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RESEARCH ARTICLE

Review on diagnosis and molecular characterization of *Toxoplasma gondii* in humans and animals

Uddin, A.H.M.M.[1¶], Hossain, D.[2¶], Ahsan, M.I.[3], Atikuzzaman, M.[1], Karim, M.R.[4*]

[1]Department of Surgery and Theriogenology, Faculty of Veterinary, Animal and Biomedical Sciences, Sylhet Agricultural University, Sylhet 3100, Bangladesh
[2]Department of Medicine and Public Health, Faculty of Animal Science and Veterinary Medicine, Sher-e-Bangla Agricultural University, Dhaka 1207, Bangladesh
[3]Department of Epidemiology and Public Health, Faculty of Veterinary, Animal and Biomedical Sciences, Sylhet Agricultural University, Sylhet 3100, Bangladesh
[4]Department of Medicine, Faculty of Veterinary Medicine and Animal Science, Bangabandhu Sheikh Mujibur Rahman Agricultural University, Gazipur 1706, Bangladesh
[¶]These authors contributed equally to this work
*Corresponding author: vet_robiul@bsmrau.edu.bd

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ABSTRACT

Toxoplasma gondii is an obligate intracellular protozoon which causes toxoplasmosis, an important zoonotic disease that is endemic worldwide. Common sources of *T. gondii* infection in humans are food or water contaminated with oocysts and raw or undercooked meat with cysts. In animals, common sources of infection include feed, water, or litter contaminated with oocysts. The diagnosis and molecular characterization of *T. gondii* infection in humans and animals is crucial due to public and veterinary health importance. Various traditional and serological methods have been used in clinical practice for toxoplasmosis diagnosis, but interpreting the results remains a challenge. Several molecular techniques have also been used for the detection and genetic characterization of *T. gondii*, but primarily in research settings. In this paper, we review the techniques that are currently used for the diagnosis and genetic characterization of *T. gondii* in humans and animals, along with their advantages and disadvantages. The techniques reviewed have laid the groundwork for the future development of more effective and precise detection and characterization of *T. gondii*. These advances will contribute to a better understanding of epidemiology, prevention and control of toxoplasmosis. Thus, this review would be of particular interest to clinical physicians, veterinarians and researchers.

Keywords: *Toxoplasma gondii*; diagnosis; genetic characterization; humans; animals.

INTRODUCTION

Toxoplasma gondii is an important obligate intracellular apicomplexan zoonotic protozoa that causes toxoplasmosis in humans and other warm blooded animals worldwide (Wana *et al*., 2020a; Matta *et al*., 2021). Like other protozoan species, *T. gondii* has three infectious stages including oocysts, bradyzoites and tachyzoites (Waldman *et al*., 2020). This parasite follows both vertical and horizontal route of transmissions (Dubey *et al*., 1998). Vertical transmission mainly occurs transplacentally during pregnancy (Webster, 2010; Sun *et al*., 2013). Horizontal transmission occurs due to consumption of raw or undercooked meat and fish containing bradyzoites; consumption of water, milk, and vegetables contaminated with oocysts; or the transfusion and transplantation of blood and organs harbouring tachyzoites from clinically infected patients (Elsheikha *et al*., 2020). Felidae family (domestic cats) are the definitive hosts; although humans, livestocks, birds, and fish can all act as intermediate hosts (Wana *et al*., 2020b; Al-Malki, 2021). During the prepatent period of approximately 18 days, a single cat can shed more than 100 million oocysts (Retmanasari *et al*., 2017).

Approximately one-third of the human population is infected with *T. gondii* through either oral, and blood or congenital transmission (Abbas *et al*., 2020; Wana *et al*., 2020b). Most animals are infected by the ingestion of food and water contaminated with oocysts from cat feces (Figure 1). In adults, primary *T. gondii* infections are often asymptomatic or may produce a mild, flu-like illness. In less than 10% of infections, a mononucleosis-like syndrome occurs with headache, malaise, and fever. Some patients may also experience fatigue, lymphadenopathy or ocular toxoplasmosis (Elsheikha *et al*., 2020). Reactivation of a latent infection can cause fatal toxoplasmatic encephalitis, myocarditis, and/orpneumonitis in immunocompromised

