcSRS domain-containing protein [*Neospora caninum* Liverpool] (Sequence IDXP_003884600.1); C: Sequence alignement of TgSAG2 with NcSRS domain-containing protein [*Neospora caninum* Liverpool] (Sequence ID XP_003884897.1); D: Sequence alignement of TgSAG2 with NcSRS domain-containing protein [*Neospora caninum* Liverpool] (Sequence ID XP_003884599.1); E: Sequence alignement of TgSAG2 with NcSRS domain-containing protein [*Neospora caninum* Liverpool] (Sequence ID XP_003884883.1).

- **Supplementary File 2**:
  https://www.sciencedirect.com/science/article/pii/S0304401722000577?via%3Dihub#ec0010
  - Table 1 S2: Results of 101 plasma samples from cats analyzed for the detection of anti-*T. gondii* antibodies by the reference techniques (MAT and ELISA) and by TgSAG1-GRA8-TRFIA, TgGRA7-TRFIA, TgGRA7t-TRFIA, TgSAG2-TRFIA, TgSAG2t-TRFIA, and for the detection of anti-*N. caninum* antibodies by IFAT.

Chapter 3b: Development and validation of a time-resolved fluorescence immunoassay for the detection of anti-*Toxoplasma gondii* antibodies in goats.



Huertas-López, A., Martínez-Subiela, S., Cerón, J. J., Vázquez-Calvo, A., Pazmiño-Bonilla, E. D., López-Ureña, N. M., Martínez-Carrasco, C. and Álvarez-García, G. (2021). Development and validation of a time-resolved fluorescence immunoassay for the detection of anti-*Toxoplasma gondii* antibodies in goats. Vet. Parasitol. 293, 109432. https://doi.org/10.1016/j.vetpar.2021.109432

ARTICLE 3: Development and validation of a time-resolved fluorescence immunoassay for the detection of anti-*Toxoplasma gondii* antibodies in goats.

JOURNAL: Veterinary Parasitology

ABSTRACT: *Toxoplasma gondii* is a worldwide distributed parasite causing abortions and fetal malformations in small ruminants. The aim of this study was to design and validate a new immunoassay based on the use of TgSAG1-GRA8 chimeric antigen for the detection of anti-*T. gondii* antibodies in serum of goats. First, a time-resolved fluorescence immunoassay (TgSAG1-GRA8-TRFIA) was developed. In addition, the diagnostic performance of TgSAG1-GRA8-TRFIA was compared with an optimized enzyme-linked immunosorbent assay (TgSALUVET-ELISA) and a Western Blot (WB), both based on whole *T. gondii* tachyzoite antigenic extract. The TgSAG1-GRA8-TRFIA has shown a high intra- and inter-assay precision, analytical sensitivity and accuracy. The ROC analysis of this assay showed an optimal cut-off of 217.4 Units of Fluorometry for *T. gondii* (UFT), with 92 % of sensitivity and 90.48 % of specificity. A positive and statistically significant Spearman's correlation with TgSALUVET-ELISA was detected, and *kappa* value was 0.83, presenting high agreement with both methods. However, TgSAG1-GRA8 protein showed cross-reactivity with specific anti-*Neospora caninum* antibodies. Thus, TgSAG-1-GRA8 chimeric antigen seems not to be an ideal option for the serodiagnosis of *T. gondii* infection in goats unless combined with the serodiagnosis of *N. caninum* infection in parallel. In the light of the results obtained, a comprehensive study on the existence of cross-reactivities between *T. gondii* antigens used in serological tests employed in animal health and specific antibodies directed against Toxoplasmatinae parasites should be performed.

URL: https://www.sciencedirect.com/science/article/abs/pii/S0304401721000923?via%3Dihub

Supplementary material not included from Huertas-López, A., Martínez-Subiela, S., Cerón, J. J., Vázquez-Calvo, A., Pazmiño-Bonilla, E. D., López-Ureña, N. M., Martínez-Carrasco, C. and Álvarez-García, G. (2021). Development and validation of a time-resolved fluorescence immunoassay for the detection of anti-*Toxoplasma gondii* antibodies in goats. Vet. Parasitol. 293, 109432. https://doi.org/10.1016/j.vetpar.2021.109432

- **Supplementary File 1:** ROC curve for TgSALUVET-ELISA in house using Western Blotting as a reference method. Receiver operating characteristic (ROC) analysis for TgSALUVET-ELISA in house using Western Blotting as a reference method. An optimal cut-off of RIPC = 10.86 was selected (bold text), with sensitivity and specificity of 98.28% and 97.7%, respectively. RIPC = relative index percent; CI = confidence interval; LR + = Positive Likelihood ratio; LR - = Negative Likelihood ratio.
  https://ars.els-cdn.com/content/image/1-s2.0-S0304401721000923-mmc1.docx

  Supplementary File 2: Positive/negative ratios obtained in the optimization procedure for TgSAG1-GRA8-TRFIA. A) With 200 ng/well of europium-labelled anti-IgG antibody fixed ratios for 50 ng, 100 ng, 200 ng and 300 ng per 200 µl well of biotinylated SAG1-GRA8 chimeric antigen and 1/100, 1/500, 1/1000, 1/2000 and 1/4000 sample dilution. B) With 50 ng/well of biotinylated SAG1-GRA8 chimeric antigen established. Ratios for 50 ng, 100 ng, 200 ng and 300 ng per 200 µl well of europium-labelled anti-IgG antibody and 1/500, 1/600, 1/700, 1/800 and 1/1000 sample dilution are shown.
  https://ars.els-cdn.com/content/image/1-s2.0-S0304401721000923-mmc2.docx

Chapter 4. Development of TRFIA for detection of anti-*Neospora caninum* antibodies in serum and milk of sheep

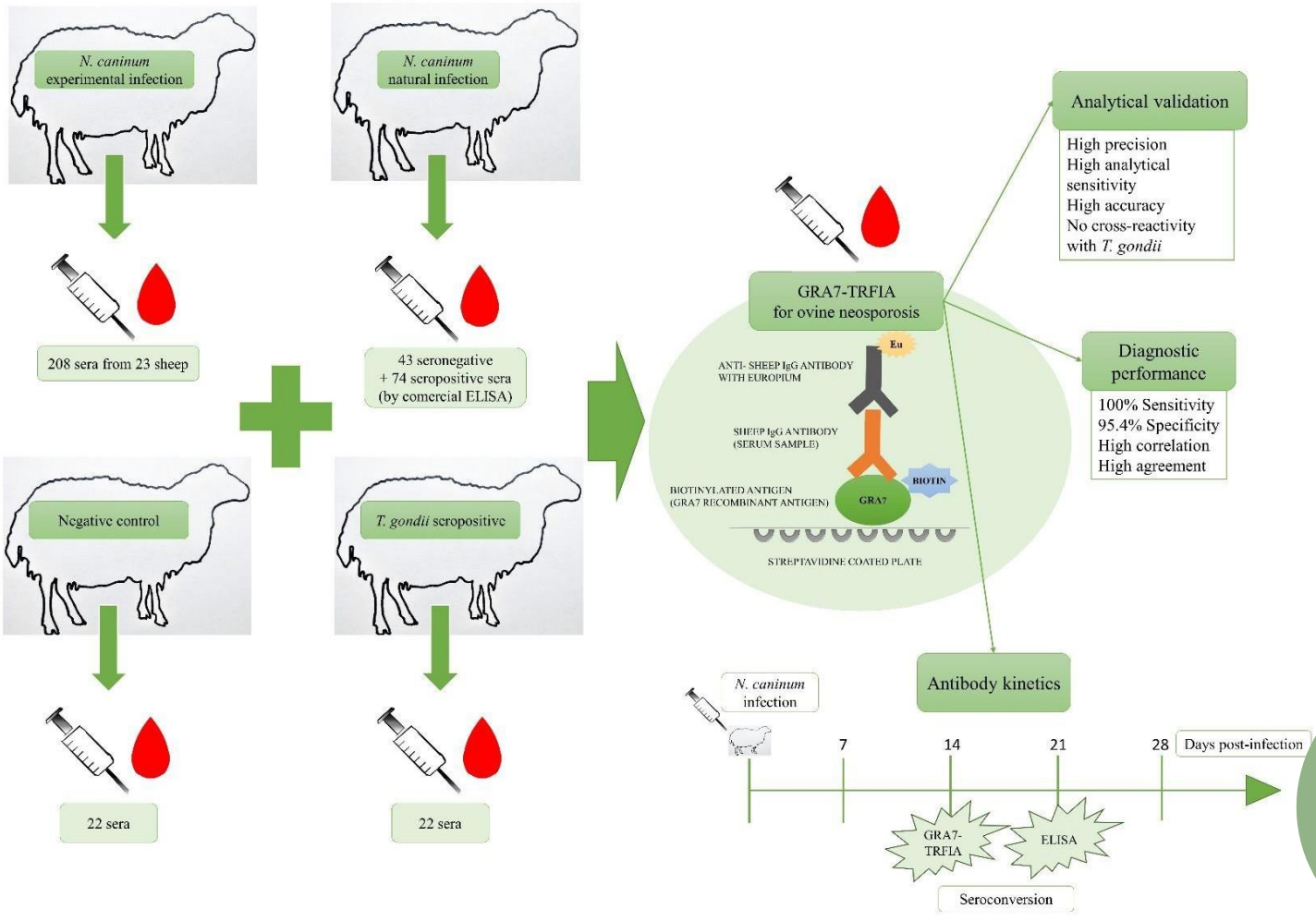
Introduction: economic impact and diagnosis of ovine neosporosis

The last chapter of this PhD thesis is focused on the serodiagnosis of the infection by the Apicomplexan parasite *Neospora caninum*. This protozoan, like *T. gondii*, causes reproductive failures in ruminants, and it is supposed to be the main cause of abortions in cattle worldwide, with an enormous economic impact (Reichel et al., 2013; Dubey et al., 2017). In sheep flocks, *N. caninum* infection also leads to important economic losses all over the world resulting from the reproductive failure (Arranz-Solís et al., 2016; Romanelli et al., 2021). The global pooled seroprevalence of ovine neosporosis has been estimated in 12% (Romanelli et al., 2021).

On the other hand, serological diagnosis has proven to be useful for the control programs based on selective culling of seropositive animals and replacement with seronegative ones (Sánchez-Sánchez et al., 2021). Nowadays, IFAT is considered the reference technique for diagnosis of *N. caninum* infection (Sinnott et al., 2017). However, this technique is subjective and quite expensive, because of the need of *N. caninum* tachyzoite cultures (Dubey and Lindsay, 1996; Sinnott et al., 2017). Moreover, several ELISAs have been developed in recent years, with a variable Se and Sp (Sinnott et al., 2017). A recent study also found high variability in the diagnostic performances of IFAT and ELISA between different laboratories (Campero et al., 2018). In this regard, the highly sensitive TRFIA could be a suitable alternative to the current serological diagnostic techniques for ovine neosporosis. Furthermore, this high analytical Se of TRFIA could be an advantage when using other types of non-invasive samples (Cantos-Barreda et al., 2017a), such as milk and saliva, which normally contain less amounts of antibodies than blood serum samples (Khaitan et al., 2015; Pritchard, 2001).

In this chapter, two TRFIA based on GRA7 antigen were developed and validated for the detection of anti-*N. caninum* antibodies in sheep. The first technique detected antibodies in blood serum samples (Chapter 4a), whereas the second one was developed for full-cream milk samples (Chapter 4b).

Chapter 4a: A time-resolved fluorescence immunoassay for the detection of anti-*Neospora caninum* antibodies in sheep.



Huertas-López, A., Martínez-Carrasco, C., Cerón, J. J., Sánchez-Sánchez, R., Vázquez-Calvo, A., Álvarez-García, G., and Martínez-Subiela, S. (2019). A time-resolved fluorescence immunoassay for the detection of anti-*Neospora caninum* antibodies in sheep. Vet. Parasitol. 276, 108944. https://doi.org/10.1016/j.vetpar.2019.108994

ARTICLE 4: A time-resolved fluorescence immunoassayfor the detection of anti-*Neospora caninum* antibodies in sheep.

