



A systematic review and meta-analysis of the validation of serological methods for detecting anti-*Toxoplasma gondii* antibodies in humans and animals

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ABSTRACT

Toxoplasma gondii is a paradigmatic zoonotic parasite from the One Health perspective, since it is broadly distributed and virtually infects all warm-blooded species. A wide variety of serological techniques have been developed to detect *T. gondii* infection in humans and animals. Our aim was to describe and compare the main characteristics of these serological tests and validation processes and to critically analyze whether these tests meet the standards required to ensure an accurate serological diagnosis. The current systematic review and meta-analysis included 134 studies that were published from 2013 to 2023. QUADAS 2 tool was used to evaluate the quality of the included studies. A total of 52 variables related to the characteristics of the techniques and analytical and diagnostic validation parameters were studied. A wider panel of tests was developed for humans, including techniques exclusively developed for humans that involve costly equipment and the measurement of different Ig isotypes that are considered biomarkers of congenital toxoplasmosis. Studies conducted in humans frequently employed commercial techniques as reference tests, measured different immunoglobulin isotypes with a predominance for IgG (>50%) and discriminated between acute and chronic infections. In animals, the most commonly used reference techniques were in-house tests, which almost exclusively detected IgG. Common limitations identified in a large number of studies were some misunderstandings of the terms “gold standard” and “reference test” and the absence of information about the negative and positive control sera used or the exact cutoff employed, which were independent of the quality of the study. There is a lack of analytical validation, with few evaluations of cross-reactivity with other pathogens. Diagnostic odds ratio values showed that indirect ELISA based on native or chimeric antigens performed better than other tests. The reproducibility of serological test results in both humans and animals is not guaranteed due to a lack of relevant information and analytical validation. Thus, several key issues should be considered in the future, including interlaboratory ring trials.

1. Introduction

Toxoplasmosis is a zoonotic disease caused by the apicomplexan parasite *Toxoplasma gondii* (Dubey, 2021), whose importance in terms of public and animal health has been widely discussed (Hoffmann et al., 2012; EFSA and ECDC, 2018; Koutsoumanis et al., 2018; Stelzer et al.,

2019). *Toxoplasma gondii* is a paradigmatic pathogen to be addressed from a One Health approach, since it is a globally distributed parasite, that affects a wide variety of warm-blooded host species; furthermore, *T. gondii* has several transmission routes. Humans and other host species can become infected by consuming raw meat containing *T. gondii* cysts (meat route), by ingesting oocysts excreted in cat feces that contaminate

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water, vegetables, and soil (environmental route), by the transplacental route (congenital infection) or less frequently by blood transfusion (Cook et al., 2000; Koutsoumanis et al., 2018; Aguirre et al., 2019; Attias et al., 2020). Thus, an interdisciplinary approach is needed to control this parasitic infection in humans, animals and the environment (Suijkerbuijk et al., 2018; Aguirre et al., 2019).

Serological techniques, which are easy to use and cost-effective, facilitate the monitoring of *T. gondii* infection and, consequently, the design and implementation of appropriate intervention strategies (Ince and McNally, 2009; Wyrosdick and Schaefer, 2015; Thrusfield et al., 2018). In humans, accurate tests are required to monitor *T. gondii* infection in pregnant women so that early treatment can be provided and acute infection can be prevented. These methods are also useful for diagnosing postnatal infections and providing information for the differential diagnosis of infections in immunosuppressed patients (Liu et al., 2015; Wyrosdick and Schaefer, 2015). In animals, serological techniques can be employed to diagnose *T. gondii*-associated abortion outbreaks in small ruminants (Dubey et al., 2020a; Dubey et al., 2020b; Lindsay and Dubey, 2020), to detect potentially infected animals that may harbor tissue cysts and that may pose a risk to humans through the consumption of raw or undercooked meat (Aguirre et al., 2019; Dubey et al., 2020a; Dubey et al., 2020b; Hatam-Nahavandi et al., 2021) and to evaluate the level of exposure of the definitive host to this parasite (Dubey et al., 2020c). Therefore, numerous serological techniques for the detection of *T. gondii* infection have been developed, particularly during the past decade (Liu et al., 2015; Wyrosdick and Schaefer, 2015; Ybañez et al., 2020; Uddin et al., 2021) based on different target species, technique formats, or *T. gondii* antigens. A recently published meta-analysis showed that the most commonly employed reference techniques in humans are enzyme immunoassays (EIAs); in animals, the most commonly employed techniques include indirect fluorescent antibody tests (IFATs), EIAs and agglutination techniques (Huertas-López et al., 2023). Huertas et al. (2023) reported that the One Health approach still needs further integration among scientific disciplines, since researchers should be familiar with the approaches followed in serodiagnosis in both humans and animals. In addition, diagnostic laboratories and professionals involved in epidemiological studies should be aware of updated diagnostic performance and validation procedures for available diagnostic tests for each host species that would contribute to improving the control of *T. gondii* infection (Huertas-López et al., 2023). According to the World Organization for Animal Health (WOAH, 2023), all diagnostic techniques should be validated for different target species. Indeed, the absence of species-specific conjugates, especially for wildlife hosts, hampers the validation process (Wyrosdick and Schaefer, 2015). Although different alternatives have been used to validate techniques for these species before they were used in epidemiological studies (Wallander et al., 2014; Elmore et al., 2016; Kornacka et al., 2016), these studies did not normally follow the previously mentioned WOAH recommendations.

Therefore, the present study aimed to provide a detailed understanding of the characteristics of the techniques and to evaluate the processes performed to validate recently developed serological assays for the diagnosis of *T. gondii* infection in human and animal hosts. This study also aimed to identify potential areas of improvement.

2. Materials and methods

2.1. Selection of articles and data extraction

The present study was performed in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement (Page et al., 2021). In brief, the PubMed, Web of Science and Scopus databases were searched from January 1, 2013, and November 20, 2023, using the following Medical Subject Headings (MeSH) terms: “toxoplasma OR toxoplasm*” and “animals OR humans OR wildlife OR companion OR livestock” and “serology OR serologic* OR antibod*” and

“diagnosis OR validation OR accuracy OR comparison OR diagnostic performance OR cutoff”. Then, the titles and abstracts of the articles were screened based on the inclusion criteria (articles that analyzed one or more serological techniques, evaluated a technique for detection of antibodies, were performed in humans and/or animals, had a sample size > 5, had an available full text in English and were not review articles). A total of 244 articles were preliminarily selected. Quality assessment was carried out with the Quality Assessment of Diagnostic Accuracy Studies (QUADAS) 2 tool, and 134 of the 244 studies met the quality criteria, with a low or medium risk of bias (Supplementary File 1). Study selection and quality assessment were performed by three independent researchers.

From the 134 selected articles, data were independently extracted by three researchers using a standardized Excel form (Microsoft® Excel for Microsoft 365 MSO, Version 2307 built 16.0.16626.20170) (Supplementary File 2). Any disagreements were resolved by discussion and consensus. In the present review, we focused on the methodological characteristics of the evaluated techniques and the validation processes. A total of 52 variables were analyzed, and they were related to i) bibliometrics (n=4); ii) study aim (n=3); iii) the studied population (n=4); iv) the type of techniques and reference tests/gold standard (n=15); v) the methodological characteristics of the evaluated serological technique(s) (n=13); vi) the validation steps (n=1); vii) the parameters studied for analytical validation (n=5); and viii) the analysis performed and the parameters studied for diagnostic validation (n=7). All studied variables and their factor labels are detailed in Table 1. Whenever possible, the number of true positives (TPs), true negatives (TNs), false positives (FPs) and false negatives (FNs) obtained by the evaluated technique with regard to the reference technique were also collected from the selected articles.

2.2. Data analysis

Statistical relationships between the “Journal quartile in JCR” (considering this variable as an approximate classification of the quality of the studies), “Final purpose of the evaluated technique (humans)”, “Final purpose of the evaluated technique (animals)”, “Humans or animals”, “Host category”, “Classification of the population (humans)” and “Classification of the population (animals)” variables and the remaining qualitative variables were analyzed by Pearson’s chi-square test when assumptions were met or Fisher’s exact test when they were not. A statistically significant relationship between variables was considered when the *p* value was <0.05 (Supplementary File 3). To cluster the selected articles according to human or animal host, a multiple correspondence analysis (MCA) (Kassambara, 2017) was carried out with the following qualitative variables: humans or animals, evaluated technique, commercial/noncommercial (evaluated technique), reference technique or method, commercial/noncommercial (reference technique), antigen employed, antibody isotype detected, discrimination between acute and chronic phases, analytical and/or diagnostic validation, and agreement. Multiple correspondence analysis was graphically represented for the “Humans or animals” variable. Finally, a diagnostic test accuracy (DTA) meta-analysis was performed in which the pooled diagnostic sensitivity (Se) and specificity (Sp) and diagnostic odds ratio (DOR) of the evaluated techniques were estimated with a random effects model (Cleophas and Zwinderman, 2009; Cota et al., 2012) that included studies with available TP, TN, FP and FN data. To perform the meta-analysis, each of the techniques evaluated in the studies was considered separately and, therefore, included independently in the analysis. If the same technique was subjected to several comparisons (e.g., comparison with different reference techniques or evaluation of different cutoffs), only the TP, TN, FP and FN data with the lowest FP + FN were considered. The evaluated techniques that showed undetermined results regarding the reference technique/s (e.g., in ELISA when two cutoff points are employed (threshold or decision limits)), for which the test results can be reduced to three (positive, intermediate

Table 1
Categories and variables studied from the 134 selected papers.