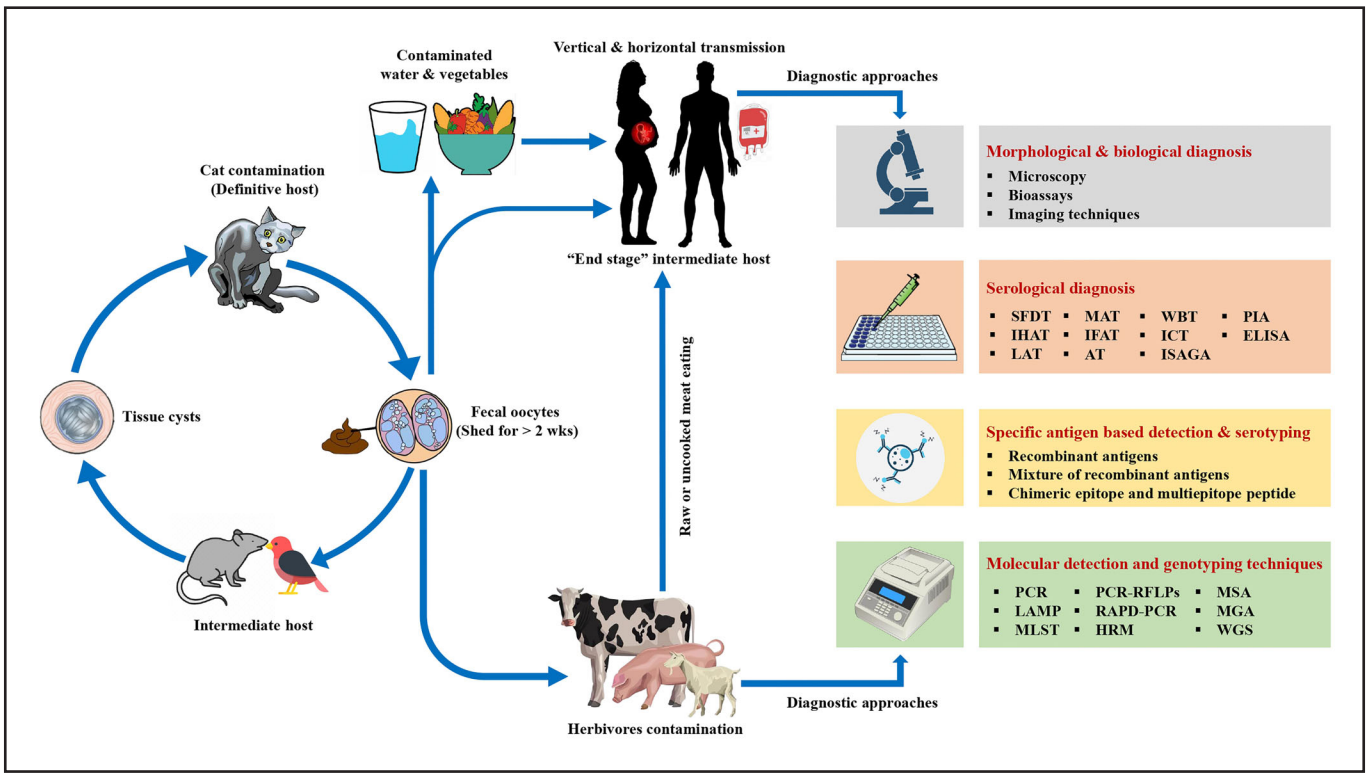

Figure 1. Transmission, life cycle and diagnostic approaches of *Toxoplasma gondii* in humans and animals. Abbreviations used: **SFDT**: Sabin-Feldman dye test, **IHAT**: Indirect hemagglutination test, **LAT**: Latex agglutination test, **MAT**: Modified agglutination test, **IFAT**: Indirect fluorescent antibody test, **AT**: Avidity test, **WBT**: Western blot test, **ICT**: Immunochromatographic test, **ISAGA**: Immunosorbent agglutination assay, **PIA**: Piezoelectric immunoagglutination assay, **ELISA**: Enzyme linked immunosorbent assay, **PCR**: Polymerase chain reaction, **LAMP**: Loop mediated isothermal amplification, **MSA**: Microsatellite analysis, **MLST**: Multilocus sequence typing, **PCR-RFLPs**: PCR-restriction fragment length polymorphisms, **RAPD-PCR**: Random amplified polymorphic DNA-PCR, **HRM**: High-resolution melting, **MGA**: Metagenomics analysis, **WGS**: Whole-genome sequencing.

individuals (Smith *et al*., 2021). The most significant clinical signs of toxoplasmosis occur in pregnant individuals, as infection can lead to abortion, stillbirth, and fetal developmental disorders (Ferra *et al*., 2020a). If infection occurs early in a pregnancy, *T. gondii* can cause a variety of newborn complications including hydrocephalus, encephalitis, chorioretinitis, intracranial calcification, and mental retardation (Onduru & Aboud, 2021).