JOURNAL: Veterinary Parasitology

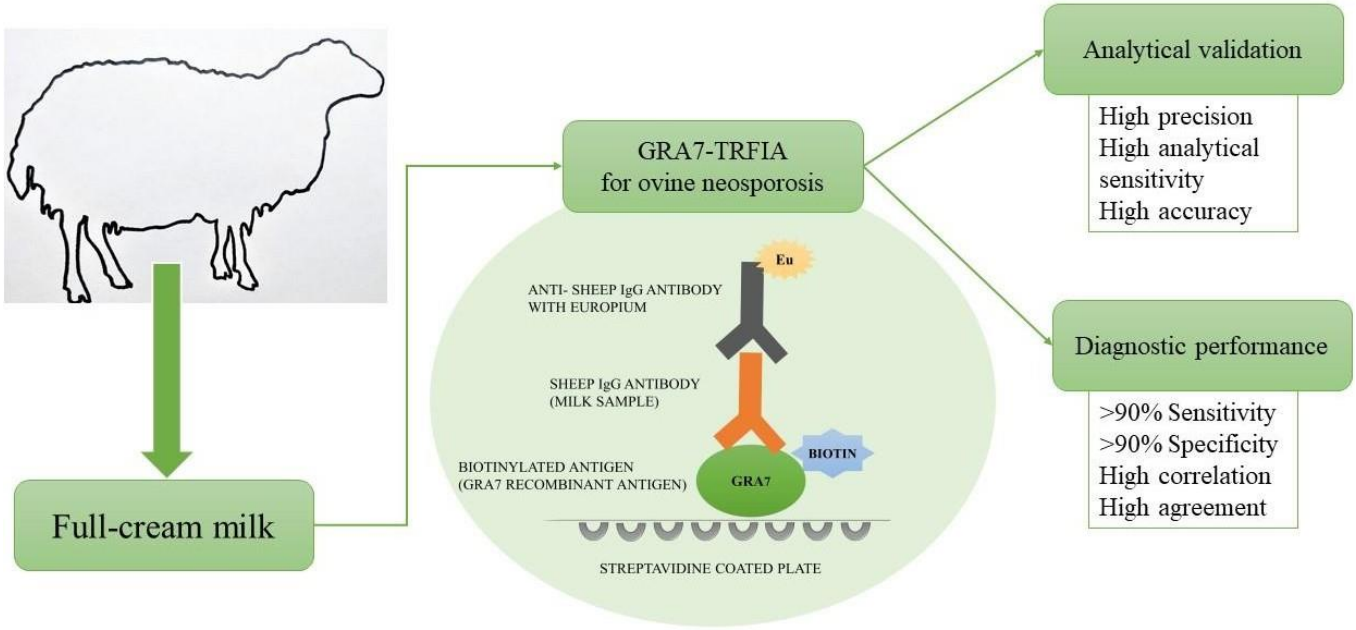
ABSTRACT: *Neospora caninum* is a protozoan parasite (Phylum Apicomplexa) that has been recently suggested as a relevant cause of reproductive disorders in small ruminants. The aim of the present study is to develop and validate a new serological test based on time resolved fluorescency using *N. caninum* GRA7 recombinant antigen (GRA7-TRFIA) for the detection of *N. caninum* antibodies in sheep. A total of 346 serum samples (208 from experimentally infected sheep, 117 from a dairy farm with a previous history of *Neospora*-associated abortion, and 21 negative sera) were used. The validation of the new assay was performed by the evaluation of assay precision, analytical sensitivity (Se), accuracy and cross reactivity. In the experimentally infected sheep, antibody kinetics was compared between GRA7-TRFIA and an in house *N. caninum* tachyzoite soluble extract-based ELISA (NcSALUVET ELISA) by Wilcoxon matched-pairs signed rank test. The cut-off and diagnostic Se and specificity (Sp) of GRA7-TRFIA was estimated by ROC analysis with field samples. In addition, concordance and correlation between GRA7-TRFIA and a commercial ELISA and NcSALUVET ELISA were assessed by *kappa* value and Spearman correlation coefficient, respectively.
Overall, GRA7-TRFIA showed an adequate precision, analytical Se and accuracy to detect anti-*N. caninum* antibodies in ovine serum, and no cross reactivity with the closely related protozoan *Toxoplasma gondii*. In naturally infected sheep, 100% Se and 95.35% Sp were obtained for a cut-off point of 62.68 Units of Fluorometry for *N. caninum* (UFN). Moreover, GRA7-TRFIA allowed earlier detection of *N. caninum* infection than NcSALUVET ELISA in experimentally infected sheep.

URL: https://www.sciencedirect.com/science/article/abs/pii/S0304401719302754?via%3Dihub

Supplementary material not included from Huertas-López, A., Martínez-Carrasco, C., Cerón, J. J., Sánchez-Sánchez, R., Vázquez-Calvo, A., Álvarez-García, G., and Martínez-Subiela, S. (2019). A time-resolved fluorescence immunoassay for the detection of anti-*Neospora caninum* antibodies in sheep. Vet. Parasitol. 276, 108944. https://doi.org/10.1016/j.vetpar.2019.108994

- **Supplementary File 1**: https://ars.els-cdn.com/content/image/1-s2.0-S0304401719302754-mmc1.docx
  - Table 6. Comparison of antibody detection by GRA7-TRFIA and NcSALUVET ELISA in sheep experimentally infected with *N. caninum* (0-14 days post-infection). GRA7-TRFIA is expressed in UFN (Units of Fluorometry for *N. caninum*) and NcSALUVET ELISA is expressed in RIPC (Relative index percent).
  - Table 7. Comparison of antibody detection by GRA7-TRFIA and NcSALUVET ELISA in experimentally infected sheep (21-56 days post-infection). GRA7-TRFIA is expressed in UFN (Units of Fluorometry for *N. caninum*) and NcSALUVET ELISA is expressed in RIPC (Relative index percent).

Chapter 4b: Detection of anti-*Neospora caninum* antibodies in sheep's full-cream milk by a time-resolved fluorescence immunoassay.



Huertas-López, A., Sánchez-Sánchez, R., Diezma-Díaz, C., Álvarez-García, G., Martínez-Carrasco, C., Martínez-Subiela, S., and Cerón, J. J. (2022). Detection of anti-*Neospora caninum* antibodies in sheep's full-cream milk by a time-resolved fluorescence immunoassay. Vet. Parasitol. 301, 109641. https://doi.org/10.1016/j.vetpar.2021.109641

ARTICLE 4: Detection of anti-*Neospora caninum* antibodies in sheep's full-cream milk by a time-resolved fluorescence immunoassay.

JOURNAL: Veterinary Parasitology

ABSTRACT: Ovine neosporosis, caused by the Apicomplexan parasite *Neospora caninum*, leads to reproductive failure worldwide. Nowadays, there is a trend to develop diagnostic techniques using non-invasive samples, such as milk, in order to reduce animal stress, sample collection effort, and costs. The objective of this study was to develop and validate a highly sensitive and specific serological technique, based on a time resolved-fluorescence immunoassay using a *N. caninum* GRA7 antigen (GRA7-TRFIA), for the detection of anti-*N. caninum* immunoglobulins G on sheep' full-cream milk samples. An analytical validation was performed, including intra- and inter-assay precision, analytical sensitivity and accuracy. The diagnostic performance of the assay was evaluated by studying the positive-negative discrimination by Mann Whitney U tests. In additon optimal cut-offs, diagnostic sensitivity and specificity, and areas under the curve were calculated by three Receiver Operating Curve (ROC) analyses, using GRA7-TRFIA and a *N. caninum* tachyzoite soluble extract-based ELISA (NcSALUVET-ELISA) in blood sera, and the coinciding results of both techniques, as reference techniques. Moreover, Spearman's correlation of GRA7-TRFIA in milk with the techniques in sera and agreement (*kappa* values) were also estimated. GRA7-TRFIA for milk samples showed an adequate precision, with high analytical sensitivity and accuracy. Regarding ROC analyses, at the optimal cut-offs, the diagnostic sensitivity and specificity were more than 90 % in all cases. In addition, GRA7-TRFIA values in milk were more positively correlated to GRA7-TRFIA values in blood sera than in the case of values obtained with NcSALUVET-ELISA. GRA7-TRFIA in milk showed an almost perfect agreement with GRA7-TRFIA in blood sera (kappa = 0.98) and with the coinciding results of GRA7-TRFIA and NcSALUVET in blood sera (kappa = 1.00), while it has a substantial agreement with NcSALUVET-ELISA (kappa = 0.69). In the light of these results, GRA7-TRFIA in full-cream milk samples is a highly sensitive technique that could be used for screening anti-*N. caninum* antibodies in sheep flocks.

URL: https://www.sciencedirect.com/science/article/pii/S0304401721003010?via%3Dihub

General discussion

General discussion

In this PhD thesis, a complete review on the current state of the development of new serological methods to detect anti-*T. gondii* antibodies has been carried out, focusing on their performance from a One Health approach and on the quality of the validation process. In this review, the need of a reference panel of sera and the validation procedure regarding the OIE protocol (OIE, 2019a) were considered key issues that were taken into account for the subsequent studies in this PhD thesis. Moreover, we conducted an epidemiological study on the seroprevalence and risk factors associated to the presence of *T. gondii* infection in dogsand cats from an anthropized area employing one of the most commonly used serological techniques in animals, the MAT. After acquiring a solid knowledge base and collecting the seropositive and seronegative reference sera necessary for subsequent use in the techniques on which this PhD thesis is focused, we successfully developed new serological techniques for the detection of anti-*T. gondii* and anti-*N. caninum* antibodies in different animal species, filling some of the gaps of knowledge detected inthe diagnosis of both parasites. The different studies performed within the framework of this thesis project have allowed to achieve the objectives stated.

Regarding **objective 1a**, a wide heterogeneity was detected when analyzing the bibliometrics characteristics and study aims of articles focused on the development of new serological techniques for the diagnosis of *T. gondii* infection. This heterogeneity was more evident when comparing the studies performed by research physicians and research veterinarians, and those carried out on humans or animals. For example, although there was not any significant relationship between the evaluated techniques and the human or animal hosts, it was detected concerning the reference technique. Studies performed in humans preferred to use ELISA as reference technique, while those conducted in animals more frequently used IFAT and agglutination tests, such as MAT. The explanation of this difference may be the high availability of commercial ELISAs for humans, that usually show high Se and Sp (Ybañez et al., 2020b), in contrast with the

shortage of commercially available test for animals, that may lead to use more time-consuming laboratorial techniques such as IFAT, as well as the lack of species-specific conjugates in some animal species, that could make necessary to select agglutination techniques as reference (de Almeida et al., 2016). Another remarkable finding of this review was the scarcity of studies with a low or medium risk of bias when assessing their quality by QUADAS 2 tool (Whiting, 2011). Finally, this study highlights the lack of implementation of the One Health approach, especially in those studies performed by research physicians and/or conducted in humans. Only one study reviewed in this research work had a One Health perspective, conducting the study on domestic (the human activity component) and wild animal species (the ecological component) (Calistri et al., 2013). This finding is particularly noteworthy considering that *T. gondii* is a cause of zoonosis, highlighting the need to intensify interdisciplinary approach (Thompson, 2013; Dantas-Torres and Otranto, 2014; Krecek et al., 2020).

To achieve **objective 1b**, a second meta-analysis of new data extracted from the previously analyzed articles was performed. In line with the previous meta-analysis, this second one confirmed the high heterogeneity in the characteristics of the *T. gondii* serological techniques and the validation process between human and veterinary medicine. Additionally, the gaps found in the description of the evaluated techniques' protocol and reagents could affect the reproducibility of the results. The meta-analysis suggests that the "gold standard" concept is misinterpreted by the authors of the articles reviewed, as some studies quoted this term to mean the reference technique they employed, insteadof a technique that could lead to an accurate diagnosis (Gardner et al., 2010). There was a significant higher use as reference of non-commercial techniques in studies conducted by RV and on animals, probably due to the difficulty for standardization of serological assays in different animal species (Liu et al., 2015; Wyrosdick and Schaefer, 2015; Uddin et al., 2021). Studies conducted by RP more often evaluated techniques for the detection of IgG and IgM; moreover, discrimination between acute and chronic phases was exclusively estimatedin studies performed on humans. These facts may be due to the importance of detectingacute toxoplasmosis in pregnant women and thus, numerous tests are developed to detect IgM antibodies or IgG avidity (Emelia et al., 2014; Smets et al., 2016; Trotta et al.,2016; Laboudi and Sadak,

2017). On the other hand, most of studies only assessed the diagnostic performance of the technique, without an analytical validation. This finding is remarkable especially considering the studies which develop new techniques, in whichintra- and inter-assay precision, analytical sensitivity and accuracy should be estimated (Jacobson, 1998; Andreasson et al., 2015). In addition, few studies analyzed the potential cross-reactivity of the evaluated technique/s with other pathogens, especially those phylogenetically related to *T. gondii* such as Apicomplexan parasites (Gondim et al., 2017). Finally, when comparing the evaluated technique with a reference one, studies conducted on humans more frequently estimated the Pearson or Spearman correlation coefficient. In contrast, articles focused on animals calculated the agreement, normally by the *kappa* value, which is stronger than calculating the agreement percentage (Hazra and Gogtay, 2017). The wide heterogeneity found in thisreview and meta-analysis highlights the need to establish a consensus-based protocol for validation of serological assays to diagnose *T. gondii* infection, similar to the standards proposed by the World Organization of Animal Health (OIE) for animals (OIE,2019a).