Category	Variable	Factor label
Bibliometrics	Year of publication	2013–2023
	Journal	*
	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; Medicine, general & internal; Medicine, research & experimental; Urology & nephrology; Zoology; Environmental sciences
Study aim	Journal quartile in JCR	Q1; Q2; Q3; Q4
	Study purpose (according to the methodology employed)	1) Design and/or validation of a new technique; 2) Validation of a previously described technique without any modification; 3) Modification of a previously described technique (with new antigens or secondary antibodies); 4) Technique designed to differentiate between acute and chronic infection; 5) Technique designed to detect more than one pathogen; 6) Technique designed to detect <i>T. gondii</i> in a different type of sample (e.g., blood serum, saliva, urine); 7) Technique designed to detect <i>T. gondii</i> in different host species; and Several.
Studied population	Final purpose of the evaluated technique (humans)	Monitor infection in pregnant women; Monitor infection in children; Monitor infection in immunocompromised patients; Other
	Final purpose of the evaluated technique (humans)	To conduct seroprevalence studies; To detect antibodies in livestock to evaluate the risk of human infection via food
	Humans or animals	Humans; Animals; Both humans and animals
	Host category	Humans; Cats; Dogs; Domestic ruminants; Pigs; Domestic birds; Wild animals (one or several species); Multiple species
Type of techniques and reference test/ Gold standard	Classification of the population (humans)	Pregnant women; Children; Immunocompromised patients; Adults (Immunocompetent, non-pregnant); Several groups, Not specified
	Classification of the population (animals)	Cats; Dogs; Domestic ruminants; Pigs; Domestic birds; Wild animals (one or several species); Multiple species
	Do they evaluate different techniques?	No (they only evaluate one 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
	Evaluated technique	EIA; IFAT; WB; Agglutination test; POC test; Avidity test; Other type; Several types
	Commercial/ Noncommercial (evaluated technique)	Commercial; Noncommercial; Commercial and noncommercial techniques
	Type of EIA (evaluated technique)	Indirect ELISA; Inhibition ELISA; Fluorescent immunosensor with chitosan-ZnO-nanoparticles; Dot-ELISA; CMIA; LIPS; QDNBs-based immunoassay; Indirect ELISA & other EIA
	Type of agglutination test (evaluated technique)	LAT; MAT; IHA; ISAGA
	Type of IgG avidity test (evaluated technique)	EIA; Flow cytometry; EIA and flow cytometry
	Other type (evaluated technique)	Multiplex bead assay; Bead-based assay (only for <i>T. gondii</i> detection); Electrochemical sensing platform; Flow cytometry; Multiplex dot-immunoassay based; SIA; Immuno-dot blot assay; Paper-based ELISA; Peptide microarray; TRFIA; Immuno-PCR; Several techniques
	Reference technique or method	EIA; IFAT; WB; Agglutination test; Dye test; POC test; Avidity test; Clinical criteria; Molecular techniques; Other type; Several types
Methodological characteristics of the evaluated technique/s	Commercial/ Noncommercial (reference technique)	Commercial; Noncommercial; Commercial and noncommercial techniques
	Type of EIA (reference technique)	Indirect ELISA; ELFA; MEIA; CMIA; Indirect ELISA & other EIA
	Type of agglutination test (reference technique)	LAT; MAT; IHA; ISAGA; Differential agglutination AC/HS; Several techniques
	Type of IgG avidity test (reference technique)	EIA; Flow cytometry; EIA and flow cytometry
	“Gold standard” quotation	Yes; No
	Method quoted as gold standard	ELISA; ELISA & IgG Avidity; IFAT; WB; MAT; IHA; Dye test; Molecular techniques; Clinical examination; Detection in sera; Authors highlight the lack of a gold standard; Several methods
	Is the reference technique the same quoted as gold standard?	Yes; No
	Type of sample	Blood sera; Blood plasma; Whole blood (filter paper-dried); Blood sera and saliva; Blood sera and meat juice; Meat juice; Milk
	Antigen employed	Native; Recombinant; Several types
	Native antigens	Not specified; Whole tachyzoite; Tachyzoite extract; Several types
Recombinant antigens	rSAG1; rSAG2; rSAG3; rSRS2; rGRA1; rGRA5; rGRA7; rGRA8; rMAG1; rROP2; rROP14; rSAG1-GRAS; rSAG1-SAG2-SAG3; rSAG1-GR1-ROP2-GR4-MIC3; Several types	
Single/Mixed use (recombinant antigens)	Single use; Mixed use; Single and mixed use; Chimera	
Parasite stage-specific antigen	Tachyzoite; Tachyzoite and bradyzoite; Tachyzoite and sporozoite; Tachyzoite, bradyzoite and sporozoite; Several antigens from different parasite stages	
Type of secondary antibody	Not specified; Non applicable; Anti-Ig secondary antibody; Protein A, G, A/G	
Antibody isotype detected	IgG; IgM; IgA; IgG and IgM; IgG, IgM and IgA; Several (other) [‡]	
Positive control	Yes; Not specified; Non applicable	
Negative control	Yes; Not specified; Non applicable	
Do they specify the cutoff?	Yes; No*; Non applicable	
Discrimination between acute and chronic phases	Yes; No	
Novelty of the method	New technique; Previously described technique; Variation of a previously described technique (e.g., new antigen); Several	

(continued on next page)

Table 1 (continued)

Category	Variable	Factor label
Validation steps	Analytical and/or diagnostic validation	Analytical validation; Diagnostic validation; Analytical and diagnostic validation
Analytical validation parameters	Intra-assay precision	Yes; No
	Interassay precision	Yes; No
	Limit of detection	Yes; No
	Cross-reactivity	Yes; No
	Pathogen/s considered for studying cross-reactivity	Other parasites (different from <i>T. gondii</i>); Virus; Bacteria; Noninfectious diseases; Several; Not specified
Diagnostic validation parameters	ROC analysis	Yes; No
	Bayesian latent class analysis	Yes; No
	AUC	Yes; No
	Se	Yes; No
	Sp	Yes; No
	Agreement	Yes; No
	Correlation coefficient (Pearson/Spearman)	Yes; Yes, but the result is not shown; No

* Journal names for each article are specified in Supplementary File 2. † Q064 detected IgG and IgA, Q079 detected IgG, IgM, IgA and IgG subclasses, and Q116 detected IgG, IgM, IgA, IgE and IgG subclasses. Multispecies = humans and animals, domestic and wild animals or different domestic species; EIA = Enzyme immunoassay; IFAT = Indirect fluorescent antibody test; WB = Western Blot; POC = Point-of-care; ELISA = Enzyme-linked immunosorbent assay; CMIA = Chemiluminescent microparticle immunoassay; LIPS = Luciferase immunoprecipitation system assay; QNDBs = Quantum dot nanobeads; LAT = Latex agglutination test; MAT = Modified agglutination test; IHA = Indirect hemagglutination test; ISAGA = Immunosorbent agglutination assay; Ig = Immunoglobulin; SIA = Suspension immunoassay; TRFIA = Time-resolved fluorescence immunoassay; PCR = Polymerase chain reaction; ELFA = Enzyme-linked fluorescence assay; MEIA = Microparticle enzyme immunoassay; SAG = Surface antigen; GRA = Dense granular antigen; MAG = Matrix antigen; ROP = Rhostry antigen; Ig = Immunoglobulin; ROC = Receiver operating characteristic; AUC = Area under de curve; Se = Sensitivity; Sp = Specificity.

[doubtful] or negative) categories), were also excluded from the analysis. DTA was performed when there were ≥ 5 entries (see the table in Supplementary File 4) for each evaluated technique for a specific host species and for a specific type of sample. Moreover, further comparisons between specific antigens employed and/or specific detected antibody isotypes were made when there was enough available data (≥ 5 entries for each type of antigen or antibody isotype).

Graphical analysis (package ggplot2, version 3.4.4.), Pearson's chi-square test/Fisher's exact test, MCA (package FactoMineR, version 2.9) and DTA (packages meta, version 6.5, and mada, version 0.5.11) were performed with R software (R Core Team, version 4.1.0, 2021–05–18).

3. Results

The flow diagram of the systematic search is shown in Fig. 1. Data were extracted from 70 articles focused on humans, 63 on animals (19/63 on domestic ruminants, 13/63 on cats, 12/63 on pigs, 8/63 on wild animals, 5/63 on domestic birds, 4/63 on dogs, and 2/63 on multiple species) and only one on both humans and animals (Study Q014). From the 71 articles that evaluated a technique for humans, 9 were conducted on pregnant women, 6 on adults (immunocompetent, nonpregnant adults), 4 on immunocompromised patients, 4 on children, 15 on several groups of population and 33 did not specify the group. The 8 articles that evaluated a technique for wild animals were concretely conducted on giant pandas (*Ailuropoda melanoleuca*), American black bears (*Ursus americanus*), wild rabbits (*Oryctolagus cuniculus*), Mexican jaguars (*Panthera onca*), wolverines (*Gulo gulo*), red foxes (*Vulpes vulpes*), raccoon dogs (*Nyctereutes procyonoides*), badgers (*Meles meles*), martens (*Martes martes*), minks (*Neovison vison*), polecats (*Mustela putorius*), and wild boar (*Sus scrofa*). These results are detailed in Supplementary File 2. All the results obtained from the chi-square and Fisher's exact tests between the "Journal quartile in JCR", "Final purpose of the evaluated technique (humans)", "Final purpose of the evaluated technique (animals)", "Humans and animals", "Host category", "Classification of the population (humans)" and "Classification of the population (animals)" variables and the other qualitative variables are included in Supplementary File 3. From these results, the most relevant findings are described as follows.

3.1. Study aim

With regard to the study purpose (according to the methodology employed), most of the studies conducted on humans (32.9%; 23/70) and animals (34.9%; 22/63) or both (100%; 1/1) aimed to evaluate a modification of a previously described technique. Only studies on humans aimed to evaluate techniques designed to detect more than one pathogen (10.0%; 7/70) and to differentiate between acute and chronic infection (8.6%; 6/70), while only articles carried out on animals evaluated a technique designed to detect *T. gondii* in different host species (3.2%; 2/63) ($p = 0.021$) (Supplementary File 3).

Focusing on the final purpose of the evaluated technique, data extracted should be taken with caution, as this information was not always explicitly mentioned by the authors. In humans, some serological techniques have been validated in specific populations (e.g., pregnant women, children, immunocompromised patients), while other studies have used samples from different population groups ($p < 0.001$) (Supplementary File 3). In animals, studies performed on livestock species normally aimed to detect antibodies to evaluate the risk of human infection via food (100%, 12/12 pigs; 60.0%, 3/5 domestic birds; 50.0% 10/20 domestic ruminants). On the other hand, studies on cats (100%; 13/13), dogs (100%; 4/4) and wild species (87.5%; 7/8) aimed to conduct seroprevalence studies ($p < 0.001$) (Supplementary File 3). The only study carried out on wild animals that highlighted the importance of detecting infection to evaluate the risk of human infection via food was performed on hunted wild boars. Noncommercial techniques were more frequently developed to conduct seroprevalence studies (73.0%; 27/37) than to evaluate the risk of human infection via food (55.6%; 15/27) ($p = 0.023$) (Supplementary File 3).