According to numerous studies, the global prevalence of *T. gondii* is 30% in humans (Moncada & Montoya, 2012), 14.0% in cattle (Retmanasari *et al*., 2017), 35.9% in sheep (Andreoletti *et al*., 2007), 17.6% in goats (Calero-Bernal & Gennari, 2019), 25.8% in horses (Tassi, 2007), 6% in pigs (Tenter *et al*., 2000), 6 to 88% in dogs and cats (Calero-Bernal & Gennari, 2019), and 12.8% in chickens (Calero-Bernal & Gennari, 2019). However, the prevalence of *T. gondii* infections has been observed to differ from study to study, country to country, host to host, and so on. Variation among the human populations largely depends on geographical parameters (climate, relative humidity, temperature, rainfall, distance among villages, population density, and polyethnicity and monoethnicity (Dubey, 1993). *T. gondii* infection prevalence also differs based on age (Kodym, 2001) and gender (Jones *et al*., 2001). Similarly, the global distribution of *T. gondii* in different animal species depends on the geographical location (Shuralev *et al*., 2018), age (Cazarotto *et al*., 2016), gender (Djokic *et al*., 2016), herd and flock size (Herrero *et al*., 2016), mixture of animal species (Deksne *et al*., 2017), biosecurity and hygiene management (Tilahun *et al*., 2018), and disease and treatment parameters (Sun *et al*., 2015).

The diagnosis and genetic characterization of *T. gondii* is important for clinical management as well as epidemiological investigation, prevention, and control of toxoplasmosis in humans and animals (Liu *et al*., 2015; Gomes *et al*., 2020; Mohamed, 2020). Toxoplasmosis cannot be diagnosed based on clinical signs and symptoms alone (Tenter *et al*., 2000). However, parasite's latent form, during which it is not ordinarily found in blood circulation, makes the parasite challenging to isolate and diagnose (Robert-Gangneux & Dardé, 2012). *T. gondii* diagnosis is usually made by biochemical, serological, histological, molecular tests, or by a combination of the above (Pal *et al*., 2014). Bioassays and serological tests are used to diagnose *T. gondii* infection in the laboratory environment (Liu *et al*., 2015). However, these tests are time consuming, laborious, expensive, health hazards, and require skilled personnel, all of which limits their routine use (Kotresha & Noordin, 2010). Over the last two decades, consistent developments in serologic and molecular diagnostic methods with increased sensitivity and specificity have been a significant achievement in the detection of *T. gondii* infections (Ybañez *et al*., 2020). There is, however, no comprehensive review of the methods of *T. gondii* diagnosis and molecular characterization (Rostami *et al*., 2018; Ferra *et al*., 2020b). Such a review would help researchers and manufacturers in considering future research avenues for *T. gondii* diagnosis and genetic characterization. In this paper, we attempt to provide an updated and comprehensive review of the diagnosis and molecular characterization of *T. gondii* in humans and animals.

Literature search strategy

Multiple science databases (Science Direct, Web of Science, PubMed, Scopus, and Google Scholar) were screened for literature on the diagnosis and genetic characterization of *T. gondii*. The following keywords, used alone or in combination: '*Toxoplasma gondii*', 'toxoplasmosis', 'diagnosis', 'serodiagnosis', 'molecular diagnosis', 'genotyping', 'serotyping', 'humans' or 'animals'. Two authors independently undertook this searh, with studies screened via titles and abstracts followed by full-text review. After removing duplicates and irrelevant papers, reference lists of full-text articles were assessed for potentially useful citations not identified through the database search. The article screening was performed with Endnote software (version X9). Articles published up to April 30, 2021 were included in this review.

Morphological and biological diagnosis

Microscopy

Toxoplasma gondii can be identified using light microscopy in body fluids (cerebrospinal fluid, amniotic fluid, aqueous humour, bronchoalveolar lavage fluid and semen) (Liu *et al*., 2015), feces (Lilly & Wortham, 2013), and biological specimens (brain tissues, lymph nodes and muscles) (McGovern & Wilson, 2013). Haematoxylin and eosin (H&E) and giemsa stains are usually used for microscopic identification of tachyzoites and bradyzoites. Periodic acid schiff (PAS) stain is commonly used for the identification of amylopection granules in bradyzoites (da Silva *et al*., 2010). However, Romanowsky stains also work well for the detection of tachyzoites in impression smears, and silver stain is a reliable option for detecting bradyzoites in tissue cysts. Electron microscopy has also been used to diagnose toxoplasmosis, although limitations of this technique include expense, low sensitivity, need for advanced training, and challenging regular operation (Liu *et al*., 2015).

Bioassays

Bioassays are the gold standard for the detection of *T. gondii* infection. They are performed by inoculating oocysts into an animal model and monitoring for infection (Hill *et al*., 2006). Murine and cat bioassays are commonly used for isolation and identification of *T. gondii* (Liu *et al*., 2015). Cat bioassays are considered standard because they are more powerful than murine bioassays (Rousseau *et al*., 2018). With cat bioassays, even low bradyzoites containing tissue samples, if fed, can lead to oocyst shedding in feces (Dubey, 2006). In the murine model bioassay; GKO mice (Opsteegh *et al*., 2016), C57BL/6 mice (Tyebji *et al*., 2020; Bergersen *et al*., 2021), WT-BALB/c mice (Bergersen *et al*., 2021), SCID mice and Swiss Webster mice (Watson & Davis, 2019) are used in various laboratories. To overcome the disadvantages of bioassays (expensive, time consuming, requires live parasite and a large number of mice), reserachers also developed serological and molecular methods to detect parasitic antigen or antibody and DNA directly from the samples (Opsteegh *et al*., 2020). Several researchers have suggested that bioassay methods do not meet animal ethical regulations (Rousseau *et al*., 2018), and that the use of bioassays in laboratories should be limited (Riaz *et al*., 2016).