The epidemiological study conducted in chapter 2 led to fulfil **objective 2**. In this study, we detected anti-*T. gondii* antibodies seroprevalence of 18.7% in cats and 7.9% in dogs from Bangkok, an anthropized area where community cat and dog keeping is a widely extended practice (Savvides, 2013; Toukhsati et al., 2015). These results proved that there is a maintenance of the urban cycle of *T. gondii* in this city over the years, as the seroprevalences obtained were similar in dogs (Jittapalapong et al., 2007, 2009) and higher in cats (Jittapalapong et al., 2007, 2010; Sukhumavasi et al., 2012) than previous epidemiological studies conducted in the same area. Moreover, it supports the need of implementing control measures for this parasitic disease, as cats could be oocysts shedders and dogs may act as mechanical vectors of oocysts, which could suppose a public health risk. On the other hand, the risk factors associated to *T. gondii* infection were older age in cats, and mixed breed and completely outdoors life in dogs. These risk factors are in line with other epidemiological studies performed in the former (reviewed in Dubey et al., 2020a) and the latter species (de Moura et al., 2009; Dantas et al., 2014; Cano-Terriza et al., 2016; Ding et al., 2017; Wang et al., 2017; Zarra-Nezhad et al., 2017).

In order to achieve **objective 3**, new TRFIA based on recombinant and chimeric

antigens were developed to detect anti-*T. gondii* antibodies in cats and goats. The results of these studies highlighted that not all antigens worked as expected. In the firstspecies, four *in-house* produced recombinant antigens (TgGRA7, truncated TgGRA7, TgSAG2 and truncated TgSAG2) and one commercial chimeric antigen (TgSAG1-GRA8) were tested by TRFIA, and the chimeric antigen obtained the best diagnostic performance. Although the protein sequences employed to produce the recombinant antigens TgGRA7 and TgSAG2 were specifically selected to avoid the potential cross- reactivity with *N. caninum* (Goodman, 2006), the poor discrimination between positive and negative samples, as well as the low sensitivity and specificity detected, limit the use of these antigens for the diagnosis of feline toxoplasmosis. The recombinant antigens TgGRA7 and TgSAG2 used in this study had been evaluated in other studies in cats, although they had better results (Huang et al., 2002; Cai et al., 2015; Abdelbaset etal., 2017; Salman et al., 2018). The worse performance of our recombinant antigens could be due to the different expression system employed, which could affect to the antigenicity and diagnostic performance of the antigens (Letourneur et al., 2001; Marti et al., 2002; Ybañez et al., 2020b). However, the chimeric antigen based on epitopes from TgSAG1 and TgGRA8 recombinant antigens had not been previously tested. In addition, another relevant finding of this study was the detection of anti-*N. caninum* antibodies in four cats, which suppose the first time that this parasite infection has beendetected in cats from Thailand. *Neospora caninum* infection had been searched in cats from this country in other study, but no positive animals were found (Arunvipas et al., 2012). Two out of four cats were co-infected with *T. gondii and N. caninum*, while the other two were only seropositive to *N. caninum*. Although the number of *N. caninum* seropositive cats was not enough to evaluate the potential cross-reactivity of the new assays with this parasite, the TRFIAs based on the recombinant antigens gave a positiveresult in one of the *N. caninum* seropositive cats, whereas both cats were seronegative by TgSAG1-GRA8-TRFIA. Cross-reactivity of TgSAG2 and TgGRA7 with *N. caninum* has been neither previously evaluated in cat sera (Huang et al., 2002; Cai et al., 2015; Abdelbaset et al., 2017; Ybañez et al., 2020a), so further research on this topic is still needed.

The same chimeric antigen TgSAG1-GRA8 was also used to develop a new TRFIA for diagnosis of toxoplasmosis in goats. This technique showed an excellent analytical

and diagnostic performance, with 92% of Se and 90.5% of Sp, comparing with the reference assay (*in-house* ELISA and WB). The lack of a gold standard technique in this species led us to use two techniques as reference for a better validation of the results. The diagnostic performance of this assay was similar to previously developed ELISAs using chimeric antigens for diagnosis of ovine toxoplasmosis (Holec-Gąsior et al., 2019), but higher compared with other techniques based on TgSAG1 or the mixture of TgSAG1 and TgGRA7 (Velmurugan et al., 2008; Bachan et al., 2018). Furthermore, and as far as authors' knowledge, this is the first time that a serological assay has been validated to detect anti-*T. gondii* antibodies in naturally infected goats (Dubey et al., 2020b). However, cross-reactivity with *N. caninum* was evaluated using sera samples from 31 *N. caninum* seropositive and *T. gondii* seronegative sheep, and some of these sera gave a positive result by this newly developed technique. The cross-reactivity of the TgSAG1-GRA8 antigen was confirmed by WB. Additionally, we detected the same cross-reactivity by WB with another commercial recombinant antigen, TgSAG1. Although it was not detected with TLA, this native antigen has other limitations, such as being difficult to standardize and the need of maintaining live cultures for its production (Holec-Gąsior et al., 2014; Rostami et al., 2018; Zhou et al., 2019; Ybañez et al., 2020b). So, we can conclude that there is cross-reactivity with *N. caninum* when using TgSAG1-GRA8 and TgSAG1 antigens. Thus, other confirmatory assays should be performed to reach a definitive diagnosis. With this regard, this study highlights the need of assessing the cross-reactivity between *T. gondii* and *N. caninum* when development new serological techniques in goats and other species, especially if they are based on recombinant and chimeric antigens, as both parasites are closely related, share antigens and can infect cats and goats (Gondim et al., 2017).

Finally, two new TRFIA based on the recombinant antigen NcGRA7 were developed for the detection of anti-*N. caninum* antibodies in blood sera and full-cream milk from sheep with excellent performances in both types of samples, allowing to achieve **objective 4**. This technique showed an outstanding analytical and diagnostic performance, with 100% of Se and 95.35% of Sp for sera samples compared with an *in-house* ELISA and a commercial ELISA based on native antigens and used as reference. These results are similar to other ELISAs developed to diagnosis ovine (Andreotti et al.,

2009; Pinheiro et al., 2015) and bovine neosporosis (Aguado-Martínez et al., 2008). Furthermore, no cross-reactivity with *T. gondii* seropositive and *N. caninum* seronegative sheep was detected with the new technique.The most significant finding in this first study performed on sera samples was that anti-*N. caninum* antibodies were detected in experimentally infected sheep one week earlierwith NcGRA7-TRFIA than with an *in-house* ELISA based on *N. caninum* native antigen.

In consequence of the successful results obtained in the study conducted in chapter 4a, we evaluated the NcGRA7-TRFIA in milk samples. This technique had proven to be very sensitive in our previous study, with a considerably low limit of detection, so it could probably detect the normally lower amounts of antibodies present in milk samples (Pritchard, 2001; Khaitan et al., 2015). Moreover, TRFIA is not affected by lipemia or hemolysis, as shown by Parra and Cerón (2007). So, after selecting the best skimming protocol, we compared the positive/negative discriminative potential of NcGRA7-TRFIA between skimmed and full-cream milk, obtaining a major difference with the full-cream milk sample. Then, we assessed the analytical and diagnostic performance of this technique using full-cream milk samples, with Se and Sp over 90% taking an *in-house* ELISA based on native antigen and the NcGRA7-TRFIA in sera as reference. Moreover, there were significant positive correlations between the results in sera and milk, and also a significant agreement with the reference techniques. As far as authors' knowledge, this is the first time that a serological technique has been successfully validated for detection of anti-*N. caninum* antibodies in ovine full-cream milk, as there is only one previous study that developed an ELISA but in ovine skimmed milk (Tamponi et al., 2015). In cattle, other ELISA was developed for full-cream milk samples, but it showed low Se (Byrem et al., 2012). Therefore, the NcGRA7-TRFIA developed in chapter 4b has not only the inherent advantages related to the use of non-invasive samples (Pritchard, 2001; Robertson and Nicholson, 2005; Samaranayake,2007; Brinkhof et al., 2010; Khaitan et al., 2015; Valinetz and Cangelosi, 2021), but also the benefits of avoiding the skimming process that could extend the study time and costs.

To sum up, this PhD thesis provides an up-to-dated review on the development of serological assays for detection of anti-*T. gondii* antibodies, an epidemiological study on the *T. gondii* serostatus of cats and dogs living in an anthropized area, as well as new

and highly sensitive serological methods that were analytically and diagnostically validated for detection of *T. gondii* infection in cat and goat sera, and *N. caninum* infection in sheep sera and milk. However, future research should include the re-evaluation of the *T. gondii*antigens used in this study with a broader panel of *N. caninum* seropositive and *T. gondii*seronegative cats, to confirm the absence of cross-reactivities in this species. Also, the assessment of new *T. gondii* recombinant or chimeric antigens, employed alone or in mixtures, by TRFIA should be performed to prevent the cross-reactivity with *N. caninum*in goats. In addition, this thesis can be the starting point for future research that will broaden and improve the currently available serological techniques for the diagnosis of *T. gondii* and *N. caninum* infections in other animal species, and human beings in the case of *T. gondii* infection. In this regard, the validation of additional TRFIAs based on recombinant or chimeric antigens in other species, such as dogs, cattle, wild animals and humans would be a compelling field of study following the One Health approach.

Conclusions

Conclusions

1. The results obtained in the systematic review and meta-analysis on the development of serological assays to detect anti-*Toxoplasma gondii* antibodies in humans and animals highlight the need of performing a quality assessment of the studies before conducting any statistical analysis to minimize the introduction of errors and biases into the analyses. Herein, biases in the selection of the study subjects, the specification of the evaluated test's threshold and the flow of study were identified by QUADAS-2 tool and 61.8% of assessed articles were discarded.
2. There is a space for improvement in the implementation of the One Health approach in the studies on the development of serological techniques to diagnose *T. gondii* infection, which is essential to design and carry out effective intervention measures to control toxoplasmosis. In this field here was only 42.5% collaboration between researchers belonging to different knowledge areas, and veterinarians were more aware than physicians of the necessity to address the study of toxoplasmosis from a One Health perspective.
3. The validation process of serological assays for *T. gondii* infection showed a great heterogeneity in both human and veterinary medicine and did not follow the World Organisation for Animal Health (OIE) guidelines. The description of the evaluated techniques' protocols and reagents could be improved, especially in the cases in which the antigen employed, the antibody isotype detected, the use of controls and the analysis of samples in duplicate were not specified, the analytical sensitivity was hardly assessed and the concept of "gold standard" was misinterpreted. Therefore, there is a need to elaborate a consensual and standardized protocol to guide the different steps of the validation process of new serological techniques for the detection of *T. gondii* infection in humans and animals.

4. The significant seroprevalence of *T. gondii* infection in cats and dogs in an anthropized area such as Bangkok (Thailand) indicates that this zoonotic parasite circulates in urban areas where there is a close contact between humans and these species. Accordingly, our results evidenced that a public health concern exists and can set the basis for designing proper intervention strategies in the study area and in synanthropic areas with similar epidemiological risk factors. Moreover, the serum biobank collected served for the development of a novel serological method for cats.
5. Novel time-resolved fluorescence immunoassays (TRFIAs) were developed for the serological diagnosis of *T. gondii* and *Neospora caninum* infections in relevant hosts such as cats, goats and ovine and showed good diagnostic performances. In order to follow a One health approach, in the case of the serodiagnosis of *T. gondii* infection, TRFIAs were employed in definitive and intermediate hosts that can be a *T. gondii* source for humans.
6. The detection of anti-*T. gondii* antibodies in cats by TRFIA indicated that not all antigens (TgSAG1-GRA8, TgGRA7, truncated TgGRA7, TgSAG2 and truncated TgSAG2) work as well as expected. On the other hand, TRFIA based on the chimeric antigen TgSAG1-GRA8 had an excellent analytical sensitivity and diagnostic performance for detection of anti-*T. gondii* antibodies in cats and goats.
7. The cross-reactivity detected between TgSAG1 and anti-*N. caninum* antibodies limits the use of serological methods based on this antigen for the diagnosis of *T. gondii* infection at least in goats. This cross-reactivity should be considered when evaluating the diagnostic usefulness of any potential antigen in those host species where both Apicomplexan parasites has been described.
8. The TRFIA based on the recombinant antigen NcGRA7 (NcGRA7-TRFIA) provided an outstanding analytical and diagnostic performance for the detection of anti-*N. caninum* antibodies in ovine sera, no cross-reactivity with anti-*T. gondii* antibodies, and an earlier detection of seroconversion in experimentally infected sheep compared with an ELISA based on whole tachyzoite extract.

9. NcGRA7-TRFIA also showed high sensitivity for the detection of anti-*N. caninum* antibodies in sheep full-cream milk samples. This technique has the advantages associated to the use of non-invasive samples that are easy and rapid to collect, allowing its use for large serological screenings. In the future, this helpful technique could be adapted for other host ruminant species, such as goats and cattle.