3.2. Methodological characteristics of the serological tests

In general, a wide variety of evaluated (Fig. 2) and reference techniques (Fig. 3) were used within each type of technique (EIA, agglutination, avidity and other tests), in both humans and animals. More tests have been developed for humans, many of which have been exclusively evaluated in this host species, such as chemiluminescent microparticle immunoassay (CMIA), quantum dot nanobead (QDNB)-based immunoassay, Western blot (WB), avidity tests or flow cytometry (Table 2). Other techniques were also evaluated exclusively in animals: IFAT,

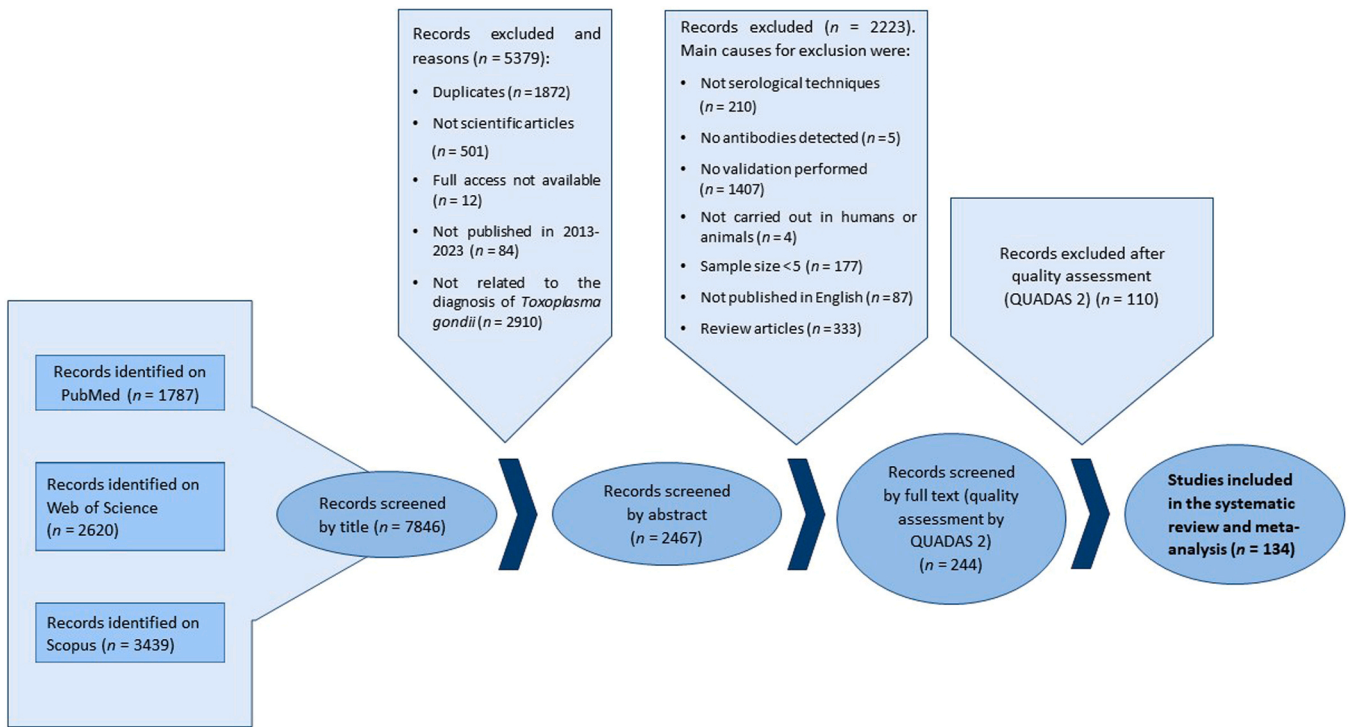


Fig. 1. Flow diagram of the systematic search. QUADAS = Quality Assessment of Diagnostic Accuracy Studies.

indirect hemagglutination test (IHA), inhibition ELISA, bead-based assay and time-resolved fluorescence immunoassay (TRFIA) (Table 2). Regarding different human target groups, most studies involving adults (immunocompetent, nonpregnant) (83.3%; 5/6), immunocompromised patients (75.0%; 3/4) and pregnant women (33.3%; 3/9) have evaluated

EIA, while several types or techniques have been evaluated in studies involving children (50.0%; 2/4) ($p = 0.041$).

Regarding the reference technique, studies performed on humans mostly used EIA (47.1%; 33/70), several types of techniques (35.7%; 25/70) and dye tests (7.1%; 5/70), while studies on animals used several

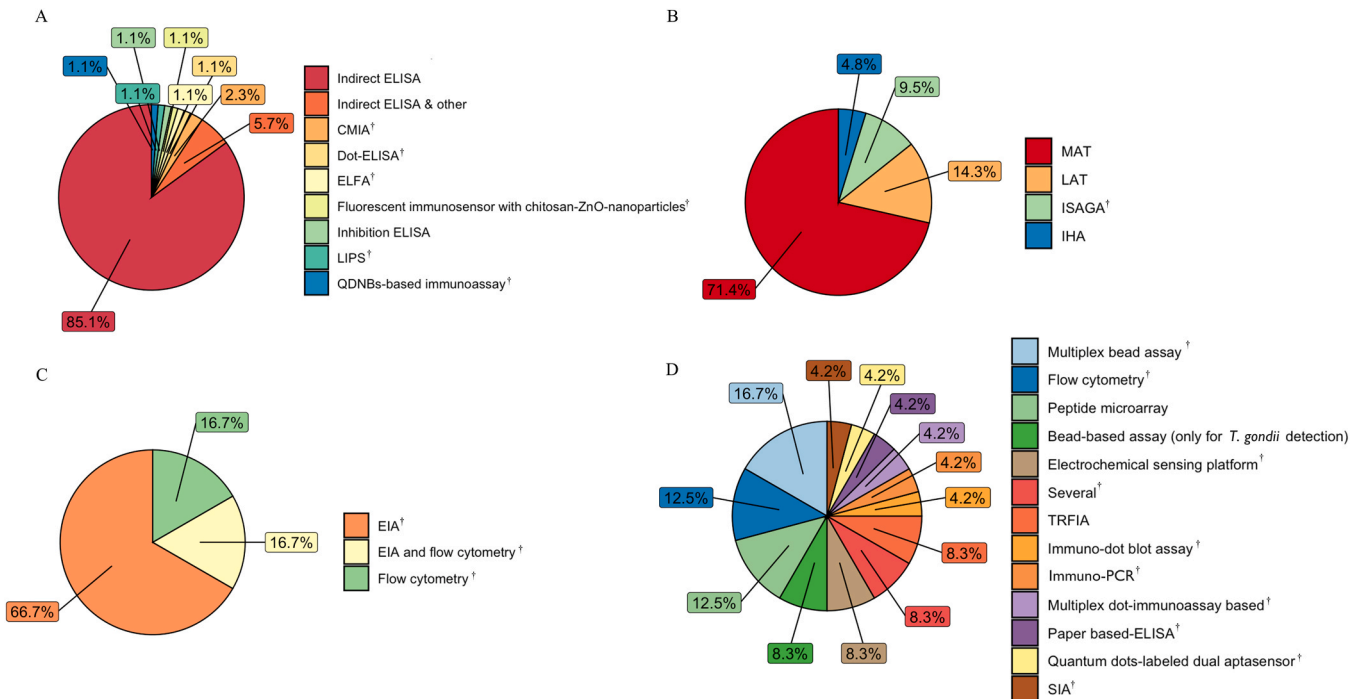


Fig. 2. Evaluated technique/s, EIA (A, n = 66 articles), agglutination tests (B, n = 16 articles), avidity tests (C, n = 6 articles) and other assays (D, n = 20 articles). † = only evaluated in studies performed on humans; EIA = enzyme immunoassay; ELISA = enzyme-linked immunosorbent assay; CMIA = chemiluminescent micro-particle immunoassay; LIPS = luciferase immunoprecipitation system assay; QDNBs = quantum dot nanobeads; MAT = modified agglutination assay; IHA = indirect hemagglutination test; LAT = latex agglutination test; ISAGA = immunosorbent agglutination assay; TRFIA = time-resolved fluorescence immunoassay; PCR = polymerase chain reaction; SIA = suspension immunoassay.

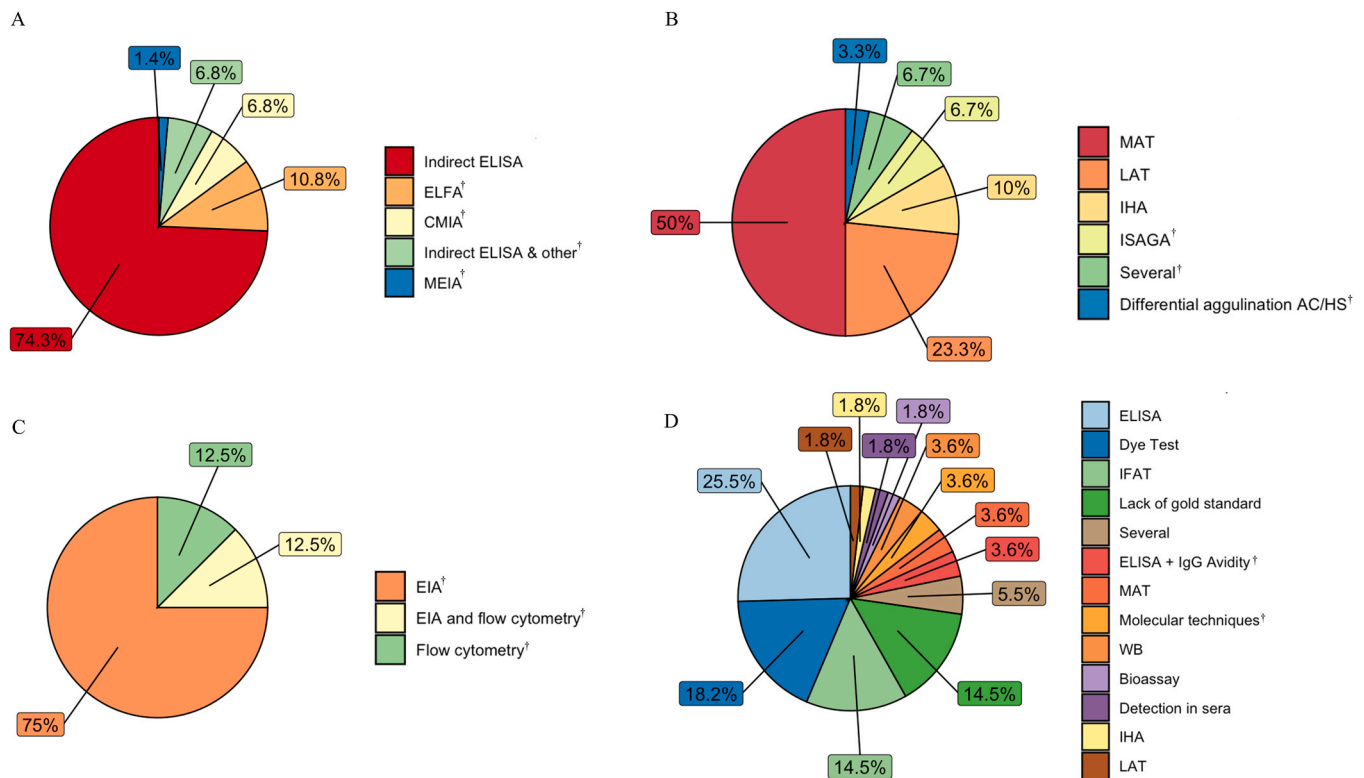


Fig. 3. Reference technique(s), EIA (A, $n = 65$ articles), agglutination test (B, $n = 22$ articles) and avidity test (C, $n = 8$ articles), and of the variable “Method quoted as gold standard” (D, $n = 42$ articles). † = only used as a reference/quoted in studies performed on humans; EIA = enzyme immunoassay; ELISA = enzyme-linked immunosorbent assay; ELFA = enzyme-linked fluorescence assay; CMIA = chemiluminescent microparticle immunoassay; MEIA = microparticle enzyme immunoassay; LAT = latex agglutination test; MAT = modified agglutination assay; IHA = indirect hemagglutination test; ISAGA = immunosorbent agglutination assay; AC/HS = acetone-fixed versus formalin-fixed tachyzoites; IFAT = indirect fluorescent antibody test; Ig = immunoglobulin; WB = Western blot.