Imaging techniques

Imaging techniques are include visual assessment of different organs, such as the brain and eye that facilitate toxoplasmosis diagnosis and can be monitored to assess therapeutic effect (Vutova *et al*., 2002). Computed tomography (CT) (Harrison & Hulette, 2017), magnetic resonance imaging (MRI) (Khalili *et al*., 2021), nuclear imaging (Rostami *et al*., 2019) and ultrasonography (US) (Rostami *et al*., 2019; De-la-Torre & Gómez-Marín, 2020) are commonly used for diagnosis of cerebral and ocular toxoplasmosis (Liu *et al*., 2015). Unfortunately, the results of imaging techniques are not yet satisfactory and this drives the researchers to explore more reliable imaging techniques (Rostami *et al*., 2018). CT scan is able to identify nodular lesions on the basal ganglion in central nervous system (CNS) toxoplasmosis. In congenital toxoplasmosis, cyst-like noduar calcified lesions develop in the periventriculus region and the choroid plexus and are appeared on CT scan (Masamed *et al*., 2009). MRI is most helpful for differentiation between various CNS lesions to determine the extent of CNS damage (Scheld *et al*., 2014). Prenatal US can also be used to identify brain lesions in congenital toxoplasmosis. Abdominal US can show hepatosplenomegaly and abdominal lymphadenopathy, which occurs with some cases of toxoplasmosis (Lago *et al*., 2007). Recently, nuclear imaging tools, such as fluorodeoxyglucose positron-emission tomography (FDG-PET), thallous chloride (201Tl) and technetium-99m (99mTc) sestamibi (MIBI), have been developed and used to differentiate the CNS toxoplasmosis from CNS lymphoma in HIV-positive patients (Rostami *et al*., 2018). Other advanced imaging techniques, such as fundus autofluorescence (FAF) (Lavinsky *et al*., 2012), confocal scanning laser ophthalmoscopy (CSLO) (Rostami *et al*., 2018), optical coherent tomography (OCT) (Lavinsky *et al*., 2012), fluorescent angiography (FA) (Rostami *et al*., 2018), ultrasound (Rostami *et al*., 2018), and indocyanine green angiography (ICG) (Lavinsky *et al*., 2012) are useful for the diagnosis and therapeutic management of ocular toxoplasmosis.

Serological diagnosis

Sabin-Feldman dye test (SFDT)

The Sabin-Feldman dye test was first developed by Sabin and Feldman (1948). The SFDT is performed by combining serum from the individual being tested with live tachyzoites (obtained from the peritoneal effusion of infected mice). This combination is then stained with methylene blue. If the serum contains anti-*Toxoplasma* antibodies, they will coat the tachyzoites and prevent them from taking up the stain (Reiter-Owona *et al*., 1999; Rorman *et al*., 2006). Although the SFDT has a high sensitivity and specificity, it is performed in very few laboratories due to the significant challenges associated with it (Udonsom *et al*., 2010). The test requires live tachyzoites (Udonsom *et al*., 2010), experienced technicians, and a sophisticated reference laboratory (Reiter-Owona *et al*., 1999).

Indirect hemagglutination test (IHAT)

Indirect hemagglutination test (IHAT) for diagnosis of toxoplasmosis was first proposed by Boyden (1951), and Stavitsky and Jarchow (1954) and later developed by Jacobs and Lunde (1957). The test uses sheep red blood cells (RBCs) to detect antigens of *T. gondii* infections. Agglutination of RBCs by *Toxoplasma gondii* antigens indicates a positive result, and lack of agglutination indicates a negative result (Su *et al*., 2010; Webster, 2010). IHAT is safe, rapid, and easy to perform (Caruana, 1980). Despite these advantages, IHAT has a low sensitivity and hence is not recommended for the diagnosis of *T. gondii* infection in chickens (Yan *et al*., 2010).

Latex agglunination test (LAT)

The latex agglunination test (LAT) detects *T. gondii* antibodies (IgG and IgM). Tachyzoites are inoculated into either cell

cultures or mice to stimulate antigen production. Antigens are then detected by commercial antigen kits (Jiang *et al*., 2008). The microneme protein 3 (MIC3) based latex agglutination test, a derivative of the traditional LAT, is considered to be more reliable for toxoplasmosis serodiagnosis (Jiang *et al*., 2008). The MIC3 surface protein is expressed in multiple stages of *T. gondii* (tachyzoites, bradyzoites and sporozoites). It facilitates attachment between host cell receptors and parasite surface proteins (Garcia-Réguet *et al*., 2000). Mice (Ismael *et al*., 2003) and human sera (Garcia-Réguet *et al*., 2000) detected by *T. gondii* microneme proteins, are used as antigens (Sager *et al*., 2003; Pietkiewicz *et al*., 2004).