References

References

Abdelbaset, A.E., Alhasan, H., Salman, D., Karram, M.H., Ellah Rushdi, M.A., Xuenan, X., Igarashi, M., 2017. Evaluation of recombinant antigens in combination and single formula for diagnosis of feline toxoplasmosis. Exp. Parasitol. 172, 1–4. https://doi.org/10.1016/j.exppara.2016.11.003

Aguado-Martínez, A., Álvarez-García, G., Fernández-García, A., Risco-Castillo, V., Arnaiz-Seco, I., Rebordosa-Trigueros, X., Navarro-Lozano, V., Ortega-Mora, L.M., 2008. Usefulness of rNcGRA7- and rNcSAG4-based ELISA tests for distinguishing primo-infection, recrudescence, and chronic bovine neosporosis. Vet. Parasitol. 157, 182–195. https://doi.org/10.1016/j.vetpar.2008.08.002

Aguirre, A.A., Longcore, T., Barbieri, M., Dabritz, H., Hill, D., Klein, P.N., Lepczyk, C., Lilly, E.L., McLeod, R., Milcarsky, J., Murphy, C.E., Su, C., VanWormer, E., Yolken, R., Sizemore, G.C., 2019. The One Health approach to toxoplasmosis: epidemiology, control, and prevention strategies. Ecohealth 16, 378–390. https://doi.org/10.1007/s10393-019-01405-7

Al-Adhami, B.H., Gajadhar, A.A., 2014. A new multi-host species indirect ELISA using protein A/G conjugate for detection of anti-*Toxoplasma gondii* IgG antibodies with comparison to ELISA-IgG, agglutination assay and Western blot. Vet. Parasitol. 200, 66–73. https://doi.org/10.1016/j.vetpar.2013.11.004

Alemayehu, D., Zou, K.H., 2012. Applications of ROC Analysis in Medical Research. Recent Developments and Future Directions. Acad. Radiol. 19, 1457–1464. https://doi.org/10.1016/j.acra.2012.09.006

Alvarez-García, G., García-Culebras, A., Gutiérrez-Expósito, D., Navarro-Lozano, V., Pastor-Fernández, I., Ortega-Mora, L.M., 2013. Serological diagnosis of bovine neosporosis: a comparative study of commercially available ELISA tests. Vet. Parasitol. 198, 85–95. https://doi.org/10.1016/j.vetpar.2013.07.033

Ammann, R.W., Battelli, G., Beard, T.C., Bessonov, A.S., Campano Diaz, S., Conraths, F.J., Craig, P.S., Dar, F.K., Deplazes, P., De Rosa, F., Eckert, J., Economides, P., Filice, C., Gasser, R., Gemmell, M.A., Giraudoux, P., Gottstein, B., Grimm, F., Heath, D.D., Jenkins, D., Kamiya, M., Kern, P., Lawson, J.R., Larrieu, E.J., Lightowlers, M.W., Liu, F., Macpherson, C.N.L., Mantovani, A., McManus, D.P., Meslin, F.X., Movsessian, S.O., Nikogossian, A.M., Nonnemaker, J.M., Orlando, D.F., Parodi, P., Pawłowski, Z.S., Rausch, R.L., Roberts, M.G., Romig, T., Sato, N., Schantz, P.M., Seimenis, A., Selhorst, T., Staubach, C., Suzuki, K., Tackmann, K., Thakur, A.S., Thompson, R.C.A., Todorov, T., Torgerson, P.R., Uchino, J., Sinner, W. von, Vuitton, D.A., Wen, H., 2002. WHO/OIE Manual on echinococcosis in humans and animals: a public health problem of global concern. Available in: https://apps.who.int/iris/bitstream/handle/10665/42427/929044522X.pdf?sequence=1&isAllowed=y

Andreasson, U., Perret-Liaudet, A., van Waalwijk van Doorn, L.J.C., Blennow, K., Chiasserini, D., Engelborghs, S., Fladby, T., Genc, S., Kruse, N., Kuiperij, H.B., Kulic, L., Lewczuk, P., Mollenhauer, B., Mroczko, B., Parnetti, L., Vanmechelen, E., Verbeek, M.M., Winblad, B., Zetterberg, H., Koel-Simmelink, M., Teunissen, C.E., 2015. A practical guide to immunoassay method validation. Front. Neurol. 6, 1–8. https://doi.org/10.3389/fneur.2015.00179

Andreotti, R., Matos, M. de F.C., Gonçalves, K.N., Oshiro, L.M., Lima-Junior, M.S. da C., Paiva, F., Leite, F.L., 2009. Comparison of indirect ELISA based on recombinant protein NcSRS2 and IFAT for detection of *Neospora caninum* antibodies in sheep. Rev. Bras. Parasitol. Vet. 18, 19–22. https://doi.org/10.4322/rbpv.01802004

Anvari, D., Saberi, R., Sharif, M., Sarvi, S., Hosseini, S.A., Moosazadeh, M., Hosseininejad, Z., Chegeni, T.N., Daryani, A., 2020. Seroprevalence of *Neospora caninum* Infection in dog population worldwide: a systematic review and meta-analysis. Acta Parasitol. 65, 273–290. https://doi.org/10.2478/s11686-019-00163-4

Arranz-Solís, D., Benavides, J., Regidor-Cerrillo, J., Horcajo, P., Castaño, P., Del Carmen Ferreras, M., Jiménez-Pelayo, L., Collantes-Fernández, E., Ferre, I., Hemphill, A., Pérez, V., Ortega-Mora, L.M., 2016. Systemic and local immune responses in sheep after *Neospora caninum* experimental infection at early, mid and late gestation. Vet. Res. 47, 1–13. https://doi.org/10.1186/s13567-015-0290-0

Arunvipas, P., Inpankaew, T., Jittapalapong, S., 2012. Risk factors of *Neospora caninum* infection in dogs and cats in dairy farms in Western Thailand. Trop. Anim. Health Prod. 44, 1117–1121. https://doi.org/10.1007/s11250-011-0048-2

Atkinson, R., Harper, P.A.W., Reichel, M.P., Ellis, J.T., 2000. Progress in the serodiagnosis of *Neospora caninum* infections of cattle. Trends Parasitol. 16, 110–114. https://doi.org/10.1016/S0169-4758(99)01604-X

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

Babo Martins, S., Rushton, J., Stärk, K.D.C., 2016. Economic assessment of zoonoses surveillance in a 'One Health' context: a conceptual framework. Zoonoses Public Health 63, 386–395. https://doi.org/10.1111/zph.12239

Bachan, M., Deb, A.R., Maharana, B.R., Sudhakar, N.R., Sudan, V., Saravanan, B.C., Tewari, A.K., 2018. High seroprevalence of *Toxoplasma gondii* in goats in Jharkhand state of India. Vet. Parasitol. Reg. Stud. Reports 12, 61–68. https://doi.org/10.1016/j.vprsr.2018.02.004

Banerjee, S., Bhatta, M., Nandi, S., Dutta, S., Saha, M.K., 2022. Diagnostic accuracy of HIV in-vitro assays evaluated by WHO prequalification evaluation laboratories: systematic review and meta-analysis. Jpn. J. Infect. Dis. JJID.2021.720. https://doi.org/10.7883/yoken.JJID.2021.720

Begley, C.G., 2013. An unappreciated challenge to oncology drug discovery: pitfalls in preclinical research. Am. Soc. Clin. Oncol. Educ. Book 33, 466–468.

https://doi.org/10.14694/edbook_am.2013.33.466
Begley, C.G., Ioannidis, J.P.A., 2015. Reproducibility in science: Improving the standard for basic and preclinical research. Circ. Res. 116, 116–126. https://doi.org/10.1161/CIRCRESAHA.114.303819

Boireau, P., Bruschi, F., Dupouy-Camet, J., Gamble, H.R., Nöckler, K., Kapel, C.M.O., Murrell, K.D., Pozio, E., 2007. FAO/WHO/OIE Guidelines for the surveillance, management, prevention and control of trichinellosis, World Organisation for Animal Health (OIE). Available in: http://www.trichinellosis.org/uploads/FAO-WHO-OIE_Guidelines.pdf

Brinkhof, J.M.A., Houwers, D.J., Moll, L., Dercksen, D., van Maanen, C., 2010. Diagnostic performance of ELISA and PCR in identifying SRLV-infected sheep and goats using serum, plasma and milk samples and in early detection of infection in dairy flocks through bulk milk testing. Vet. Microbiol. 142, 193–198. https://doi.org/10.1016/j.vetmic.2009.09.060

Byrem, T.M., Bartlett, P.C., Donohue, H., Voisinet, B.D., Houseman, J.T., 2012. Performance of a commercial serum ELISA for the detection of antibodies to *Neospora caninum* in whole and skim milk samples. Vet. Parasitol. 190, 249–253. https://doi.org/10.1016/j.vetpar.2012.06.005

Cai, Y., Wang, Z., Li, J., Li, N., Wei, F., Liu, Q., 2015. Evaluation of an indirect ELISA using recombinant granule antigen GRA7 for serodiagnosis of *Toxoplasma gondii* infection in cats. J. Parasitol. 101, 37–40. https://doi.org/10.1645/14-575.1

Calero-Berna, R., Gennari, S.M., 2019. Clinical toxoplasmosis in dogs and cats: an update. Front. Vet. Sci. 6, 1–9. https://doi.org/10.3389/fvets.2019.00054

Calistri, P., Iannetti, S., L. Danzetta, M., Narcisi, V., Cito, F., Di Sabatino, D., Bruno, R., Sauro, F., Atzeni, M., Carvelli, A., Giovannini, A., 2013. The components of "One World - One Health" approach. Transbound. Emerg. Dis. 60, 4–13. https://doi.org/10.1111/tbed.12145

Campero, L.M., Moreno-Gonzalo, J., Venturini, M.C., Moré, G., Dellarupe, A., Rambeaud, M., Echaide, I.E., Valentini, B., Campero, C.M., Moore, D.P., Cano, D.B., Fort, M., Mota, R.A., Serrano-Martínez, M.E., Cruz-Vázquez, C., Ortega-Mora, L.M., Álvarez-García, G., 2018. An Ibero-American inter-laboratory trial to evaluate serological tests for the detection of anti-*Neospora caninum* antibodies in cattle. Trop. Anim. Health Prod. 50, 75–84. https://doi.org/10.1007/s11250-017-1401-x

Cano-Terriza, D., Puig-Ribas, M., Jiménez-Ruiz, S., Cabezón, Ó., Almería, S., Galán-Relaño, Á., Dubey, J.P., García-Bocanegra, I., 2016. Risk factors of *Toxoplasma gondii* infection in hunting, pet and watchdogs from southern Spain and northern Africa. Parasitol. Int. 65, 363–366. https://doi.org/10.1016/j.parint.2016.05.001

Cantos-Barreda, A., Escribano, D., Bernal, L.J., Cerón, J.J., Martínez-Subiela, S., 2017a. Quantification of anti-*Leishmania* antibodies in saliva of dogs. Vet. Parasitol. 242, 54–58. https://doi.org/10.1016/j.vetpar.2017.05.017

Cantos-Barreda, A., Escribano, D., Bernal, L.J., Pardo-Marín, L., Cerón, J.J., Martínez-

Subiela, S., 2017b. New wide dynamic range assays for quantification of anti-*Leishmania* IgG2 and IgA antibodies in canine serum. Vet. Immunol. Immunopathol. 189, 11–16. https://doi.org/10.1016/j.vetimm.2017.05.007

Chaves, C.E.V., de Oliveira, S.M. do V.L., Venturini, J., Grande, A.J., Sylvestre, T.F., Mendes, R.P., Paniago, A.M.M., 2020. Accuracy of serological tests for diagnosis of chronic pulmonary aspergillosis: a systematic review and meta-analysis. PLoS One 15, 1–17. https://doi.org/10.1371/journal.pone.0222738

Claassen, J.A.H.R., 2005. The gold standard: not a golden standard. BMJ. 330, 1121. https://doi.org/10.1136/bmj.330.7500.1121

Cohen, J., 1960. A coefficient of agreement for nominal scales. Educ. Psychol. Meas. 20, 37–46. https://doi.org/10.1177/001316446002000104

Cong, W., Elsheikha, H.M., Zhou, N., Peng, P., Qin, S.Y., Meng, Q.F., Qian, A.D., 2018. Prevalence of antibodies against *Toxoplasma gondii* in pets and their owners in Shandong province, Eastern China. BMC Infect. Dis. 18, 430. https://doi.org/10.1186/s12879-018-3307-2

Cook, A.J.C., Gilbert, R.E., Buffolano, W., Zufferey, J., Petersen, E., Jenum, P.A., Foulon, W., Semprini, A.E., Dunn, D.T., 2000. Sources of *Toxoplasma* infection in pregnant women: European multicentre case-control study. Br. Med. J. 321, 142–147. https://doi.org/10.1136/bmj.321.7254.142