types of techniques (25.4%; 16/63%), IFAT (20.6%; 13/63), agglutination tests (19.0%; 12/63) and EIA (15.9%; 10/62) instead ($p < 0.001$) (Table 2 and Supplementary File 3). When studies performed in humans specified the target age groups 75.0% (3/4), 66.7% (4/6) and 50.0% (2/4) of studies conducted on adults, children and immunocompromised patients, respectively, used EIA as a reference technique, while most studies on pregnant women (66.7%; 6/9) used several techniques as a reference ($p = 0.004$) (Supplementary File 3). In animals, agglutination tests were more frequently used in studies carried out on cats (38.5%; 5/13), IFATs on domestic ruminants (26.3%; 5/19), molecular techniques on domestic birds (40.0%; 2/5). EIA and several types of techniques were more frequently used in studies conducted on pigs (25.0% each; 3/12), and EIA and IFAT were more frequently used in studies on wild animals (25.0% each; 2/8). Studies on dogs used agglutination tests, IFATs, WBs and several techniques (25.0% each; 1/4), and studies on multiple species used EIAs (33.3%; 1/3) and several types of techniques (66.7%; 2/3) ($p < 0.001$) (Supplementary File 3). In humans, the most commonly employed reference techniques were commercial (64.3%; 45/70) ($p < 0.001$) (Supplementary File 3), while in-house (noncommercial) techniques were predominantly used in animals (42.9%; 27/63). These latter techniques were more commonly used for dogs (100%; 4/4), domestic birds (100%; 5/5) and domestic ruminants (52.6%; 10/19). However, studies performed on pigs (70.0%; 7/10) and wild animals (57.1%; 4/7) predominantly used commercial techniques ($p < 0.001$) (Supplementary File 3). Studies conducted on cats used interchangeably commercial techniques (36.4%; 4/11), noncommercial techniques (36.4%; 4/11) or both (27.3%; 3/11) as references. Two out of eight (25.0%) articles that considered agglutination techniques as the reference techniques in humans used the immunosorbent agglutination assay (ISAGA), 2/8 (25.0%) used MAT and 2/8 (25.0%) used several agglutination tests, while differential agglutination AC/HS and latex

agglutination test (LAT) were used in one study each ($p = 0.005$) (Supplementary File 3). More articles focused on animals that used agglutination tests as a reference: 59.1% (13/22) used a modified agglutination test (MAT), 27.3% (6/22) used LAT, and 13.6% (3/22) used an indirect hemagglutination assay (IHA).

We found a lack of consensus regarding the gold standard or the reference test employed across studies performed in humans and animals. A total of 56 articles explicitly mentioned the “gold standard” (41.8%; 56/134) (Fig. 3D and Supplementary File 2), and only eight studies (14.3%) conducted on animals highlighted the lack of a gold standard technique for serodiagnosis of toxoplasmosis (Table 2). In contrast, 47 articles mentioned a specific method as the gold standard (humans: $n=27$; animals: $n=20$), matching this chosen method with the reference technique employed for 39 of the articles analyzed (69.6%). In humans, most of these studies considered ELISA (40.7%; 11/27) and the dye test (29.6%; 8/27) to be the gold standard techniques, whereas IFAT (35%; 7/20) was considered the gold standard technique in animal studies ($p = 0.002$) (Table 2 and Supplementary File 3).

Regarding the type of sample, most articles evaluated a technique for blood sera (89.6%; 120/134), while few articles evaluated a technique for other types of samples (meat juice 2.2%, 3/134; whole blood -filter paper-dried- 1.5%, 2/134; plasma 1.5%, 2/134; milk 0.7%, 1/134; and saliva 0.7%, 1/134) or for several types (blood sera and saliva 2.2%, 3/134; and blood sera and meat juice 1.5%, 2/134). Although there were no significant differences between humans and animals (Supplementary File 3), saliva was exclusively used in studies conducted on humans, while meat juice, milk and whole blood (filter paper-dried) were exclusively used in studies on animals (Table 2).

Finally, other relevant differences found between the tests evaluated for humans and animals were related to the antigens and the secondary antibodies used and to the immunoglobulin isotypes measured. In

Table 2
Serological tests developed for the diagnosis of *Toxoplasma gondii* in humans and animals.

		Humans (n)	Animals (n)	Humans and animals (n) [†]
Evaluated technique [‡]	EIA	Fluorescent immunosensor with chitosan-ZnO-nanoparticles (1); Dot-ELISA (1); CMIA (2); LIPS (1); QDNBs-based immunoassay (1); ELFA (1)	Inhibition ELISA (1)	Indirect ELISA (74); Indirect ELISA & other EIA (5)
	IFAT	(0)	IFAT (5)	(0)
	WB	(0)	(0)	WB (4)
	POC test	(0)	(0)	Lateral flow immunoassay (13)
	Agglutination test	ISAGA (2)	IHA (1)	LAT (3); MAT (15)
	Avidity test	EIA (4); Flow cytometry (1); EIA and flow cytometry (1)	(0)	(0)
	Other type	Multiplex bead assay (4); Electrochemical sensing platform (2); Flow cytometry (3); Multiplex dot-immunoassay based (1); Paper-based ELISA (1); Immuno-dot blot assay (1); SIA (1); Immuno-PCR (1); Quantum dots-labeled dual aptasensor (1); Several techniques (2)	Bead-based assay (2); TRFIA (2)	Peptide microarray (3)
Reference technique or method [‡]	EIA	ELFA (9); MEIA (1); CMIA (6); Indirect ELISA & other EIA (4)	(0)	Indirect ELISA (56)
	IFAT	(0)	(0)	IFAT (29)
	WB	(0)	(0)	WB (13)
	Agglutination test	ISAGA (3); Differential agglutination AC/HS (1); ISAGA and DAT (1); HSDA (1)	IHA (7); DAT (4)	LAT (7); MAT (12)
	Dye test	(0)	(0)	Dye test (8)
	POC test	POC test (2)	(0)	(0)
	Avidity test	EIA (6); ELFA (1); Flow cytometry (1); EIA and flow cytometry (1)	(0)	(0)
	Clinical criteria	Clinical criteria (2)	(0)	(0)
	Molecular techniques	(0)	Magnetic-capture-real-time PCR (1); PCR (5)	(0)
	Other type	Imaging techniques (2)	Bayesian analysis (4)	Mouse bioassay (2)
Method quoted as gold standard	Clinical examination (1); ELISA & IgG Avidity (2); Molecular techniques (2); Several methods (2)	Lack of gold standard (9); Detection in sera (1); IHA (1); MAT (2); Bioassay (1); LAT (1)	Dye test (10); ELISA (14); IFAT (8); WB (3)	
Type of sample	Blood sera and saliva (3); Saliva (1)	Blood sera and meat juice (2); Meat juice (3); Milk (1); Whole blood (filter paper-dried) (2)	Blood sera (120); Blood plasma (2)	
Antigen employed [§]	Parasite stage-specific antigen	(0)	(0)	Tachyzoite (45); Tachyzoite and bradyzoite (10); Tachyzoite and sporozoite (8); Tachyzoite, bradyzoite and sporozoite (2); Several antigens from different parasite stages (26)
	Native antigen	(0)	(0)	Native antigen (50)
	Recombinant antigens	rGRA5 (1); rROP2 (1); rSAG1-SAG2-SAG3 (1)	rSAG3 (1); rSRS2 (1); rGRA1 (1); rGRA8 (1); rMAG1 (1); rROP14 (1); rSAG1-GRAS (1); rSAG1-GRAS1-ROP2-GRAS4-MIC3 (1)	rSAG1 (17); rSAG2 (5); rGRA7 (6); Several types (29)
Antibody isotype detected	IgM (2); IgA (1); IgG, IgM and IgA (3); IgG and IgA (1); IgG, IgM, IgA and IgG subclasses (1); IgG, IgM, IgA, IgE and IgG subclasses (1)	(0)	IgG (98); IgG and IgM (24)	

[†]Total number of articles without differentiating between studies conducted on humans or animals. [‡]Techniques included in “Several types” within “Evaluated technique” and “Reference technique” variables were also classified in this table. [§]The only article that evaluated a nonprotein antigen was Q050, which used a synthetic glycosylphosphatidylinositol (GPI) glycans on a bead-based multiplex assay. n = number of articles; EIA = Enzyme immunoassay; IFAT = Indirect fluorescent antibody test; WB = Western Blot; POC = Point-of-care; ELISA = Enzyme-linked immunosorbent assay; CMIA = Chemiluminescent microparticle immunoassay; LIPS = Luciferase immunoprecipitation system assay; QDNBs = Quantum dot nanobeads; LAT = Latex agglutination test; MAT = Modified agglutination test; IHA = Indirect hemagglutination test; ISAGA = Immunosorbent agglutination assay; DAT = Direct agglutination test; SIA = Suspension immunoassay; TRFIA = Time-resolved fluorescence immunoassay; ELFA = Enzyme-linked fluorescence assay; MEIA = Microparticle enzyme immunoassay; PCR = Polymerase chain reaction; SAG = Surface antigen; GRA = Dense granule antigen; MAG = Matrix antigen; ROP = Rhoptry antigen; Ig = Immunoglobulin

general terms, the use of native and recombinant antigens was quite similar, although slightly greater in the case of the former (Figs. 4A, 5A and 5B). Within human populations, studies conducted on children exclusively used native antigens (100%; 3/3), while those on pregnant women and immunocompromised patients used recombinant antigens (66.7% 4/6 and 2/3, respectively) and several types of antigens (33.3%; 2/6 and 1/3, respectively) ($p = 0.041$) (Supplementary File 3). Studies on adults (immunocompetent, nonpregnant) used native (40.0%; 2/5), recombinant (40.0%; 2/5) and several types of antigens (20.0%; 1/5). Moreover, recombinant antigens are used alone (single use), in combination (mixed use) or as chimeras in both humans and animals (Figs. 4C and 4D), including antigens from all parasite stages (Fig. 4B), but the use of bradyzoite- and sporozoite-specific antigens is scarce (Table 2). There was a greater diversity of recombinant antigens exclusively used in tests developed for animals (surface, dense granule, matrix and rhoptry proteins) than for humans (dense granule and rhoptry proteins). Moreover, surface antigen (rSAG) 1, rSAG2 and dense granule antigen 7 (rGRA7) are commonly used in tests developed for humans and animals (Fig. 4C, Table 2).

The secondary antibody used was significantly related to the host category ($p < 0.001$) (Supplementary File 3). Specifically, anti-immunoglobulin (Ig) antibodies directed against target species were usually employed on humans (78.6%; 55/70), cats (61.5%; 8/13), dogs (75%; 3/4), domestic ruminants (78.9%; 15/19), pigs (58.3%; 7/12) and domestic birds (80.0%; 4/5), while anti-Ig antibodies (25.0%; 2/8), protein A, G or A/G (12.5%; 1/8) and several types (37.5%; 3/8) were used on wild animals (Supplementary File 2). Of the studies that developed a technique for serodiagnosis of *T. gondii* infection on several host species, 66.7% (2/3 articles) used an anti-Ig antibody directed against each target species (goats, sheep and humans in study Q023; horses, pigs and sheep in study Q0106), while one study (33.3%) conducted on pigs, cats, mice and seals (Q127) used protein A/G (Supplementary File 2).