Modified agglutination test (MAT)

The modified agglutination test (MAT) was first developed by Fulton and Turk (1959). It was later modified by several researchers for the detection of *T. gondii* antibodies in humans (Su *et al*., 2010; Al-Adhami *et al*., 2016) and animals (Dubey & Desmonts, 1987). The test is more specific, rapid, and cost effective than other serological tests (Webster, 2010). Tachyzoites, produced by intraperitoneal inoculation in mice, are used for MAT. Positive results are confirmed by agglutination of the tachyzoites (Fernandes *et al*., 2019). 2-mercaptoethanol, alkaline buffer, Evan's blue dye, and TgMAT antigen are added to the antigen mixture, which diminishes the IgM antibodies in serum samples (Dubey, 1997).

Indirect fluorescent antibody test (IFAT)

Indirect fluorescent antibody test (IFAT) is an easy and reliable test for *T. gondii* (Rorman *et al*., 2006). It was first described by Camargo (1964). The test is a two-step immunologic reaction. First, tachyzoites bind with anti-*Toxoplasma* immunoglobulins in a patient's serum. Second, the bound complex is identified by a fluorescein labelled anti-whole globulin. The reaction is examined by fluorescence microscopy and the result is expressed in IU/mL as recommended by World Heath Organization (WHO). Fluorescence indicates a positive result, while lack of it indicates a negative result. If serum contains rheumatoid factors or antinuclear antibodies, there is a high chance of false-positive results. So caution must be taken when interpretting IFAT results (Rorman *et al*., 2006).

Enzyme linked immunosorbent assay (ELISA)

Enzyme linked immunosorbent assay (ELISA) is a widely used diagnostic tool for the detection of antigenic markers and active antibodies with satisfactory sensitivity and specificity (Liu *et al*., 2015). There are three basic components of an ELISA: a solid phase antigen or antibody, an enzyme-coated antigen or antibody, and a substrate for the enzymatic reaction (Liu *et al*., 2015). Different types of ELISA tests, including indirect ELISA and sandwich ELISA, can be used in the serodiagnosis of *T. gondii*. Indirect ELISA is performed using a microtitre plate to which the antigen of interest directly adheres, while sandwich ELISA uses antibodies already adhered to the plate to bind the antigen of interest. The antigen is then identified by a primary antibody to which an enzyme-conjugated secondary antibody binds. A substrate is added, allowing the enzyme to create a color reaction. IgG, IgM and IgA anti-*Toxoplasma* antibodies are frequently identified using ELISA. Tachyzoites lysate antigen (TLA) is typically used for indirect ELISA diagnosis to *T. gondii* infections (Liu *et al*., 2015). However, due to lack of standardization, the results of TLA-based ELISA are contradictory among different laboratories, experiments, and batches. Recently, a number of recombinant antigens have been identified: surface antigens SAG1 (Burg *et al*., 1988; Kotresha *et al*., 2012) and SAG2 (Hiszczyñska-Sawicka *et al*., 2005); matrix protein (MAG1) (Holec *et al*., 2007); microneme proteins MIC2 (Beghetto *et al*., 2006), MIC3 (Beghetto *et al*., 2003), MIC4 (Sardinha-Silva *et al*., 2019), and MIC5 (Saouros *et al*., 2012); rhoptry proteins ROP1 (Holec-Gasior *et al*., 2009) and ROP2 (Aubert *et al*., 2000); and granule antigens GRA1 (Hiszczyñska-Sawicka *et al*., 2003; Pietkiewicz *et al*., 2004), GRA2 (Holec-Gasior *et al*., 2009; Lau *et al*., 2012; Ching *et al*., 2013), GRA4 (Lau *et al*., 2010a; Hanafiah *et al*., 2020), GRA6 (Redlich & Müller, 1998; Hiszczyñska-Sawicka *et al*., 2005), GRA7 (Hiszczyñska-Sawicka *et al*., 2003; Selseleh *et al*., 2012), GRA8 (Hiszczyñska-Sawicka *et al*., 2003) and GRA9 (Nockemann *et al*., 1998). These recombinant antigens have higher sensitivity and specificity for *T. gondii* diagnosis (Aubert *et al*., 2000; Li *et al*., 2000). The sandwich ELISA with TLA and recombinant P35 is commonly used for the detection of human IgM antibodies and acute infection (Suzuki *et al*., 2000; Lu *et al*., 2006). With all ELISA tests, positive or negative results are determined by the correlation between the optical densities of the control serum. The modified ELISA, also called the dot-ELISA, is more sensitive, specific, easier to perform, as it does not require sophisticated equipment.