Cota, G.F., de Sousa, M.R., Demarqui, F.N., Rabello, A., 2012. The diagnostic accuracy of serologic and molecular methods for detecting visceral leishmaniasis in HIV infected patients: meta-analysis. PLoS Negl. Trop. Dis. 6, e1665. https://doi.org/10.1371/journal.pntd.0001665

Dabritz, H.A., Conrad, P.A., 2010. Cats and *Toxoplasma*: implications for public health. Zoonoses Public Health. 57, 34–52. https://doi.org/10.1111/j.1863-2378.2009.01273.x

Dantas-Torres, F., Otranto, D., 2014. Dogs, cats, parasites, and humans in Brazil: opening the black box. Parasites Vectors 7, 1–25. https://doi.org/10.1186/1756-3305-7-22

Dantas, S.B.A., Da Fonsêca Fernandes, A.R., De Souza Neto, O.L., Mota, R.A., Alves, C.J., De Azevedo, S.S., 2014. Fatores de risco para a ocorrência de anticorpos contra *Toxoplasma gondii* e *Neospora caninum* em cães domiciliados no Nordeste do Brasil. Semin. Agrar. 35, 875–881. https://doi.org/10.5433/1679-0359.2014v35n2p875

de Almeida, J.C., Frehse, M.S., Navarro, I.T., Garcia, J.L., Biondo, A.W., Freire, R.L., 2016. Comparison of indirect fluorescent antibody test and the modified agglutination test for the detection of *Toxoplasma gondii* antibodies in stray dogs from Southern Brazil. Acta Parasitol. 61, 694–696. https://doi.org/10.1515/ap-2016-0097

de Moura, A.B., de Souza, A.P., Sartor, A.A., Bellato, V., Teixeira, E.B., Pisetta, G.M., Heusser Junior, A., 2009. Ocorrência de anticorpos e fatores de risco para infecção por *Toxoplasma gondii* em cães, nas cidades de Lages e Balneário Camboriú, Santa Catarina, Brasil. Rev. Bras. Parasitol. Vet. 18, 52–56.

https://doi.org/10.4322/rbpv.01803009

de Paula Dreer, M., Gonçalves, D., da Silva Caetano, I., Gerônimo, E., Menegas, P., Bergo, D., Ruiz Lopes-Mori, F., Benitez, A., de Freitas, J., Evers, F., Navarro, I., Martins, L. de, 2013. Toxoplasmosis, leptospirosis and brucellosis in stray dogs housed at the shelter in Umuarama municipality, Paraná, Brazil. J. Venom. Anim. Toxins Incl. Trop. Dis. 19, 23. https://doi.org/10.1186/1678-9199-19-23

de Wit, L.A., Kilpatrick, A.M., VanWormer, E., Croll, D.A., Tershy, B.R., Kim, M., Shapiro, K., 2020. Seasonal and spatial variation in *Toxoplasma gondii* contamination in soil in urban public spaces in California, United States. Zoonoses Public Health 67, 70–78. https://doi.org/10.1111/zph.12656

Ding, H., Gao, Y.-M., Deng, Y., Lamberton, P.H.L., Lu, D.-B., 2017. A systematic review and meta-analysis of the seroprevalence of *Toxoplasma gondii* in cats in mainland China. Parasit. Vectors 10, 27. https://doi.org/10.1186/s13071-017-1970-6

Ding, J., Banai, M., Yu, S., Ting, X., 2018. Editorial: veterinary bacterial zoonoses. Front. Vet. Sci. 5, 1–2. https://doi.org/10.3389/fvets.2018.00162

Donahoe, S.L., Lindsay, S.A., Krockenberger, M., Phalen, D., Šlapeta, J., 2015. A review of neosporosis and pathologic findings of *Neospora caninum* infection in wildlife. Int. J. Parasitol. Parasites Wildl. 4, 216–238. https://doi.org/10.1016/j.ijppaw.2015.04.002

Dubey, J.P., 1982. Repeat transplacental transfer of *Toxoplasma gondii* in dairy goats. J. Am. Vet. Med. Assoc. 180, 1220–1221.

Dubey, J.P., Lappin, M.R., Thulliez, P., 1995. Long-term antibody responses of cats fed *Toxoplasma gondii* tissue cysts. J. Parasitol. 81, 887–893.

Dubey, J.P., Lindsay, D.S., 1996. A review of *Neospora caninum* and neosporosis. Vet. Parasitol. 67, 1–59. https://doi.org/10.1016/S0304-4017(96)01035-7

Dubey, J.P., Barr, B.C., Barta, J.R., Bjerkås, I., Björkman, C., Blagburn, B.L., Bowman, D.D., Buxton, D., Ellis, J.T., Gottstein, B., Hemphill, A., Hill, D.E., Howe, D.K., Jenkins, M.C., Kobayashi, Y., Koudela, B., Marsh, A.E., Mattsson, J.G., McAllister, M.M., Modrý, D., Omata, Y., Sibley, L.D., Speer, C.A., Trees, A.J., Uggla, A., Upton, S.J., Williams, D.J.L., Lindsay, D.S., 2002. Redescription of *Neospora caninum* and its differentiation from related coccidia. Int. J. Parasitol. 32, 929–946. https://doi.org/10.1016/S0020-7519(02)00094-2

Dubey, J.P., 2003. Review of *Neospora caninum* and neosporosis in animals. Korean J. Parasitol. 41, 1–16. https://doi.org/10.3347/kjp.2003.41.1.1

Dubey, J.P., Schares, G., Ortega-Mora, L.M., 2007. Epidemiology and control of neosporosis and *Neospora caninum*. Clin. Microbiol. Rev. 20, 2. https://doi.org/10.1128/CMR.00031-06

Dubey, J.P., Schares, G., 2011. Neosporosis in animals-The last five years. Vet. Parasitol. 180, 90–108. https://doi.org/10.1016/j.vetpar.2011.05.031

Dubey, J.P., 2016. Toxoplasmosis of animals and humans, second ed. CRC Press, Boca Raton, p. 336. https://doi.org/10.1201/9781420092370

Dubey, J.P., Hemphill, A., Calero-Bernal, R., Schares, G., 2017. Neosporosis in animals. CRC Press, Boca Raton , p. 548. https://doi.org/10.1201/9781315152561

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

Dubey, J.P., Murata, F.H.A., Cerqueira-Cézar, C.K., Kwok, O.C.H., 2020b. Public health and economic importance of *Toxoplasma gondii* infections in goats: the last decade. Res. Vet. Sci. 132, 292–307. https://doi.org/10.1016/j.rvsc.2020.06.014

Dubey, J. P., Murata, F.H.A., Cerqueira-Cézar, C.K., Kwok, O.C.H., Su, C., 2020c. Economic and public health importance of *Toxoplasma gondii* infections in sheep: 2009–2020. Vet. Parasitol. 286, 109195. https://doi.org/10.1016/j.vetpar.2020.109195

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

EFSA, ECDC, 2018. The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2017. EFSA J. 16, e05500. https://doi.org/10.2903/j.efsa.2018.5500

Emelia, O., Rahana, A.R., Mohamad Firdaus, A., Cheng, H.S., Nursyairah, M.S., Fatinah, A.S., Azmawati, M.N., Siti, N.A.M., Aisah, M.Y., 2014. IgG avidity assay: A tool for excluding acute toxoplasmosis in prolonged IgM titer sera from pregnant women. Trop. Biomed. 31, 633–640.

Erdman, L.K., Kain, K.C., 2008. Molecular diagnostic and surveillance tools for global malaria control. Travel Med. Infect. Dis. 6, 82–99. https://doi.org/10.1016/j.tmaid.2007.10.001

Fábrega, L., Restrepo, C.M., Torres, A., Smith, D., Chan, P., Pérez, D., Cumbrera, A., Caballero E., Z., 2020. Frequency of *Toxoplasma gondii* and risk factors associated with the infection in stray dogs and cats of Panama. Microorganisms 8, 927. https://doi.org/10.3390/microorganisms8060927

Feldmann, H., Czub, M., Jones, S., Dick, D., Garbutt, M., Grolla, A., Artsob, H., 2002. Emerging and re-emerging infectious diseases. Med. Microbiol. Immunol. 191, 63–74. https://doi.org/10.1007/s00430-002-0122-5

Ferra, B., Holec-Gąsior, L., Grążlewska, W., 2020. *Toxoplasma gondii* recombinant antigens in the serodiagnosis of toxoplasmosis in domestic and farm animals. Animals 10, 1–27. https://doi.org/10.3390/ani10081245

Ferreira González, I., Urrútia, G., Alonso-Coello, P., 2011. Systematic reviews and meta-analysis: scientific rationale and interpretation. Rev. Esp. Cardiol. 64, 688–696. https://doi.org/10.1016/j.recesp.2011.03.029

Finbarr Duggan, P., 1992. Time to abolish “gold standard”. Br. Med. J. 304, 1568. https://doi.org/10.1136/bmj.304.6841.1568-b

Frenkel, J.K., Lindsay, D.S., Parker, B.B., Dobesh, M., 2003. Dogs as possible mechanical carriers of *Toxoplasma*, and their fur as a source of infection of young children. Int. J. Infect. Dis. 7, 292–293. https://doi.org/10.1016/S1201-9712(03)90112-3

Freppel, W., Ferguson, D.J.P., Shapiro, K., Dubey, J.P., Puech, P.H., Dumètre, A., 2019. Structure, composition, and roles of the *Toxoplasma gondii* oocyst and sporocyst walls. Cell Surf. 5, 100016. https://doi.org/10.1016/j.tcsw.2018.100016

Frössling, J., Bonnett, B., Lindberg, A., Björkman, C., 2003. Validation of a *Neospora caninum* iscom ELISA without a gold standard. Prev. Vet. Med. 57, 141–153. https://doi.org/10.1016/S0167-5877(02)00216-7

Galat, M., Must, K., Rissanen, K., Jokelainen, P., 2019. Comparison of a commercial modified direct agglutination test and a commercial enzyme-linked immunosorbent assay for screening for antibodies against *Toxoplasma gondii* in naturally exposed domestic cats. Parasitol. Res. 118, 2437–2441. https://doi.org/10.1007/s00436-019-06368-w

Gardner, I.A., Greiner, M., 2006. Receiver-operating characteristic curves and likelihood ratios: improvements over traditional methods for the evaluation and application of veterinary clinical pathology tests. Vet. Clin. Pathol. 35, 8–17.

Gardner, I.A., Greiner, M., Dubey, J.P., 2010. Statistical evaluation of test accuracy studies for *Toxoplasma gondii* in food animal intermediate hosts. Zoonoses Public Health 57, 82–94. https://doi.org/10.1111/j.1863-2378.2009.01281.x

Genchi, M., Vismarra, A., Zanet, S., Morelli, S., Galuppi, R., Cringoli, G., Lia, R., Diaferia, M., Frangipane di Regalbono, A., Venegoni, G., Solari Basano, F., Varcasia, A., Perrucci, S., Musella, V., Brianti, E., Gazzonis, A., Drigo, M., Colombo, L., Kramer, L., 2021. Prevalence and risk factors associated with cat parasites in Italy: a multicenter study. Parasites Vectors 14, 1–11. https://doi.org/10.1186/s13071-021-04981-2

Gibbs, E.P.J., 2014. The evolution of one health: a decade of progress and challenges for the future. Vet. Rec. 174, 85–91. https://doi.org/10.1136/vr.g143

Gilman, R.H., Gonzalez, A.E., Llanos-Zavalaga, F., Tsang, V.C.W., Garcia, H.H., 2012. Prevention and control of *Taenia solium* taeniasis/cysticercosis in Peru. Pathog. Glob. Health 106, 312–318. https://doi.org/10.1179/2047773212Y.0000000045

Godard, B., Schmidtke, J., Cassiman, J.J., Aymé, S., 2003. Data storage and DNA banking for biomedical research: informed consent, confidentiality, quality issues, ownership, return of benefits. A professional perspective. Eur. J. Hum. Genet. 11, 88–122. https://doi.org/10.1038/sj.ejhg.5201114

Gomez, C.A., Budvytyte, L.N., Press, C., Zhou, L., McLeod, R., Maldonado, Y., Montoya, J.G., Contopoulos-Ioannidis, D.G., 2018. Evaluation of three point-of-care tests for detection of *Toxoplasma* immunoglobulin IgG and IgM in the United States: Proof

of concept and challenges. Open Forum Infect. Dis. 5, ofy215. https://doi.org/10.1093/ofid/ofy215

Gondim, L.F.P., Mineo, J.R., Schares, G., 2017. Importance of serological cross-reactivity among *Toxoplasma gondii*, *Hammondia* spp., *Neospora* spp., *Sarcocystis* spp. and *Besnoitia besnoiti*. Parasitology 144, 851–868 https://doi.org/10.1017/S0031182017000063