Techniques developed for humans measured different Ig isotypes (IgG: 40/70; IgM: 2/70; IgA: 1/70; several isotypes: 26/70) ($p = 0.001$)

(Supplementary File 3), while IgG tests predominated in animals (92.0%; 58/63) (Table 2; Figs. 5C and 5D). In addition, only 6.7% (9/134) of the articles discriminated between acute and chronic phases of *T. gondii* infection that were exclusively performed on humans ($p = 0.012$) (Supplementary File 3).

Relevant data on the reagents used were not always available. The antigen employed and the antibody isotype detected were not specified in 9.7% (13/134; 12 in humans and 1 in animals) and 2.2% (3/134; 1 in humans and 2 in animals) of the articles, respectively. The use of positive and negative controls was not specified in 47.0% (63/134; 43 in humans and 20 in animals) and 44.0% (59/134; 39 in humans and 20 in animals) of the studies, respectively (Supplementary File 2).

3.3. Validation of the serological test results

Most articles exclusively performed diagnostic validation (85.8%; 115/134) vs. 3.0% of articles (4/134) that carried out analytical validation and 11.2% (15/134) that performed both analytical and diagnostic validations. The validation steps and the parameters studied for analytical and diagnostic validation in humans and animals are summarized in Table 3.

Regarding analytical validation, there were no statistically significant differences between humans and animals (Supplementary File 3). Only 10.4% (14/134), 12.7% (17/134) and 9.7% (13/134) of the articles determined the intra- and interassay precision (defined as the coefficients of variation of the results of replicates of a sample within and between runs of the same test, respectively) (WOAH, 2023) and the limit of detection, respectively. Thirty-one articles (23.1%) evaluated cross-reactivity with other parasites ($n=12$), viruses ($n=2$), and several pathogens ($n=16$) (including viruses, bacteria, parasites and fungi) (Table 4 and Supplementary File 2). In articles conducted on humans, cross-reactivity was evaluated using positive sera to a wide variety of pathogens: protists (e.g., *Plasmodium* spp., *Leishmania* spp., *Trypanosoma cruzi*), helminths (e.g., *Echinococcus* spp., *Schistosoma* spp., *Fasciola* spp.), viruses (e.g., Cytomegalovirus, Rubella virus, Herpes simplex

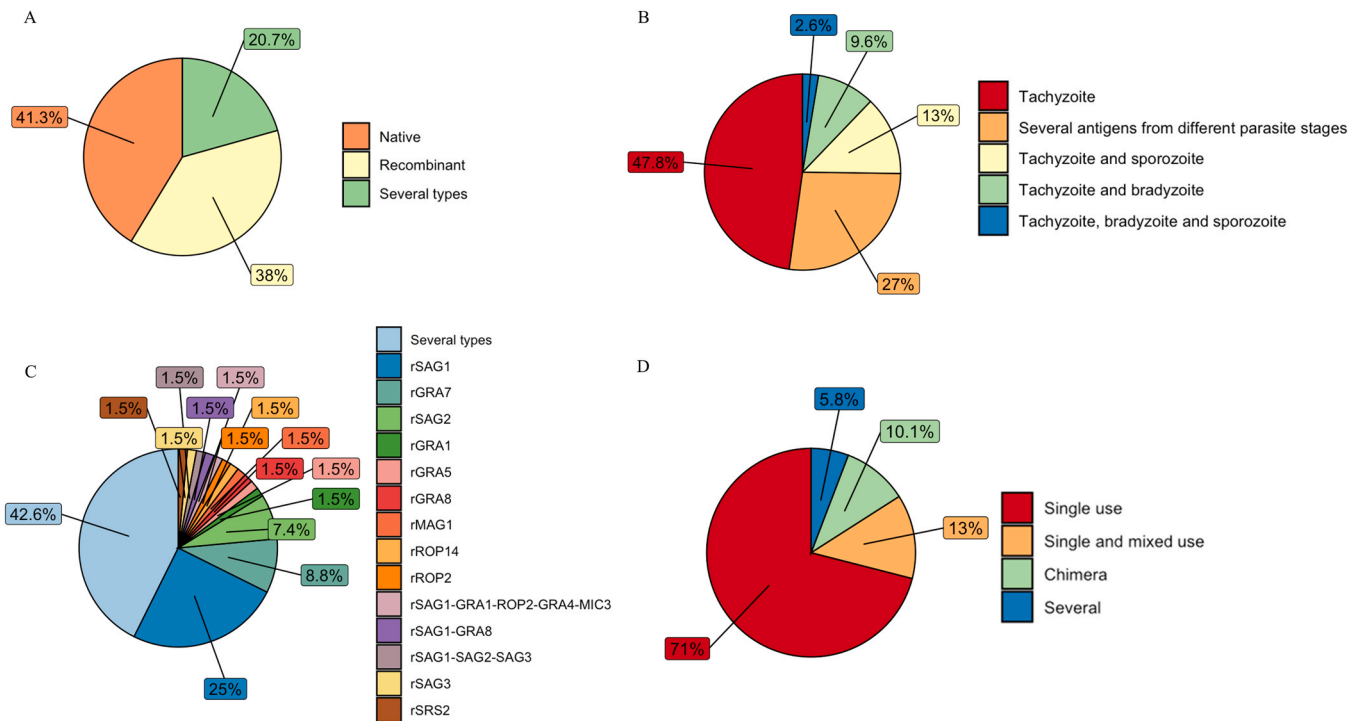


Fig. 4. Antigens used in the evaluated techniques: type of antigens (native/recombinant) (A, $n = 98$ articles), parasite stage-specific antigens (B, $n = 93$ articles), type of recombinant antigens (C, $n = 57$ articles) and single/mixed-use/chimera (recombinant antigens) (D, $n = 58$ articles). SAG = surface antigen; GRA = dense granule antigen; MAG = matrix antigen; ROP = rhoptry antigen.

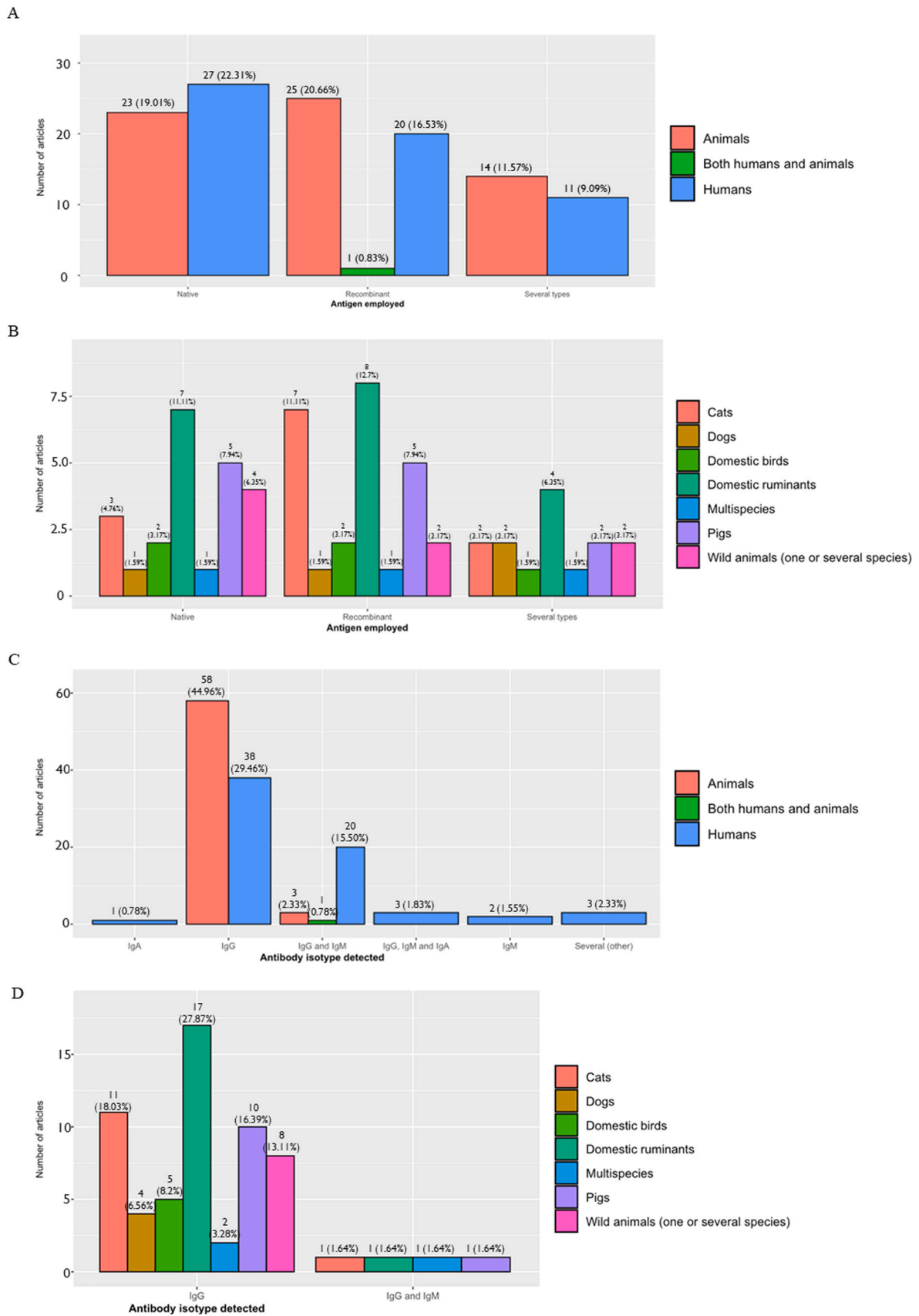


Fig. 5. Antigens (n = 98 articles) and antibody isotypes (n = 96 articles) employed in the evaluated techniques regarding “Humans or animals” (A, C) and “Host category” (B, D) variables. [†]Q057 detected IgG and IgA, and Q069 detected IgG, IgM, IgA and IgG subclasses. Multispecies = humans and animals, domestic and wild animals or different domestic species; Ig = immunoglobulin.

Table 3

Validation procedure followed and parameters determined throughout the development process of the serological tests implemented in humans and animals.