Immunosorbent agglutination assay (ISAGA)

The immunosorbent agglutination assay (ISAGA) is the most precise test for detecting IgM, IgA, and IgE antibodies against *T. gondii* (Stepick-Biek *et al*., 1990). *Toxoplasma gondii* tachyzoites are used as the antigen for this test. This test is named based on the antibody that it identifies, such as IgM-ISAGA, IgA-ISAGA, and IgE-ISAGA (Desmonts & Thulliez, 1985; Stepick-Biek *et al*., 1990). IgM-ISAGA is used for the diagnosis of acute and congenital toxoplasmosis (Desmonts & Thulliez, 1985). IgE-ISAGA is used for the diagnosis of acute toxoplasmosis (Pinon *et al*., 1990), toxoplasmic encephalitis and chorioretinitis (Remington *et al*., 2004). IgA-ISAGA is used for the diagnosis of prenatal, neonatal, and postnatal congenital toxoplasmosis (Stepick-Biek *et al*., 1990).

Immunochromatographic test (ICT)

Immunochromatographic test (ICT) is a rapid, easy, and cost-effective assay with high sensitivity and specificity. ICT identifies target antigens (or sometimes antibodies) from serum or blood samples. ICT uses colloidal gold-labeled antibody (CGLA) dipped on a nitrocellulose membrane as a test surface. CGLA binds with the target antigen (mobile phase), then progresses through an immobile phase using capillary flow, and, finally, antibody-antigen complexes show a color reaction (Wang *et al*., 2011). Sensitivity and specificity of this test have been reported as 100% in the USA, and 97% and 96%, in France (Begeman *et al*., 2017). Due to high sensitivity and specificity, ICT is an important tool for diagnosing toxoplasmosis in humans (Wassef & Abdel-Malek, 2019) and animals (Khan & Noordin, 2020). A modified ICT, based on recombinant TgGRA7, has been developed for rapid detection of *T. gondii* in the field setting (Terkawi *et al*., 2013). A dynamic flow immunochromatographic test (DFICT) has also been developed for the rapid detection of *T. gondii* infection in dogs and cats (Jiang *et al*., 2015). This test combines techniques from immunochromatography and fluid dynamics. DFICT is attractive due to its efficiency, low sample volume requirements, and high selectivity. A list of ICTs used for the diagnosis of *T. gondii* infections in humans and animals is presented in Table 1.

Table 1. Summary of immunochromatographic (ICT) tests used for diagnosis of toxoplasmosis in humans and animals

Antigen/ antibody	Target molecules	Reference test	Host	Results	References
Whole-cell lysate	➢ IgG ➢ IgM	➢ SFDT ➢ IgM ELISA	Human	Sensitivity: 100% Specificity: 100%	(Begeman *et al*., 2017)
Recombinant antigen	➢ IgG ➢ IgM	➢ SFDT ➢ IgM ELISA	Human	IgG-Sensitivity: 100% IgG-Specificity: 96.3% IgM-Sensitivity: 62.2% IgM-Specificity: 88.5%	(Gomez *et al*., 2018)
Recombinant antigen	➢ IgG ➢ IgM	➢ SFDT ➢ IgM ELISA	Human	IgG-Sensitivity: 100% IgG-Specificity: 97.5% IgM-Sensitivity: 28% IgM-Specificity: 97.6%	(Gomez *et al*., 2018)
N-terminal rSAG1A + GRA2	➢ IgG	➢ ELISA	Human	Sensitivity: 97.1% Specificity: 100%	(Song *et al*., 2013)
rSAG1	➢ IgG	➢ ELISA	Cat	Sensitivity: 100% Specificity: 99.4%	(Chong *et al*., 2011)
rtSAG2	➢ IgG	➢ ELISA ➢ LAT	Cat	ELISA-Sensitivity:97.2% ELISA-Specificity:95.8% LAT-Sensitivity: 100% LAT-Specificity:94.5%	(Huang *et al*., 2004)
SAG3	➢ IgG	➢ ELISA	Pig	Sensitivity: 100% Specificity: 99.65%	(Luo *et al*., 2018)

Western blot (WB)

In Western blot, gel electrophoresis (12% polyacrylamide gel) is used to separate serum proteins according to their molecular weight. A primary antibody is added which binds to the protein of interest. Then, a second antibody, conjugated with an enzyme, is added which binds to the primary antibody and facilitates identification of the protein of interest. For toxoplasmosis diagnosis, the proteins of interest are *T. gondii* antibodies (Meek *et al*., 2003). Western blot is useful for the diagnosis of acute and congenital toxoplasmosis because of its ability to detect low antibody levels (Sardinha-Silva *et al*., 2019).