Goodman, R.E., 2006. Practical and predictive bioinformatics methods for the identification of potentially cross-reactive protein matches. Mol. Nutr. Food Res. 50, 655–660. https://doi.org/10.1002/mnfr.200500277

Gutiérrez-Expósito, D., Ferre, I., Ortega-Mora, L.M., Álvarez-García, G., 2017. Advances in the diagnosis of bovine besnoitiosis: current options and applications for control. Int. J. Parasitol. 47, 737–751. https://doi.org/10.1016/j.ijpara.2017.08.003

Hatam-Nahavandi, K., Calero-Bernal, R., Rahimi, M.T., Pagheh, A.S., Zarean, M., Dezhkam, A., Ahmadpour, E., 2021. *Toxoplasma gondii* infection in domestic and wild felids as public health concerns: a systematic review and meta-analysis. Sci. Rep. 11, 1–11. https://doi.org/10.1038/s41598-021-89031-8

Hazra, A., Gogtay, N., 2017. Biostatistics series module 7: the statistics of diagnostic tests. Indian J. Dermatol. 62, 18. https://doi.org/10.4103/0019-5154.198047

Hemelaar, J., 2012. The origin and diversity of the HIV-1 pandemic. Trends Mol. Med. 18, 182–192. https://doi.org/10.1016/j.molmed.2011.12.001

Hoffmann, S., Batz, M.B., Morris, J.G., 2012. Annual cost of illness and quality-adjusted life year losses in the United States due to 14 foodborne pathogens. J. Food Prot. 75, 1292–1302. https://doi.org/10.4315/0362-028X.JFP-11-417

Holec-Gąsior, L., Ferra, B., Hiszczyńska-Sawicka, E., Kur, J., 2014. The optimal mixture of *Toxoplasma gondii* recombinant antigens (GRA1, P22, ROP1) for diagnosis of ovine toxoplasmosis. Vet. Parasitol. 206, 146–152. https://doi.org/10.1016/j.vetpar.2014.09.018

Holec-Gąsior, L., Ferra, B., Grądzlewska, W., 2019. *Toxoplasma gondii* tetravalent chimeric proteins as novel antigens for detection of specific immunoglobulin G in sera of small ruminants. Animals 9, 1146. https://doi.org/10.3390/ani9121146

Horton, R., Lo, S., 2015. Planetary health: a new science for exceptional action. Lancet 386, 1921–1922. https://doi.org/10.1016/S0140-6736(15)61038-8

Hu, J., Ferroglio, E., Trisciuoglio, A., 2011. Immunoblot diagnosis of infection with *Neospora caninum* in cattle based on recombinant NcSAG4 Antigen. Parasitol. Res. 108, 1055–1058. https://doi.org/10.1007/s00436-011-2286-7

Huang, X., Xuan, X., Kimbita, E.N., Battur, B., Miyazawa, T., Fukumoto, S., Mishima, M., Makala, L.H., Suzuki, H., Sugimoto, C., Nagasawa, H., Fujisaki, K., Mikami, T., Igarashi, I., 2002. Development and evaluation of an Enzyme-linked immunosorbent assay with recombinant SAG2 for diagnosis of *Toxoplasma gondii* infection in cats. J. Parasitol. 88, 804–807. https://doi.org/10.1645/0022-

3395(2002)088[0804:DAEOAE]2.0.CO;2

Humboldt-Dachroeden, S., Rubin, O., Sylvester Frid-Nielsen, S., 2020. The state of One Health research across disciplines and sectors – a bibliometric analysis. One Heal. 10, 100146. https://doi.org/10.1016/j.onehlt.2020.100146

Ince, J., McNally, A., 2009. Development of rapid, automated diagnostics for infectious disease: Advances and challenges. Expert Rev. Med. Devices 6, 641–651. https://doi.org/10.1586/erd.09.46

Innes, E.A., Bartley, P.M., Buxton, D., Katzer, F., 2009. Ovine toxoplasmosis. Parasitology 136, 1887–1894. https://doi.org/10.1017/S0031182009991636

Jacobson, R.H., 1998. Validation of serological assays for diagnosis of infectious diseases. OIE Rev. Sci. Tech. 17, 469–486. https://doi.org/10.20506/rst.17.2.1119

Jiang, H.H., Li, M.W., Xu, M.J., Cong, W., Zhu, X.Q., 2015. Prevalence of *Toxoplasma gondii* in dogs in Zhanjiang, Southern China. Korean J. Parasitol. 53, 493–496. https://doi.org/10.3347/kjp.2015.53.4.493

Jittapalapong, S., Nimsupan, B., Pinyopanuwat, N., Chimnoi, W., Kabeya, H., Maruyama, S., 2007. Seroprevalence of *Toxoplasma gondii* antibodies in stray cats and dogs in the Bangkok metropolitan area, Thailand. Vet. Parasitol. 145, 138–141. https://doi.org/10.1016/j.vetpar.2006.10.021

Jittapalapong, S., Sittisan, P., Sakpuaram, T., Kabeya, H., Maruyama, S., Inpankaew, T., 2009. Coinfection of *Leptospira* spp and *Toxoplasma gondii* among stray dogs in Bangkok, Thailand. Southeast Asian J. Trop. Med. Public Health 40, 247–252.

Jittapalapong, S., Inpankaew, T., Pinyopanuwat, N., Chimnoi, W., Kengradomkij, C., Wongnarkpet, S., Maruyama, S., Lekkla, A., Sukthana, Y., 2010. Epidemiology of *Toxoplasma gondii* infection of stray cats in Bangkok, Thailand. Southeast Asian J. Trop. Med. Public Health 41, 13–18.

Kassambara, A., 2017. Practical guide to principal component methods in R (Multivariate Analysis II), first edn. CreateSpace Independent Publishing Platform, Scotts Valley, CA.

Kaufman, E., Lamster, I.B., 2002. The diagnostic applications of saliva — A review. Crit. Rev. Oral Biol. Med. 13, 197–212. https://doi.org/10.1177/154411130201300209

Keusch, G.T., Pappaioanou, M., Gonzalez, M.C., Scott, K.A., Tsai, P., 2009. Sustaining global surveillance and response to emerging zoonotic diseases, first edn. National Academies Press, Washington, D.C, p. 312. https://doi.org/10.17226/12625

Khaitan, T., Kabiraj, A., Bhattacharya, P.T., Ginjupally, U., Jha, H., 2015. Diagnostic efficacy of saliva in oral and systemic health. Int. J. Stomatol. Occlusion Med. 8, 83–86. https://doi.org/10.1007/s12548-015-0133-7

Koutsoumanis, K., Allende, A., Alvarez-Ordóñez, A., Bolton, D., Bover-Cid, S., Chemaly, M., Davies, R., De Cesare, A., Herman, L., Hilbert, F., Lindqvist, R., Nauta, M., Peixe, L., Ru, G., Simmons, M., Skandamis, P., Suffredini, E., Cacciò, S., Chalmers, R.,

Deplazes, P., Devleesschauwer, B., Innes, E., Romig, T., van der Giessen, J., Hempen, M., Van der Stede, Y., Robertson, L., 2018. Public health risks associated with food-borne parasites. EFSA J. 16, e05495. https://doi.org/10.2903/j.efsa.2018.5495

Krecek, R.C., Rabinowitz, P.M., Conrad, P.A., 2020. Demystifying and demonstrating the value of a One Health approach to parasitological challenges. Vet. Parasitol. 287, 109202. https://doi.org/10.1016/j.vetpar.2020.109202

Kruse, H., Kirkemo, A.M., Handeland, K., 2004. Wildlife as source of zoonotic infections. Emerg. Infect. Dis. 10, 2067–2072. https://doi.org/10.3201/eid1012.040707

Laboudi, M., Sadak, A., 2017. Serodiagnosis of toxoplasmosis: the effect of measurement of IgG avidity in pregnant women in Rabat in Morocco. Acta Trop. 172, 139–142. https://doi.org/10.1016/j.actatropica.2017.04.008

Lappin, M.R., 2010. Update on the diagnosis and management of *Toxoplasma gondii* infection in cats. Top. Companion Anim. Med. 25, 136–141. https://doi.org/10.1053/j.tcam.2010.07.002

Laurenti, P., Raponi, M., de Waure, C., Marino, M., Ricciardi, W., Damiani, G., 2016. Performance of interferon-γ release assays in the diagnosis of confirmed active tuberculosis in immunocompetent children: a new systematic review and meta-analysis. BMC Infect. Dis. 16, 131. https://doi.org/10.1186/s12879-016-1461-y

Letourneur, O., Gervasi, G., Gaïa, S., Pagès, J., Watelet, B., Jolivet, M., 2001. Characterization of *Toxoplasma gondii* surface antigen 1 (SAG1) secreted from *Pichia pastoris*: evidence of hyper O-glycosylation. Biotechnol. Appl. Biochem. 33, 35. https://doi.org/10.1042/ba20000069

Li, B., Zhong, N., Peng, W., Shang, L., Jin, H., Liu, Q., 2012. Seroprevalence of *Toxoplasma gondii* infection in dogs in Sichuan Province, southwestern China. J. Parasitol. 98, 209–210. https://doi.org/10.1645/GE-2942.1

Li, X., Pomares, C., Peyron, F., Press, C.J., Ramirez, R., Geraldine, G., Cannavo, I., Chapey, E., Levigne, P., Wallon, M., Montoya, J.G., Dai, H., 2019. Plasmonic gold chips for the diagnosis of *Toxoplasma gondii*, CMV, and rubella infections using saliva with serum detection precision. Eur. J. Clin. Microbiol. Infect. Dis. 38, 883–890. https://doi.org/10.1007/s10096-019-03487-1

Lindsay, D.S., Dubey, P., Butler, J.M., Blagburn, B.L., 1997. Mechanical transmission of *Toxoplasma gondii* oocysts by dogs. Vet. Parasitol. 73, 27–33. https://doi.org/10.1016/s0304-4017(97)00048-4

Lindsay, D.S., Dubey, J.P., Duncan, R.B., 1999. Confirmation that the dog is a definitive host for *Neospora caninum*. Vet. Parasitol. 82, 327–333. https://doi.org/10.1016/S0304-4017(99)00054-0

Lindsay, D.S., Dubey, J.P., 2020. Neosporosis, toxoplasmosis, and sarcocystosis in ruminants: an update. Vet. Clin. North Am. - Food Anim. Pract. 36, 205–222. https://doi.org/10.1016/j.cvfa.2019.11.004

Lisboa Bastos, M., Tavaziva, G., Abidi, S.K., Campbell, J.R., Haraoui, L.P., Johnston, J.C., Lan, Z., Law, S., MacLean, E., Trajman, A., Menzies, D., Benedetti, A., Khan, F.A.,

2020. Diagnostic accuracy of serological tests for covid-19: systematic review and meta-analysis. BMJ 370, m2516. https://doi.org/10.1136/bmj.m2516

Liu, Y., He, G., Cheng, Z., Qi, Y., Liu, J., Zhang, H., Liu, G., Shi, D., Yang, D., Wang, S., Wang, Z., 2012. Seroprevalence of *Toxoplasma gondii* in dogs in Shandong, Henan, and Heilongjiang Provinces, and in the Xinjiang Uygur Autonomous Region, People's Republic of China. J. Parasitol. 98, 211–212. https://doi.org/10.1645/GE-2892.1

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

Liyanage, K.L.D.T.D., Wiethoelter, A., Hufschmid, J., Jabbar, A., 2021. Descriptive comparison of elisas for the detection of *Toxoplasma gondii* antibodies in animals: a systematic review. Pathogens 10, 605. https://doi.org/10.3390/pathogens10050605

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

Mahittikorn, A., Thammasonthijarern, N., Roobthaisong, A., Udonsom, R., Popruk, S., Siri, S., Mori, H., Sukthana, Y., 2017. Development of a loop-mediated isothermal amplification technique and comparison with quantitative real-time PCR for the rapid visual detection of canine neosporosis. Parasit. Vectors 10, 394. https://doi.org/10.1186/s13071-017-2330-2

Marti, M., Li, Y., Köhler, P., Hehl, A.B., 2002. Conformationally correct expression of membrane-anchored *Toxoplasma gondii* SAG1 in the primitive protozoan *Giardia duodenalis*. Infect. Immun. 70, 1014–1016. https://doi.org/10.1128/IAI.70.2.1014-1016.2002

McHugh, M.L., 2012. Interrater reliability: the *kappa* statistic. Biochem. medica 22, 276–82.