		Humans (n = 70)	Animals (n = 6363)
Analytical and/or diagnostic validation	Analytical validation	4.3% (3/70)	1.6% (1/63)
	Diagnostic validation	88.6% (62/70)	82.5% (52/63)
Analytical validation parameters	Analytical and diagnostic validation	7.1% (5/70)	15.9% (10/63)
	Intra-assay precision	8.6% (6/70)	12.7% (8/63)
	Interassay precision	11.4% (8/70)	14.3% (9/63)
	Limit of detection	10% (7/70)	9.5% (6/63)
Diagnostic validation parameters	Cross-reactivity	18.6% (13/70)	28.6% (18/63)
	ROC analysis	37.1% (26/70)	41.3% (26/63)
	Bayesian latent class analysis	0% (0/70)	9.5% (6/63)
	AUC	27.1% (19/70)	38.1% (24/63)
	Se	95.7% (67/70)	98.4% (62/63)
	Sp	95.7% (67/70)	98.4% (62/63)
	Agreement	41.4% (29/70)	84.1% (53/63)
	Correlation coefficient (Pearson/Spearman)	25.7% (18/70)	7.9% (5/63)

The only article carried out in both humans and animals (Q023) exclusively performed diagnostic validation and estimated the diagnostic Se and Sp. Bold = percentage of articles > 80%; n = number of articles; ROC = Receiver operating characteristic; AUC = Area under de curve; Se = Sensitivity; Sp = Specificity.

virus), bacteria (e.g., *Treponema pallidum*, *Mycobacterium tuberculosis*) and fungi (*Paracoccidioides brasilienses*) (Table 4). In contrast, in studies performed on animal species, except for pigs and cats, the number of evaluated pathogens was limited, with a focus mainly on Apicomplexa parasites and helminths. Notably, in the case of the only study in mice in which cross-reactivity was analyzed, the target species was a nematode (*Trichinella spiralis*) and not a protist (Table 4).

Regarding the diagnostic validation parameters, although most of the studies calculated the diagnostic Se and Sp (97.0%; 130/134), only 38.8% (52/134) performed a receiver operating characteristic (ROC) curve analysis, and 4.5% (6/134) performed a Bayesian latent class analysis (Table 3). All the articles that performed a Bayesian latent class analysis were focused on animals (three on pigs, one on domestic ruminants, one on cats and one on wild animals) ($p = 0.029$) (Supplementary File 3). Finally, the agreement and correlation between the evaluated and reference techniques were only estimated in 61.2% (82/134) and 17.2% (23/134) of the studies, respectively; in this respect, agreement was more frequently evaluated in tests developed for animals (84.1%; 53/63) than in tests developed for humans (41.4%; 29/70) ($p < 0.001$) (Table 3 and Supplementary File 3), while the correlation coefficient was more frequently determined in tests developed for humans (25.7%; 18/70) than in tests developed for animals (7.9%; 5/63) ($p = 0.023$) (Table 3 and Supplementary File 3). Regarding the specific host category, agreement was determined in 100% (13/13) of the articles focused on cats, 100% (4/4) on dogs, 87.5% (7/8) on wild animals, 84.2% (16/19) on domestic ruminants, 80.0% (4/5) on domestic birds and 66.7% (8/12) on pigs ($p < 0.001$) (Supplementary Files 2 and 3).

Remarkably, six studies (4 in humans and 2 in animals) did not specify the established cutoff despite the estimation of this value being described (excluding point-of-care tests).

3.4. Multiple correspondence analysis (MCA)

Fig. 6 represents the MCA for the “Humans or animals” variable. Specifically, there is a clear separation of the articles depending on their performance in human or animal hosts. In particular, studies performed in humans showed a positive influence of variables from dimensions 1 and 2, in contrast with those carried out in animal hosts, which are negatively influenced by variables from both dimensions. These results agree with the significant differences reported above between tests developed for humans and for animals. Factor labels with higher contributions for dimension 1 (10.1% variability) were “No” (from the variable “Agreement”), “EIA” (from “Reference technique or method”), “Humans” (from “Humans or animals”) and “ELISA” (from “Method quoted as gold standard”) (Fig. 1A in Supplementary File 5). On the other hand, for dimension 2 (8.2% variability), “Several types” (from the

variable “Reference technique or method”), “Commercial and noncommercial” (from “Commercial/noncommercial (reference technique)”), “IgG and IgM” (from “Antibody isotype detected”), and “Several methods” (from “Method quoted as gold standard”) were the most contributory factor labels (Fig. 1B in Supplementary File 5).

3.5. Diagnostic test accuracy meta-analysis

Data on TP, TN, FP and FN were available for 79.1% (106/134) of the articles. Among them, 19 techniques yielded undetermined results and were not included in the analysis. The raw data included in the DTA meta-analysis are collected in Supplementary File 4, and the results of the DTA meta-analysis are summarized in Table 5.

The DORs showed that all diagnostic tests included in the analysis were correctly discriminated. However, the DORs markedly differed among the tests. The highest DOR values corresponded to the recombinant (chimera)-based indirect ELISA and the lateral flow immunoassay developed for humans and the indirect ELISA (based on native and recombinant -mixed use and chimera- antigens) developed for sheep. The lowest DOR values corresponded to TRFIA and LACA tests developed for cats and pigs, respectively. Relevant differences were also observed regarding the pooled Se and Sp values, with the highest Se values (>90.0%) corresponding to indirect ELISAs developed for humans, cats, cattle, sheep, and goats. In general, pooled Sp values were greater; in contrast, several tests showed low Se values (e.g., tests developed for pigs). In those host species where IgG- and IgM-based indirect ELISAs could be compared (humans, sheep and goats), IgG-based tests showed higher Se values. Moreover, in humans, tests based on native antigens performed similarly to tests based on chimeric antigens and better than tests based on a single recombinant antigen. Finally, when the single or mixed recombinant antigen- and chimeric recombinant antigen-based tests were compared in sheep, the latter showed better performance, similar to the native antigen-based tests (Table 5).

4. Discussion

Herein an updated report of the characteristics of recently developed serological assays for the diagnosis of *T. gondii* infection and validation procedure followed in humans and animals is provided. The final aim of this study was to provide valuable information for physicians, veterinarians and other diagnostic specialists regarding serological methods developed for humans and animals and to identify areas of improvement for accurate and reproducible diagnostic results (Lebov et al., 2017). The breadth of the literature search, the quality assessment and the large number of included studies enabled us to offer a representative picture

Table 4Pathogen/s considered for studying cross-reactions when developing serological techniques for *Toxoplasma gondii* infection on the different host species.

		Other parasites		Viruses	Bacteria	Fungi
		Protists	Helminths			
Humans (n)		<i>Cryptosporidium</i> spp. (1), <i>Plasmodium</i> spp. (1), microsporidia (1), amoeba (2), <i>Giardia</i> spp. (1), <i>Leishmania</i> spp. (4), <i>Trypanosoma cruzi</i> (4)	<i>Echinococcus</i> spp. (5), <i>Schistosoma</i> spp. (1) <i>Fasciola</i> spp. (2), <i>Toxocara</i> spp. (1), <i>Ascaris lumbricoides</i> (1)	CMV (5), rubella virus (4), HSV (4), HIV (2), EBV (2), VZV (1); HBV (2); DENV (1)	<i>Treponema pallidum</i> (3), <i>Mycobacterium tuberculosis</i> (2), <i>Helicobacter pylori</i> (1), <i>Salmonella typhi</i> (1), <i>Shigella</i> spp.	<i>Paracoccidioides brasilienses</i> (2)
Animals	Cats (n)	<i>Hammondia hammondi</i> (1), <i>Isospora</i> spp. (1), <i>Leishmania infantum</i> (1)	<i>Trichinella spiralis</i> (2), <i>Echinococcus</i> spp. (1), hookworms (1), <i>Platynosomum</i> spp. (1), <i>Toxocara</i> spp. (1), <i>Dirofilaria immitis</i> (1), <i>Spirometra mansonii</i> (1), <i>Clonorchis sinensis</i> (1), <i>Paragonimus kellicotti</i> (1)	FPV (2), FIV (2), FeLV (1), FHV-1 (1), FCV (1)	<i>Eperythrozoon</i> spp. (1), <i>Bartonella henselae</i> (1)	(0)
	Dogs (n)	<i>Neospora caninum</i> (1)	(0)	CPV (1)	<i>Escherichia coli</i> (1), <i>Salmonella enterica</i> (1), <i>Listeria monocytogenes</i> (1)	(0)
	Domestic ruminants (n)	<i>Neospora caninum</i> (5)	(0)	(0)	(0)	(0)
	Horses (n)	<i>Neospora caninum</i> (1)	(0)	(0)	(0)	(0)
	Pigs (n)	<i>Neospora caninum</i> (2), <i>Cystoisospora suis</i> (2), <i>Cryptosporidium suis</i> (2),	<i>Toxocara cati</i> (1), <i>Ascaris suum</i> (1), <i>Cysticercus cellulosae</i> (1), <i>Trichinella spiralis</i> (1)	SFV (2), PRRSV (2), PrV (1), PCV (1), FMDV (2)	<i>Mycoplasma suis</i> (1), <i>Streptococcus suis</i> (1), <i>Salmonella choleraesuis</i> (1)	(0)
	Domestic birds (n)	<i>Neospora caninum</i> (1)	(0)	(0)	(0)	(0)
	Wolverines (n)	<i>Neospora caninum</i> (1), <i>Hammondia hammondi</i> (1)				
	Mice (n)	(0)	<i>Trichinella spiralis</i> (1)	(0)	(0)	(0)

Article Q127 developed a multispecies test for cats, pigs, mice and seals, but it only evaluated cross-reactivity with *Trichinella spiralis* positive sera from cats and mice. Therefore, cross-reactivity studied with *Besnoitia besnoiti*, *B. tarandi* and *Neospora caninum* positive sera from other species (cattle, rabbits and rats) was not included in this table. n = number of articles; CMV = Cytomegalovirus; HSV = Herpes simplex virus (1 and/or 2 in article Q006; type not specified in Q084, Q092 and Q124); HIV = human immunodeficiency virus (type 1 in Q101; type not specified in Q006); EBV = Epstein-Barr virus; VZV = Varicella zoster virus; HBV = hepatitis B virus; DENV = Dengue virus; FPV = feline parvovirus; FIV = feline immunodeficiency virus; FeLV = feline leukemia virus; FHV-1 = feline herpesvirus type-1; FCV = feline calicivirus; CPV = canine parvovirus; SFV = swine fever virus (African or classic type not specified in Q018 and Q032); PRSSV = porcine reproductive and respiratory syndrome virus; PrV = pseudorabies virus; PCV = porcine circovirus; FMDV = swine foot-and-mouth disease virus (types A and O in Q018; type not specified in Q032).