Piezoelectric immunoagglutination assay (PIA)

Piezoelectric immunoagglutination assay is a new development in serodiagnosis of *T. gondii* infection. It is based on the LAT principle (Wang *et al*., 2004). For this method, a piezoelectric quartz crystal (PQC) is responsible for the bio-molecular interaction and quantification. Gold nanoparticles are substituted for latex particles to make the test easier, more reliable, and more sensitive for toxoplasmosis diagnosis. The agglutination properties of gold nanoparticle suspensions create large variations in absorption spectrum and are used to develop agglutination based diagnostic methods. The agglutination complex of antigen-coated gold nanoparticles and antibodies is viewed by transmission electron microscopy (TEM). PIA can be used to detect *Toxoplasma* antibody in both blood and serum samples (Wang *et al*., 2004).

Avidity test

The term avidity indicates 'functional affinity', which is the binding potency of antigens and antibodies (Hedman *et al*., 1993). This test is performed by different serological procedures, such as enzyme linked immunosorbent assay (ELISA) and Western blot (WB) (Liu *et al*., 2015). The results of avidity tests are low during the early stage of infection and higher with progression of infection. Thus, the avidity test can distinguish acute and chronic infection of *T. gondii* (de Ory *et al*., 1995). However, there are limitations to the avidity test. *Toxoplasma gondii*-specific low-avidity IgG antibodies may persist for months pregnant women and treatment of *T. gondii* may delay the avidity maturation during pregnancy (Meroni *et al*., 2009). High concentrations of antibodies in serum samples may also affect the results of avidity testing. It is necessary to improve detection methods of antibody avidity (Bonyadi & Bastani, 2013).

Specific antigen-based detection and serotyping

Recombinant antigens

Recombinant antigens are developed based on specific proteins from *T. gondii* that have been encoded, cloned, and expressed by different expression systems. Many of these proteins were originally identified as antibodies in human and animal serum samples (Rostami *et al*., 2018; Ferra *et al*., 2020a). The antigens include surface antigens SAG1 (P30) (Kotresha *et al*., 2012; Bachan *et al*., 2018), SAG2 (P22) (Singh *et al*., 2014; Sudan *et al*., 2019), and SAG3 (P43) (Khanaliha *et al*., 2012); dense granule antigens GRA1 (P24) (Hiszczyñska-Sawicka *et al*., 2003), GRA2 (P28) (Holec-Gasior *et al*., 2009; Lau *et al*., 2012; Ching *et al*., 2013), GRA4 (Lau *et al*., 2010a), GRA5 (Holec-Gasior & Kur, 2010), GRA6 (P32) (Redlich & Müller, 1998; Hiszczyñska-Sawicka *et al*., 2005), GRA7 (P29) (Selseleh *et al*., 2012; Wang *et al*., 2014a; Cai *et al*., 2015), GRA8 (P35) (Aubert *et al*., 2000; Hiszczyñska-Sawicka *et al*., 2005; Lu *et al*., 2006; Duong *et al*., 2020), and GRA9 (B10/P41) (Nockemann *et al*., 1998); the rhoptry antigens ROP1 (P66) (Aubert *et al*., 2000; Holec-Gasior *et al*., 2009), ROP2 (P54) (Nigro *et al*., 2003), ROP5 (Grzybowski *et al*., 2015), ROP8 (Sonaimuthu *et al*., 2014) and ROP18 (Grzybowski *et al*., 2015); the matrix antigen MAG1 (Holec *et al*., 2007; Zhuo *et al*., 2017); M2AP (Beghetto *et al*., 2006); and the micronene protein MIC1 (Holec *et al*., 2008) (Table 2). In earlier studies, TLA was a commonly used antigen in the serodiagnosis of *T. gondii* (Holec-Gasior, 2013). However, several limitations of TLA (high expense, time-consuming to make and health hazards for laboratory personel) restrict its current use. Nonetheless, the recombinant antigen-based diagnosis of toxoplasmosis mazimizes output. It is also easy to handle and able to differentiate disease phases that accelerate the diagnosis and treatment.

Table 2. Recombinant antigens used for the diagnosis of *T. gondii* infection in humans and animals