Nagai, K., Horita, N., Yamamoto, M., Tsukahara, T., Nagakura, H., Tashiro, K., Shibata, Y., Watanabe, H., Nakashima, K., Ushio, R., Ikeda, M., Narita, A., Kanai, A., Sato, T., Kaneko, T., 2016. Diagnostic test accuracy of loop-mediated isothermal amplification assay for *Mycobacterium tuberculosis*: systematic review and meta-analysis. Sci. Rep. 6, 3–9. https://doi.org/10.1038/srep39090

Narrod, C., Zinsstag, J., Tiongco, M., 2012. One health framework for estimating the economic costs of zoonotic diseases on society. Ecohealth 9, 150–162. https://doi.org/10.1007/s10393-012-0747-9

Okello, A.L., Gibbs, E.P.J., Vandersmissen, A., Welburn, S.C., 2011. One Health and the neglected zoonoses: turning rhetoric into reality. Vet. Rec. 169, 281–285.

https://doi.org/10.1136/vr.d5378

Page, M.J., McKenzie, J.E., Bossuyt, P.M., Boutron, I., Hoffmann, T.C., Mulrow, C.D., Shamseer, L., Tetzlaff, J.M., Akl, E.A., Brennan, S.E., Chou, R., Glanville, J., Grimshaw, J.M., Hróbjartsson, A., Lalu, M.M., Li, T., Loder, E.W., Mayo-Wilson, E., McDonald, S., McGuinness, L.A., Stewart, L.A., Thomas, J., Tricco, A.C., Welch, V.A., Whiting, P., Moher, D., 2021. The PRISMA 2020 statement: an updated guideline for reporting systematic reviews. PLoS Med. 18, e1003583. https://doi.org/10.1371/journal.pmed.1003583

Parra, M.D., Martínez-Subiela, S., Cerón, J.J., 2004. Fluorometría en tiempo retardado: conceptos generales y aplicaciones en medicina veterinaria. An. Vet. Murcia 20, 35–47.

Parra, M.D., Cerón, J.J., 2007. Effects of haemolysis, lipaemia and bilirubinaemia in canine C-reactive protein and haptoglobin determination by time-resolved fluorometry: short communication. Acta Vet. Hung. 55, 295–299. https://doi.org/10.1556/AVet.55.2007.3.3

Pereira, H.S., Tavares e Almeida, L., Fernandes, V., Senra, R.L., Fontes, P.P., Bittar, E.R., de Oliveira Barros Ribon, A., Rotta, P.P., Menezes-Souza, D., Bittar, J.F.F., de Oliveira Mendes, T.A., 2020. Chimeric protein designed by genome-scale immunoinformatics enhances serodiagnosis of bovine neosporosis. J. Clin. Microbiol. 58, 1–10. https://doi.org/10.1128/JCM.01343-19

Pinheiro, A.F., Borsuk, S., Berne, M.E.A., Da Pinto, L.S., Andreotti, R., Roos, T., Roloff, B.C., Leite, F.P.L., 2015. Utilização de um ELISA baseado em NcSRS2 de *Neospora caninum* expressa em *Pichia pastoris* para diagnóstico de neosporose em ovinos e cães. Rev. Bras. Parasitol. Vet. 24, 148–154. https://doi.org/10.1590/S1984-29612015015

Polo, T.C.F., Miot, H.A., 2020. Use of ROC curves in clinical and experimental studies. J. Vasc. Bras. 19, 1–4. https://doi.org/10.1590/1677-5449.200186

Pritchard, G., 2001. Milk antibody testing in cattle. In Pract. 23, 542–549. https://doi.org/10.1136/inpract.23.9.542

Qian, W.F., Yan, W.C., Wang, T.Q., Zhai, K., Han, L.F., Lv, C.C., 2015. Prevalence and genetic characterization of *Toxoplasma gondii* in pet dogs in central China. Korean J. Parasitol. 53, 125–128. https://doi.org/10.3347/kjp.2015.53.1.125

Rabinowitz, P.M., Natterson-Horowitz, B.J., Kahn, L.H., Kock, R., Pappaioanou, M., 2017. Incorporating One Health into medical education. BMC Med. Educ. 17, 1–7. https://doi.org/10.1186/s12909-017-0883-6

Rahmanian, V., Rahmanian, K., Jahromi, A., Bokaie, S., 2020. Seroprevalence of *Toxoplasma gondii* infection: an umbrella review of updated systematic reviews and meta-analyses. J. Fam. Med. Prim. Care 9, 3848. https://doi.org/10.4103/jfmpc.jfmpc_753_20

Reichel, M.P., Alejandra Ayanegui-Alcérreca, M., Gondim, L.F.P., Ellis, J.T., 2013. What is the global economic impact of *Neospora caninum* in cattle - the billion dollar

question. Int. J. Parasitol. 43, 133–142. https://doi.org/10.1016/j.ijpara.2012.10.022

Ribeiro, C.M., Soares, I.R., Mendes, R.G., de Santis Bastos, P.A., Katagiri, S., Zavilenski, R.B., de Abreu, H.F.P., Afreixo, V., 2019. Meta-analysis of the prevalence and risk factors associated with bovine neosporosis. Trop. Anim. Health Prod. 51, 1783–1800. https://doi.org/10.1007/s11250-019-01929-8

Robert-Gangneux, F., Dardé, M.L., 2012. Epidemiology of and diagnostic strategies for toxoplasmosis. Clin. Microbiol. Rev. 25, 264–296. https://doi.org/10.1128/CMR.05013-11

Robertson, B.H., Nicholson, J.K.A., 2005. New microbiology tools for public health and their implications. Annu. Rev. Public Health 26, 281–302. https://doi.org/10.1146/annurev.publhealth.26.021304.144522

Rodrigues, A.A., Reis, S.S., Sousa, M.L. de, Moraes, E. da S., Garcia, J.L., Nascimento, T.V.C., Cunha, I.A.L. da, 2020. A systematic literature review and meta-analysis of risk factors for *Neospora caninum* seroprevalence in goats. Prev. Vet. Med. 185, 105176. https://doi.org/10.1016/j.prevetmed.2020.105176

Romanelli, P.R., Caldart, E.T., Martins, F.D.C., Martins, C.M., de Matos, A.M.R.N., Pinto-Ferreira, F., Mareze, M., Mitsuka-Breganó, R., Freire, R.L., Navarro, I.T., 2021. Seroprevalence and associated risk factors of ovine neosporosis worldwide: a systematic review and meta-analysis. Semin. Agrar. 42, 2111–2125. https://doi.org/10.5433/1679-0359.2021v42n3Supl1p2111

Rostami, A., Karanis, P., Fallahi, S., 2018. Advances in serological, imaging techniques and molecular diagnosis of *Toxoplasma gondii* infection. Infection 46, 303–315. https://doi.org/10.1007/s15010-017-1111-3

Rostami, A., Riahi, S.M., Contopoulos-Ioannidis, D.G., Gamble, H.R., Fakhri, Y., Shiadeh, M.N., Foroutan, M., Behniafar, H., Taghipour, A., Maldonado, Y.A., Mokdad, A.H., Gasser, R.B., 2019. Acute *Toxoplasma* infection in pregnant women worldwide: A systematic review and meta-analysis. PLoS Negl. Trop. Dis. 13, 1–20. https://doi.org/10.1371/journal.pntd.0007807

Ruffolo, B.B., Toledo, R. dos S., Martins, F.D.C., Bugni, F.M., da Costa, L., Marana, E.R.M., Navarro, I.T., Garcia, J.L., Su, C., Freire, R.L., 2016. Isolation and genotyping of *Toxoplasma gondii* in seronegative urban rats and presence of antibodies in communicating dogs in Brazil. Rev. Inst. Med. Trop. Sao Paulo 58, 28. https://doi.org/10.1590/S1678-9946201658028

Salman, D., Pumidonming, W., Oohashi, E., Igarashi, M., 2018. Prevalence of *Toxoplasma gondii* and other intestinal parasites in cats in Tokachi sub-prefecture, Japan. J. Vet. Med. Sci. 80, 960–967. https://doi.org/10.1292/jvms.17-0713

Samaranayake, L., 2007. Saliva as a diagnostic fluid. Int. Dent. J. 57, 295–299. https://doi.org/10.1111/j.1875-595X.2007.tb00135.x

Sánchez-Sánchez, R., Vázquez, P., Ferre, I., Ortega-Mora, L.M., 2018. Treatment of toxoplasmosis and neosporosis in farm ruminants: state of knowledge and future

trends. Curr. Top. Med. Chem. 18, 1304–1323. https://doi.org/10.2174/1568026618666181002113617

Sánchez-Sánchez, R., Vázquez-Calvo, Á., Fernández-Escobar, M., Regidor-Cerrillo, J., Benavides, J., Gutiérrez, J., Gutiérrez-Expósito, D., Crespo-Ramos, F.J., Ortega-Mora, L.M., Álvarez-García, G., 2021. Dynamics of *Neospora caninum*-associated abortions in a dairy sheep flock and results of a test-and-cull control programme. Pathogens 10, 1518. https: //doi.org/10.3390/pathogens10111518

Savvides, N., 2013. Living with dogs: alternative animal practices in Bangkok, Thailand. Anim. Stud. J. 2, 28–50.

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

Schmiege, D., Maria, A., Arredondo, P., Ntajal, J., Minetto, J., Paris, G., Koissi, M., Patel, K., Yasobant, S., 2020. One Health in the context of coronavirus outbreaks : A systematic literature review. One Heal. 10, 100170. https://doi.org/10.1016/j.onehlt.2020.100170

Schober, P., Schwarte, L.A., 2018. Correlation coefficients: appropriate use and interpretation. Anesth. Analg. 126, 1763–1768. https://doi.org/10.1213/ANE.0000000000002864

Schweon, S.J., Vitale, L.J., 2020. Zoonoses: preventing disease transmission from animals. Nursing (Lond). 50, 13–16. https://doi.org/10.1097/01.NURSE.0000697200.87222.94Seefeldt, S.L., Kirkbride, C.A., Dubey, J.P., 1989. Comparison of Enzyme-linked immunosorbent assay, indirect fluorescent antibody test, and direct agglutination test for detecting *Toxoplasma gondii* antibodies in naturally aborted ovine fetuses. J. Vet. Diagnostic Investig. 1, 124–127. https://doi.org/10.1177/104063878900100206

Silaghi, C., Knaus, M., Rapti, D., Kusi, I., Shukullari, E., Hamel, D., Pfister, K., Rehbein, S., 2014. Survey of *Toxoplasma gondii* and *Neospora caninum*, haemotropic mycoplasmas and other arthropod-borne pathogens in cats from Albania. Parasites Vectors 7, 62. https://doi.org/10.1186/1756-3305-7-62 1–10.

Silva, R., Machado, G., 2016. Canine neosporosis: perspectives on pathogenesis and management. Vet. Med. Res. Reports 2016:7, 59–70. https://doi.org/10.2147/vmrr.s76969

Sinnott, F.A., Monte, L.G., Collares, T.F., Silveira, R.M., Borsuk, S., 2017. Review on the immunological and molecular diagnosis of neosporosis (years 2011–2016). Vet. Parasitol. 239, 19–25. https://doi.org/10.1016/j.vetpar.2017.04.008

Smets, A., Fauchier, T., Michel, G., Marty, P., Pomares, C., 2016. Comparison of *Toxoplasma gondii* IgG avidity Architect and Vidas assays with the estimated date of infection in pregnant women. Parasite 23, 45.

https://doi.org/10.1051/parasite/2016056

Sokol-Borrelli, S.L., Coombs, R.S., Boyle, J.P., 2020. A comparison of stage conversion in the coccidian Apicomplexans *Toxoplasma gondii*, *Hammondia hammondi*, and *Neospora caninum*. Front. Cell. Infect. Microbiol. 10, 1–14. https://doi.org/10.3389/fcimb.2020.608283

Sroka, J., Cencek, T., Ziomko, I., Karamon, J., Zwoliński, J., 2008. Preliminary assessment of ELISA, MAT, and LAT for detecting *Toxoplasma gondii* antibodies in pigs. Bull. Vet. Inst. Pulawy 52, 545–549.

Suijkerbuijk, A.W.M., van Gils, P.F., Bonačić Marinović, A.A., Feenstra, T.L., Kortbeek, L.M., Mangen, M.J.J., Opsteegh, M., de Wit, G.A., van der Giessen, J.W.B., 2018. The design of a Social Cost-Benefit Analysis of preventive interventions for toxoplasmosis: an example of the One Health approach. Zoonoses Public Health 65, 185–194. https://doi.org/10.1111/zph.12417

Sukhumavasi, W., Bellosa, M.L., Lucio-Forster, A., Liotta, J.L., Lee, A.C.Y., Pornmingmas, P., Chungpivat, S., Mohammed, H.O., Lorentzen, L., Dubey, J.P., Bowman, D.D., 2012. Serological survey of *Toxoplasma gondii*, *Dirofilaria immitis*, Feline Immunodeficiency Virus (FIV) and Feline Leukemia Virus (FeLV) infections in pet cats in Bangkok and vicinities, Thailand. Vet. Parasitol. 188, 25–30. https://doi.org/10.1016/j.vetpar.2012.02.021

Sun, X., Ji, Y., Liu, X., Xiang, M., He, G., Xie, L., Suo, J., Suo, X., 2017. Improvement and Evaluation of Loop-mediated isothermal amplification for rapid detection of *Toxoplasma gondii* infection in human blood samples. PLoS One 12, e0169125. https://doi.org/10.1371/journal.pone.0169125

Tamponi, C., Varcasia, A., Pipia, A.P., Zidda, A., Panzalis, R., Dore, F., Dessì, G., Sanna, G., Salis, F., Björkman, C., Scala, A., 2015. ISCOM ELISA in milk as screening for *Neospora caninum* in dairy sheep. Large Anim. Rev. 21, 213–216.