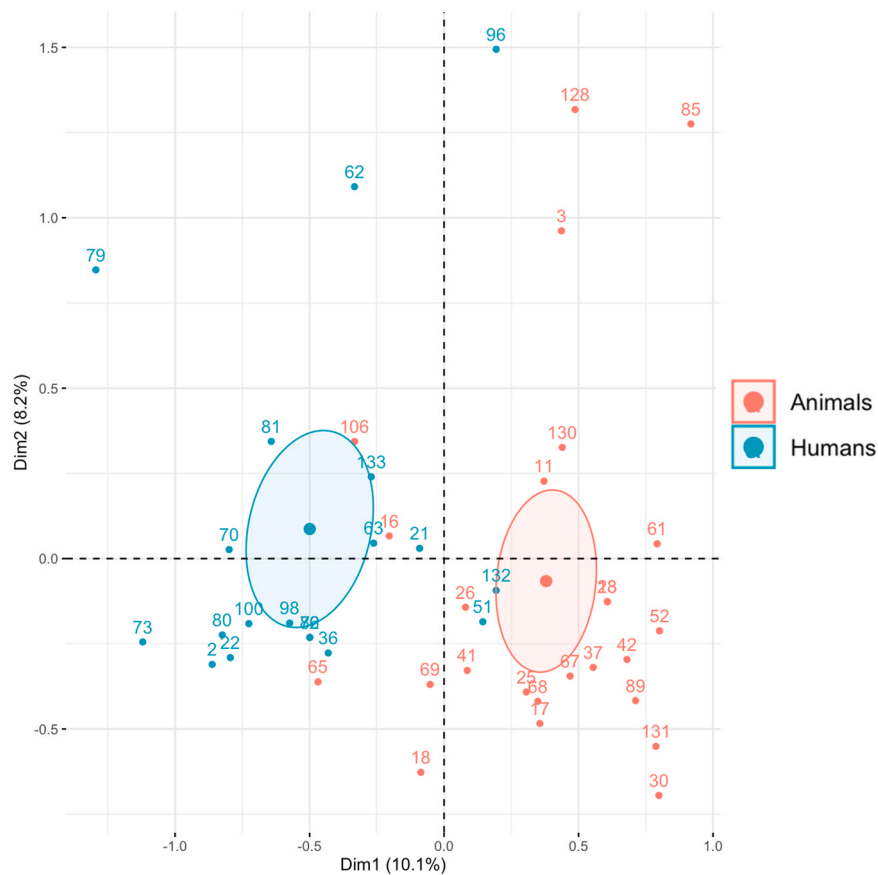


Fig. 6. Graphical depiction of the individual distribution of the studies within dimension 1 and dimension 2 in multiple correspondence analysis (MCA) for “Humans or animals”, with Dim-1 (9.9% variability) = dimension 1 (factor labels with higher contribution: “Agreement – No”, “Reference technique or method – ELISA”, “Humans or animals – Humans” and “Method quoted as gold standard - ELISA”) and Dim-2 (8.5% variability) = dimension 2 (factor labels with higher contribution: “Reference technique or method – Several types”, “Commercial/noncommercial (reference technique) – Commercial and noncommercial techniques”, “Antibody isotype detected – IgG and IgM”, and “Method quoted as gold standard – Several methods”).

of recently developed serological methods. In fact, the large number of articles indicates the great effort invested by research groups to develop accurate tests. Moreover, the high heterogeneity related to the test formats and reagents employed facilitates the use of different tests according to the available equipment and objective of the technique. However, the limitations identified in the validation process, the low Se values obtained on some occasions and variable DOR values among tests may complicate the reproducibility and hamper the comparison of results among different studies.

We extracted a large number of variables related to the characteristics of the serological techniques and the analytical and diagnostic validation steps followed according to the World Health Organization (WHO) and the World Organization for Animal Health (WOAH) guidelines. A protocol for the validation of serological assays in animals was established by WOAH that consists of five stages with different levels of evaluation: analytical characteristics (stage 1), diagnostic characteristics (stage 2), reproducibility (stage 3), implementation (stage 4) field validation (stage 5) and validation status retention (WOAH, 2023). Although the WHO has not published any official protocol for the evaluation of serological assays to diagnose *T. gondii* infection in humans, several protocols for evaluating diagnostic assays for other diseases, such as malaria or human immunodeficiency virus (HIV), have been established (WHO, 2016; Banerjee et al., 2022).

The present study confirmed that a wide variety of techniques (i.e., different formats, antibodies and antigens used) have been developed for the serodiagnosis of *T. gondii*. A wider panel of tests developed for humans, including those regarded as references, could be explained by greater accessibility to more sophisticated technologies and expensive

equipment in human medicine (e.g., CMIA, QDNB-based immunoassay or flow cytometry) than in veterinary medicine and the different diagnostic approaches followed regarding the target population (monitoring IgG/IgM during pregnancy or measuring IgM and IgA as the best biomarkers of congenital toxoplasmosis at birth) (Bollani et al., 2022). The more frequent use of commercial tests in human medicine could be explained by the fact that serology is usually employed to monitor *T. gondii* infection in a health care setting, for example, in pregnant women on a regular basis in national programs (Prusa et al., 2014), to establish early treatment for acute infection (Hotop et al., 2012), and to facilitate the differential diagnosis of infection in immunosuppressed patients (DuPont et al., 2021). In fact, we found a few studies focused exclusively on these target populations. In contrast, the lower availability of commercial tests adapted to animals could be explained by the less frequent use of these tests in the routine diagnosis of *T. gondii* infection in both domestic and wild animal species, in which epidemiological studies are usually carried out for research purposes. This assumption is consistent with the finding of a predominance of tests developed for seroprevalence studies in several animal species (cats, dogs and wildlife). In addition, this reduced presence of commercial tests is likely to be due to the lack of available reference sera or species-specific conjugates (Wyrosdick and Schaefer, 2015). Moreover, reference techniques more commonly used in animals, such as in-house IFATs or agglutination tests, normally employ native antigens that are costly and more difficult to standardize than recombinant antigens (Liu et al., 2015; Rostami et al., 2018; Uddin et al., 2021). The use of commercial techniques could facilitate the repeatability of the results, but for this purpose, it is necessary that these tests are accurately and

Table 5

Pooled sensitivity, specificity and diagnostic odds ratio of the serological techniques developed for the diagnosis of *Toxoplasma gondii* infection in humans and animals.

Host category	Evaluated technique	Antibody isotype detected	Antigen employed	Number of evaluated techniques included in the analysis	Pooled Se (95% CI)	Pooled Sp (95% CI)	Pooled DOR (95% CI)		
Humans	Indirect ELISA	All types	All types	59	93.73% (89.88–96.70%)	97.42% (95.37–98.88%)	462.22 (232.20–920.11)		
			IgG	All types	43	94.09% (90.23–97.02%)	98.30% (96.05–99.63%)	635.35 (275.82–1463.54)	
		IgM	Native	All types	9	98.69% (96.59–99.81%)	96.60% (95.08–100%)	996.54 (154.08–6445.22)	
				Recombinant (single use)	21	88.21% (80.03–94.45%)	97.97% (94.32–99.80%)	222.98 (85.90–578.83)	
			Recombinant (chimera)	All types	8	97.47% (90.23–100%)	99.19% (95.56–100%)	4952.52 (285.85–85807.13)	
				Recombinant (single use)	11	89.83% (70.66–99.41%)	96.67% (94.82–98.12%)	210.72 (47.50–934.80)	
			Lateral flow immunoassay	All types	All types	6	90.81% (72.69–99.56%)	96.27% (92.97–98.56%)	179.60 (36.34–887.52)
					IgG	10	82.77% (59.31–97.32%)	96.32% (92.87–98.66%)	252.98 (25.49–2510.42)
				IgG	All types	5	95.48% (68.62–100%)	98.30% (93.95–99.98%)	2941.79 (65.92–131283.42)
					Recombinant (single use)	7	87.28% (73.27–96.58%)	97.12% (95.51–98.38%)	130.16 (57.26–295.87)
Cats	Indirect ELISA	IgG	All types	5	67.28% (53.01–80.09%)	69.71% (53.22–83.94%)	4.64 (1.26–17.08)		
			TRFIA	5	90.90% (77.33–98.65%)	95.92% (91.23–98.88%)	128.30 (27.29–603.24)		
Cattle	Indirect ELISA	IgG	All types	5	90.90% (77.33–98.65%)	95.92% (91.23–98.88%)	128.30 (27.29–603.24)		
			Indirect ELISA	All types	All types	39	94.61% (90.09–97.82%)	99.40% (98.74–99.82%)	725.39 (324.78–1620.13)
Sheep	Indirect ELISA	IgG	All types		34	95.30% (90.82–98.34%)	99.48% (98.78–99.89%)	864.40 (355.88–2099.59)	
			Native	7	99.52 (98.34–99.99%)	99.70 (98.51–100%)	2514 (945.05–6687.66)		
		Recombinant (single use)	All types	14	83.99% (70.94–93.69%)	98.75% (96.57–99.86%)	134.57 (43.83–413.16)		
			Recombinant (mixed use)	7	98.75% (93.38–100%)	98.94% (95.79–100%)	1505.47 (216.23–10481.90)		
		Recombinant (chimera)	All types	6	99.23% (97.21–99.99%)	100% (99.72–100%)	7226.78 (1850.15–28228.20)		
			IgM	5	88.63% (63.97–99.81%)	98.82% (96.11–99.96%)	248.03 (45.20–1360.99)		
		Goats	Indirect ELISA	All types	All types	17	90.62% (82.19–96.55%)	98.22% (96.49–99.38%)	547.41 (169.24–1770.57)
					IgG	12	91.46% (83.02–97.19%)	97.88% (95.39–99.43%)	475.02 (132.02–1700.97)
				Recombinant (single use)	All types	7	85.53% (70.79–95.68%)	96.98% (93.71–99.08%)	194.31 (59.51–634.44)
					IgM	5	88.61% (62.40–99.92%)	98.91% (96.47–99.96%)	864.58 (46.08–16220.74)
Chickens	Indirect ELISA	IgG	All types	6	89.20% (69.76–99.23%)	92.12% (80.39–98.79%)	84.93 (19.95–361.57)		
Dogs	Indirect ELISA	IgG	All types	6	85.09% (79.32–90.06%)	96.69% (94.92–98.09%)	121.14 (49.84–294.45)		
Pigs	Indirect ELISA	All types	All types	16	76.74% (60.62–89.62%)	99.15% (97.46–99.94%)	196.46 (56.22–686.54)		
			IgG	13	75.25% (55.05–90.89%)	99.65% (98.45–100%)	314.15 (67.04–1471.98)		
		Recombinant (chimera)	All types	6	75.93% (50.61–94.05%)	99.65% (96.97–100%)	312.59 (51.88–1883.32)		
			LACA	5	67.27% (31.44–94.23%)	82.11% (71.19–90.88%)	7.58 (1.94–29.53)		
Horses	Indirect ELISA	IgG	All types	9	84.61% (70.47–94.73%)	100% (99.79–100%)	469.85 (169.66–1301.20)		
			Recombinant (chimera)	5	82.14% (59.83–96.57%)	100% (99.62–100%)	403.22 (103.86–1565.49)		
Jaguars	Indirect ELISA	IgG	All types	6	95.68% (92.75–97.88%)	89.17% (81.01–95.24%)	93.23 (40.11–216.70)		

Bold = highest Se, Sp and DOR results; ELISA = Enzyme-linked immunosorbent assay; TRFIA = Time-resolved fluorescence immunoassay; LACA = Luciferase-linked antibody capture assay; Ig = immunoglobulin; Se = Sensitivity, Sp = Specificity; DOR = Diagnostic odd ratio; CI = Confidence interval.

completely validated for all target species. Thus, our study shows that, on the contrary, there are often gaps in the development of serological techniques for the detection of *T. gondii*. For example, two articles used IFAT as a reference technique in pigs, but the test was partially marketed

since only the slides were commercially available (Macaluso et al., 2019; Athanasiou et al., 2022). Moreover, a study conducted on wild carnivores used the multispecies ID Screen Toxoplasmosis Indirect kit (IDvet, France) as a reference (Kornacka et al., 2016), which employs an

anti-multispecies IgG-HRP conjugate. However, this commercial test has not been validated for wild animals (Liyanage et al., 2021). The lack of information provided about the validation process of some commercial tests used as references, such as the IHA kit employed in pigs by Yang et al. (2022) or the LAT kit used in cats by Gao et al. (2020), is also a relevant limitation and the results should be interpreted with caution.