Tenter, A.M., Vietmeyer, C., Johnson, A.M., Janitschke, K., Rommel, M., Lehmacher, W., 1994. ELISAs based on recombinant antigens for sero-epidemiological studies on *Toxoplasma gondii* infections in cats. Parasitology 109, 29–36. https://doi.org/10.1017/S0031182000077738

Thebault, A., Kooh, P., Cadavez, V., Gonzales-Barron, U., Villena, I., 2021. Risk factors for sporadic toxoplasmosis: a systematic review and meta-analysis. Microb. Risk Anal. 17, 100133. https://doi.org/10.1016/j.mran.2020.100133

Thompson, R.C.A., 2013. Parasite zoonoses and wildlife: One health, spillover andhuman activity. Int. J. Parasitol. 43, 1079–1088. https://doi.org/10.1016/j.ijpara.2013.06.007

Thrusfield, M., Christley, R., Brown, H., Diggle, P.J., French, N., Howe, K., Kelly, L., O'Connor, A., Sargeant, J., Wood, H., 2018. Veterinary Epidemiology, fourth edn. John Wiley & Sons, Ltd, Chichester, UK, p.864. https://doi.org/10.1002/9781118280249

Tiwari, R., Dhama, K., Sharun, K., Yatoo, M.I., Malik, S., Singh, R., Michalak, I., Sah, R., Katterine, D., Rodriguez-morales, A.J., 2020. COVID-19 : animals , veterinary and zoonotic links. Vet. Q. 40, 169–182. https://doi.org/10.1080/01652176.2020.1766725

Toukhsati, S.R., Phillips, C.J.C., Podberscek, A.L., Coleman, G.J., 2015. Companion animals in Thailand: human factors that predict sterilization of cats and dogs. Soc. Anim. 23, 569–593. https://doi.org/10.1163/15685306-12341381

Trees, A.J., Williams, D.J.L., 2005. Endogenous and exogenous transplacental infection in *Neospora caninum* and *Toxoplasma gondii*. Trends Parasitol. 21, 558–561. https://doi.org/10.1016/j.pt.2005.09.005

Trotta, M., Borchi, B., Zammarchi, L., Sterrantino, G., Brogi, M., Kiros, S.T., Lorini, C., Bonaccorsi, G., Colao, M.G., Bartoloni, A., 2016. Immunoglobulin M indirect-fluorescent antibody test for the diagnosis of acute toxoplasmosis during pregnancy in the avidity era: A 14-year experience at the Tuscany Reference Center for Infectious Diseases in Pregnancy, Florence, Italy. J. Obstet. Gynaecol. Res. 42, 1699–1703. https://doi.org/10.1111/jog.13144

Uddin, A.H.M.M., Hossain, D., Ahsan, M.I., Atikuzzaman, M., Karim, M.R., 2021. Review on diagnosis and molecular characterization of *Toxoplasma gondii* in humans and animals. Trop. Biomed. 38, 511–539. https://doi.org/10.47665/tb.38.4.091

Valinetz, E.D., Cangelosi, G.A., 2021. A look inside: oral sampling for detection of non-oral infectious diseases. J. Clin. Microbiol. 59, 10. https://doi.org/10.1128/JCM.02360-20

Varcasia, A., Capelli, G., Ruiu, A., Ladu, M., Scala, A., Bjorkman, C., 2006. Prevalence of *Neospora caninum* infection in Sardinian dairy farms (Italy) detected by iscom ELISA on tank bulk milk. Parasitol. Res. 98, 264–267. https://doi.org/10.1007/s00436-005-0044-4

Velmurugan, G.V., Tewari, A.K., Rao, J.R., Baidya, S., Kumar, M.U., Mishra, A.K., 2008. High-level expression of SAG1 and GRA7 gene of *Toxoplasma gondii* (Izatnagar isolate) and their application in serodiagnosis of goat toxoplasmosis. Vet. Parasitol. 154, 185–192. https://doi.org/10.1016/j.vetpar.2008.03.032Wang, F., Zhang, W., Niu, D., 2021. Editorial: foodborne Enterobacteriaceae of animal origin. Front. Cell. Infect. Microbiol. 11, 10–12. https://doi.org/10.3389/fcimb.2021.772359

Wang, S., Zhou, Y.H., Xie, Q., Zheng, B., Zhang, H.Z., Yao, Z.J., Wang, D., Liu, S.G., Zhang, Z.C., 2017. Prevalence of *Toxoplasma gondii* antibodies in dogs in central China. Isr. J. Vet. Med. 72, 11–15.

Whiting, P.F., 2011. QUADAS-2: a revised tool for the quality assessment of diagnostic accuracy studies. Ann. Intern. Med. 155, 529. https://doi.org/10.7326/0003-4819-155-8-201110180-00009

WHO, 2016. Recommended selection criteria for procurement of malaria rapid diagnostic tests. World Heal. Organ. 16. Available in:

https://apps.who.int/iris/bitstream/handle/10665/259870/WHO-CDS-GMP-2018.01-eng.pdf?sequence=21&isAllowed=y

Williams, D.J.L., Hartley, C.S., Björkman, C., Trees, A.J., 2009. Endogenous and exogenous transplacental transmission of *Neospora caninum* - How the route of transmission impacts on epidemiology and control of disease. Parasitology 136, 1895–1900. https://doi.org/10.1017/S0031182009990588

World Organisation for Animal Health (OIE), 2019a. Principles and methods of validation of diagnostic assays for infectious diseases, in: OIE Manual of Diagnostic Tests for Aquatic Animals s (the Aquatic Manual). Available in: https://www.oie.int/fileadmin/Home/eng/Health_standards/aahm/current/chapitre_validation_diagnostics_assays.pdf

World Organisation for Animal Health (OIE), 2019b. World Animal Health Information Database (WAHIS Interface): Animal population. Available in: https://www.oie.int/wahis_2/public/wahid.php/Countryinformation/Animalpopulation

Wyrosdick, H.M., Schaefer, J.J., 2015. *Toxoplasma gondii*: history and diagnostic test development. Anim. Heal. Res. Rev. 16, 150–162. https://doi.org/10.1017/S1466252315000183

Xie, Y., Ma, Y., Cai, L., Jiang, S., Li, C., 2022. Reconsidering meat intake and human health: a review of current research. Mol. Nutr. Food Res. 2022, 2101066. https://doi.org/10.1002/mnfr.202101066

Ybañez, R.H.D., Kyan, H., Nishikawa, Y., 2020a. Detection of antibodies against *Toxoplasma gondii* in cats using an immunochromatographic test based on GRA7 antigen. J. Vet. Med. Sci. 82, 441–445. https://doi.org/10.1292/jvms.19-0654

Ybañez, R.H.D., Ybañez, A.P., Nishikawa, Y., 2020b. Review on the current trends of toxoplasmosis serodiagnosis in humans. Front. Cell. Infect. Microbiol. 10, 1–18. https://doi.org/10.3389/fcimb.2020.00204

Yin, Q., El-Ashram, S., Liu, X.Y., Suo, X., 2015. Early detection of *Toxoplasma gondii*-infected cats by interferon-gamma release assay. Exp. Parasitol. 157, 145–149. https://doi.org/10.1016/j.exppara.2015.08.015

Zarra-Nezhad, F., Borujeni, M.P., Mosallanejad, B., Hamidinejat, H., 2017. A seroepidemiological survey of *Toxoplasma gondii* infection in referred dogs to Veterinary Hospital of Ahvaz, Iran. Int. J. Vet. Sci. Med. 5, 148–151. https://doi.org/10.1016/j.ijvsm.2017.08.006

Zhang, Z.L., Hou, Y.L., Li, D.T., Li, F.Z., 2021. Diagnostic efficacy of anti-SARS-CoV-2 IgG/IgM test for COVID-19: A meta-analysis. J. Med. Virol. 93, 366–374. https://doi.org/10.1002/jmv.26211

Zhou, N., Fu, H., Wang, Z., Shi, H., Yu, Y., Qu, T., Wang, Longlong, Zhang, X., Wang, Lin, 2019. Seroprevalence and risk factors of *Toxoplasma gondii* infection in children with leukemia in Shandong Province, Eastern China: A case-control prospective study. PeerJ 7, e6604. https://doi.org/10.7717/peerj.6604

Zhuo, X., Sun, H., Zhang, Z., Luo, J., Shan, Y., Du, A., 2017. Development and application of an Indirect Enzyme-linked immunosorbent assay using recombinant MAG1 for serodiagnosis of *Toxoplasma gondii* in dogs. J. Parasitol. 103, 237–242. https://doi.org/10.1645/16-89

Zulpo, D.L., Sammi, A.S., dos Santos, J.R., Sasse, J.P., Martins, T.A., Minutti, A.F., Cardim, S.T., de Barros, L.D., Navarro, I.T., Garcia, J.L., 2018. *Toxoplasma gondii*: A study of oocyst re-shedding in domestic cats. Vet. Parasitol. 249, 17–20. https://doi.org/10.1016/j.vetpar.2017.10.021

APPENDIX I

Scientific publications at international conferences related to the thesis project:

1. **Title:** Diseño de nuevas técnicas de diagnóstico para la detección de *Toxoplasma gondii* y *Neospora caninum* en el campo de la veterinaria.
   Authors: Huertas López, Ana; Álvarez García, Gema; Martínez Subiela, Silvia; Cerón Madrigal, José Joaquín; Martínez-Carrasco Pleite, Carlos.
   Conference: IV Jornadas Doctorales de la Universidad de Murcia
   Organizer: Escuela Internacional de Doctorado de la Universidad de Murcia
   Type of publication: oral communication.
   Date: 29/05/2018
   DOI: 10.6018/editum.2736
   Available in: https://publicaciones.um.es/publicaciones/public/obras/ficha.seam?numero=2736&edicion=1
2. **Title:** Design and validation of a time-resolved fluorescence immunoassay for the detection of anti- *Neospora caninum* antibodies in sheep.
   Authors: Huertas López, Ana; Álvarez García, Gema; Cerón, José Joaquín; Martínez-Carrasco, Carlos; Martínez Subiela, Silvia.
   Conference: The 19th International Symposium of the World Association of Veterinary Laboratory Diagnosticians (ISWAVLD 2019) and OIE Seminar
   Organizer: World Association of Veterinary Laboratory Diagnosticians (WAVLD)
   Type of publication: poster presentation.
   Date: 19/06/2019 - 22/06/2019
   Available in: https://www.tavld.org/iswavld/abstract-book.pdf
3. **Title:** The systematic review and meta-analysis on the serological diagnosis of *Toxoplasma gondii* infection highlights the lack of a One health approach.
   Authors: Huertas López, Ana; Martínez-Carrasco Pleite, Carlos; Sánchez,Roberto; Cantos Barreda, Ana; Ibáñez López, Francisco Javier; Martínez Subiela,Silvia; Cerón, José Joaquín; Álvarez García, Gema.
   Conference: VI Jornadas Doctorales de la Universidad de Murcia
   Organizer: Escuela Internacional de Doctorado de la Universidad de Murcia

Type of publication: oral communication.

Date: 29/05/2021 - 31/05/2021

4. **Title:** Analytical validation of a new time-resolved fluorescence immunoassay based on NcGRA7 for detection of anti-*Neospora caninum* antibodies in sheep milk.

   Authors: Huertas López, Ana; Martínez Subiela, Silvia; Álvarez García, Gema; Martínez-Carrasco, Carlos; Sánchez, Roberto; Cerón, José Joaquín.

   Conference: EAVLD Virtual meeting 2021

   Organizer: European Association of Veterinary Laboratory Diagnosticians (EAVLD)

   Type of publication: poster presentation.

   Date: 17/11/2021

APPENDIX II

Awards related to the thesis project:

- Best communication of a PhD thesis in the field of health sciences awardin the contest “Thesis in 3 minutes”, organized by the University of Murcia (Unidad de Cultura Científica). 10/12/2020
  https://www.youtube.com/watch?v=p1XIhvmdHng
- Finalist in the “I Edition of the contest #HiloTesis” organized by the Spanish University Rectors' Conference (CRUE) and the University of Murcia (Unidad de Cultura Científica). 02/07/2021
  https://twitter.com/AnaHuertasVet/status/1385574783572664325?s=20