There were also considerable differences between human and veterinary medicine in the use of agglutination tests as reference techniques. The revolutionary appearance of agglutination tests in the 1980s (Desmonts and Remington, 1980; Dubey and Desmonts, 1987), which did not require species-specific conjugates (Wyrosdick and Schaefer, 2015), led to the generalized use of these techniques in numerous species, including humans. They are especially useful in wildlife, where it is difficult to find species-specific conjugates. Therefore, among the included studies, agglutination techniques were used more as a reference technique in animals than in humans. However, these tests are frequently performed in-house, and since the interpretation of the results could be subjective, data on the validation process followed for each species should be reviewed before their use as reference tests, or an additional technique should be used to verify the results (Donahoe et al., 2015).

Another noticeable finding is that most of the evaluated techniques used blood serum samples, even when there is a current tendency to use noninvasive samples, such as milk from dairy livestock or saliva from humans, which offer several advantages, such as simplicity of collection, less stressful and painful sampling (Pritchard, 2001; Robertson and Nicholson, 2005; Samaranyake, 2007; Khaitan et al., 2015; Valinetz and Cangelosi, 2021) or a reduction in the costs of screening programs (Pritchard, 2001; Brinkhof et al., 2010; Khaitan et al., 2015). On the other hand, meat juice samples were rarely used in animals among the included studies, even when they are useful when other samples are not accessible (e.g., hunted animals) (Ranucci et al., 2012; Coelho et al., 2015).

The validation of serological assays should be carried out by comparison with a previously validated technique or, ideally, with a gold standard (Jacobson, 1998; Thrusfield et al., 2018; WOA, 2023). In the present review, we found some misunderstanding between the terms “gold standard” and “reference test”. The “gold standard” can be defined as the technique that could lead to a perfect diagnosis of an infection status (Gardner et al., 2010). In this regard, the Sabin-Feldman dye test has historically been considered the gold standard for the diagnosis of *T. gondii* infection in humans (Liu et al., 2015; Wyrosdick and Schaefer, 2015; Ybañez et al., 2020; Uddin et al., 2021) but, according to our results, it was only employed in three studies performed in humans and one study conducted in pigs. Similarly, in studies performed in animals, Liu et al. (2015) suggested that laboratory animal bioassays are generally considered the gold standard, but they were not used in any of the articles analyzed in this study. However, despite the high Se of cat bioassays (Dubey, 2001, 2006, 2021), these techniques are not appropriate for routine validation processes due to their well-known limitations, such as the requirement of live zoites and ethical concerns related to the use of laboratory animals (Wyrosdick and Schaefer, 2015; Uddin et al., 2021). Therefore, some authors have suggested that there is no reliable gold standard in veterinary medicine (Wyrosdick and Schaefer, 2015). In this regard, in the present study, only 4 of the 47 studies conducted on animals used Bayesian latent class analysis, which allows the evaluation of the diagnostic accuracy of tests in the absence of a gold standard method with an accurate approach (Kostoulas et al., 2017, 2021; Johnson et al. 2019b; Cheung et al., 2021). Nevertheless, we found that several studies considered different techniques, such as ELISA, as the “gold standard”, probably due to the inappropriate use of these terms. In this sense, some authors considered that any of the available techniques meet high Se and Sp requirements, and they proposed changing the “gold standard” term to the “best available technique” (Finbarr Duggan, 1992; Claassen, 2005).

Regarding the employed antigens, native *T. gondii* antigens were predominant, as expected since they have been traditionally used since

the end of the 1950 s, with the first serological tests developed to detect this parasite (Frenkel and Jacobs, 1958; Fulton and Turk, 1959). However, recent reviews highlight the disadvantages of using native antigens (e.g., high cost, length of time required and difficulty of interlaboratory standardization) (Liu et al., 2015; Wyrosdick and Schaefer, 2015; Uddin et al., 2021), which could be the reason for the increasing search for recombinant antigens as alternatives, especially in animals but also in some human population groups (e.g., pregnant women and immunocompromised patients). In fact, rSAG1, rSAG2 and rGRA7 are frequently used in both humans and animals since they have been shown to be highly immunogenic (Jacobs et al., 1999; Lau et al., 2011). Specifically, rSAG1 is mostly expressed in the tachyzoite and sporozoite stages (Radke et al., 2004) and has been widely used in commercial tests (Douet et al., 2019; Liyanage et al., 2021), while rSAG2 and rGRA7 are mostly expressed in the tachyzoite and bradyzoite stages (Lekutis et al., 2000; Pfrepper et al., 2005). In addition, the combination of recombinant antigens and chimeric antigens composed of epitopes from different proteins showed better diagnostic performance than the single use of recombinant antigens (Liu et al., 2015; Wyrosdick and Schaefer, 2015; Uddin et al., 2021), which was confirmed by DTA analysis in the present meta-analysis. On the other hand, the detection of specific IgM and the use of avidity tests that allow discrimination between acute and chronic infection predominated in humans, as expected. In human medicine, the use of these tests is well established for the early detection of acute toxoplasmosis in pregnant women, with potential for therapeutic control (Emelia et al., 2014; Smets et al., 2016; Trotta et al., 2016; Laboudi and Sadak, 2017). In contrast, these tests have hardly been adapted to animals since discrimination between acute and chronic phases is not a real need in routine diagnosis of relevant host species. Clinical signs are often undetected, and when they are detected, for instance, *T. gondii*-associated abortions in sheep, they are generally attributed to recent oocyst exposure (Dubey et al., 2020b). In addition, results the MCA results are in line with previous findings regarding the differences between articles conducted on humans and those carried out on animals, showing a clear separation between the two groups of articles. This separation is influenced mainly by the variables “Agreement” and “Reference technique or method” (Dimension 1) and “Reference technique or method” and “Commercial/noncommercial (reference technique)” (Dimension 2).

This review also highlights the considerable proportion of studies that did not explain in detail the whole procedure followed and reagents employed (e.g., control sera, antigens, secondary antibodies, or the exact cutoff), which hampers the reproducibility of the study (Begley and Ioannidis, 2015). Moreover, relevant gaps related to the validation process were identified in both animals and humans. Most studies have evaluated the diagnostic performance of the developed tests by calculating their diagnostic Se and Sp values. However, only a few of them have evaluated parameters related to analytical validation (Jacobson, 1998; Andreasson et al., 2015; WOA, 2023). The limit of detection was scarcely estimated, despite its importance during proficiency testing (Jacobson, 1998; WOA, 2023). Moreover, cross-reactions between *T. gondii* antigens and specific antibodies directed against other pathogens, especially phylogenetically similar pathogens, such as apicomplexan parasites (e.g., *Neospora caninum* and *Sarcocystis* spp.) (Gondim et al., 2017), other relevant parasites and, to a lesser extent, bacteria and viruses, have not been frequently evaluated. The most relevant pathogens should be selected based on the host species to evaluate potential cross-reactivities. For example, *N. caninum* and *T. gondii* are relevant abortifacient parasites in sheep, and there is evidence for the existence of false-positive reactions between anti-*N. caninum* antibodies and the *T. gondii* SAG1 antigen (Huertas-López et al., 2021; Sánchez-Sánchez et al., 2021).

Finally, the results of DTA analysis should be considered with caution since the number of evaluated techniques was lower than 10 in some of the analyses (Cleophas and Zwinderman, 2009). Moreover, the TP, TN, FP and FN values were obtained from comparisons with a

reference technique as an assumed gold standard, which may limit the accuracy of the estimated pooled results. However, marked differences in DOR values and low Se values observed in some cases indicated different test performances that should be considered. In summary, indirect ELISA performed better in most host species evaluated; techniques that measured IgG vs. IgM showed higher Se values in humans and small ruminants, probably due to the lower persistence of detectable IgMs after *T. gondii* infection compared to IgGs (Vargas-Villavicencio et al., 2022), and tests based on chimeric antigens performed very well, which is consistent with previous studies (Liu et al., 2015; Wyrosdick and Schaefer, 2015; Uddin et al., 2021). Thus, in the future, it seems feasible that native antigens could be replaced with chimeric antigens.

The present review has several limitations. First, regarding data extraction, some variables were not easily recognizable because they were not explicitly mentioned by the authors (e.g., final purpose of the study), and this fact could make their analysis difficult. In addition, some analyses (e.g., DTA analysis for pooled Se, Sp and DOR calculations) are influenced by the number of included articles, as previously mentioned (Cleophas and Zwinderman, 2009). In this regard, humans were easily grouped, unlike animals, where a larger number of species were included. Consequently, the results of pooled Se, Sp and DOR in the present meta-analysis may be more robust in humans than in certain animal species. Moreover, DOR depends on criteria used to define infection in the examined group or species (Simundić, 2009), and as stated below, the terms “reference technique” and “gold standard” are usually misunderstood, which could influence the diagnostic performance results.

5. Conclusions

The present review confirms the wide heterogeneity of formats and reagents used in recently developed serological tests adapted for humans and animals. In principle, this finding is positive since researchers have available a wide spectrum of methodologies that can be chosen based on the target species, purpose of the technique, DOR values and laboratory facilities. However, the lack of relevant data and of a harmonized validation approach highlights the areas for improvement in the development and validation of serological techniques for the diagnosis of *T. gondii* infection to obtain more precise DOR values (at least for less frequently employed tests) and provide accurate, reproducible, and comparable results. Accordingly, several key issues should be considered in the future: i) all steps and reagents should be described in detail to ensure the reproducibility of the serological test results; ii) recombinant antigens seem to be a good alternative to native antigens, in that case a combination of recombinant antigens or chimeric antigens seem to offer better diagnostic performance; iii) a panel of well-characterized *T. gondii* seropositive and seronegative reference sera from multispecies biobanks should be ideally created and used to facilitate the development of novel tools and adaptation to different host species; iv) parameters of analytical and diagnostic validation should be assessed and appropriately documented, and with these premises, validated commercial tests would be a preferred option to be employed for comparisons as the best available tests (alone or in combination), although the most accurate method in the absence of a gold standard test would be the use of Bayesian latent class analysis; and v) ring trials and proficiency tests should be systematically implemented.

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CRedit authorship contribution statement

Ana Huertas-López: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Roberto Sánchez-Sánchez:** Writing – review & editing, Methodology, Investigation, Data curation. **Ana Cantos-Barreda:** Writing – review & editing, Methodology, Investigation, Data curation. **Carlos Martínez-Carrasco:** Writing – review & editing, Supervision, Conceptualization. **Silvia Martínez-Subiela:** Writing – review & editing, Conceptualization. **Francisco Javier Ibáñez-López:** Writing – review & editing, Software, Methodology, Formal analysis. **Gema Álvarez-García:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Conceptualization. **José Joaquín Cerón:** Writing – review & editing, Funding acquisition, Conceptualization.

Declaration of Competing Interest

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

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.vetpar.2024.110173](https://doi.org/10.1016/j.vetpar.2024.110173).

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