Antigen	Expression system	Assay	Type of infection	Sensitivity (%)	Specificity (%)	Host	References
GRA1	*E. coli*	IgG	➢ Acute ➢ Chronic	Acute: 83.3 Chronic: 77.8	-	Human	(Pietkiewicz *et al*., 2004)
GRA2	*E. coli*	IgG ELISA	➢ Acute ➢ Chronic	Acute (France): 95.8 Chronic (France): 65.7 Acute (Iran): 100 Chronic (Iran): 71.4	-	Human	(Saadatnia & Golkar, 2012)
	E. coli	IgG ELISA	➢ Acute ➢ Chronic	Acute: 100 Chronic: 22.5	-	Human	(Holec-Gasior *et al*., 2009)
GRA3	*E. coli*	IgG ELISA	➢ Congenital	82	100	Human	(Beghetto *et al*., 2006)
GRA4	*E. coli*	IgG ELISA	➢ Acute ➢ Chronic	Acute: 58.3 Chronic: 18.2	-	Human	(Nigro *et al*., 2003)
GRA5	*E. coli*	IgG ELISA	➢ Acute ➢ Post-acute ➢ Chronic	Acute: 63 Post-acute: 50 Chronic: 75	-	Human	(Holec-Gasior & Kur, 2010)
GRA6	*E. coli*	IgG ELISA	➢ Acute ➢ Chronic	89	99.6	Human	(Redlich & Müller, 1998)
	E. coli	IgG ELISA	➢ Acute ➢ Chronic	Acute: 93.9 Chronic: 63.1	-	Human	(Hiszczyńska-Sawicka *et al*., 2005)
	E. coli	IgG ELISA	➢ Acute ➢ Chronic	Cutoff mean +2SD Acute: 95.8 Chronic: 44.1 Cutoff mean + 3SD Acute:87.5 Chronic:5.9	-	Human	(Golkar *et al*., 2008)
GRA7	*E. coli*	IgG ELISA	-	96.4	98.6	Cattle	(Udonsom *et al*., 2021)
GRA7	*E. coli*	IgG ELISA	➢ Acute ➢ Chronic	Acute: 94 Chronic:79	98	Human	(Jacobs *et al*., 1998)
	E. coli	IgG ELISA	➢ Acute ➢ Chronic ➢ Recent seroconversion	83.4	-	Human	(Aubert *et al*., 2000)
	E. coli	IgG ELISA	➢ Acute ➢ Chronic	Acute: 75 Chronic: 36.3	-	Human	(Nigro *et al*., 2003)
	E. coli	IgG ELISA	➢ Acute ➢ Chronic	Acute: 95.9 Chronic: 68.9	-	Human	(Pietkiewicz *et al*., 2004)
	E. coli	IgG ELISA	➢ Congenital	88	100	Human	(Beghetto *et al*., 2006)
	E. coli	IgG immunoblot	➢ Acute ➢ Chronic	Acute: 100 Chronic: 40	-	Human	(Kotresha *et al*., 2012)
GRA7	*E. coli*	TLA-ELISA	-	84.6	99.3	Cat	(Cai *et al*., 2015)
	E. coli	GRA7-ELISA	-	89.7	92.5		

GRA7	*E. coli*	IgG ICT IgG ELISA	-	100	100	Cat	(Ybañez *et al.*, 2020)
GRA7	*E. coli*	GRA7-ELISA	-	96.4	98.6	Dog	(Wang *et al.*, 2014a)
	E. coli	TLA-ELISA	-	95.5	91.1	Dog	
GRA8	*E. coli*	IgG ELISA	➢ Acute ➢ Chronic ➢ Recent seroconversion	70	-	Human	(Aubert *et al.*, 2000)
	E. coli	IgM ELISA	➢ Chronic ➢ Recent seroconversion	54.9	-		
	E. coli	IgM ELISA	➢ Acute ➢ Chronic	Acute: 90	-	Human	(Suzuki *et al.*, 2000)
	E. coli	IgG ELISA	➢ Acute ➢ Chronic	Acute: 85.3 Chronic: 8	-	Human	(Li *et al.*, 2000)
	E. coli	IgG ELISA	➢ Acute ➢ Chronic	Acute: 86.7 Chronic: 54.5	-	Human	(Hiszczyńska-Sawicka *et al.*, 2005)
	E. coli	IgM ELISA	➢ Acute ➢ Recently seroconverted ➢ Persistent IgM-positive ➢ Chronic	Acute: 100 Recently seroconverted: 88 Persistent IgM-positive: 16 Chronic: 4	-	Human	(Lu *et al.*, 2006)
GRA8	*E. coli*	Purified Nluc-GRA8-LACA	-	90.5	95.4	Chicken	(Duong *et al.*, 2020)
	E. coli	Unpurified Nluc-GRA8-LACA	-	85.7	96.9		
	E. coli	GRA8-ELISA	-	85.7	84.6		
H11	*E. coli*	H11-ELISA	-	34	89	Sheep	(Sager *et al.*, 2003)
	E. coli	P30-ELISA	-	96	100		
	E. coli	TEA-ELISA	-	96	100		
M2AP	*E. coli*	IgG ELISA	➢ Congenital	78	100	Human	(Beghetto *et al.*, 2006)
MAG1	*E. coli*	IgG ELISA	➢ Acute ➢ Chronic	Acute: 97.3 Chronic: 7.5	-	Human	
MAG1	*E. coli*	MAG1-ELISA	-	93.9	98.3	Dog	(Zhuo *et al.*, 2017)
	E. coli	TLA-ELISA	-	87.8	96.7		
MIC2	*E. coli*	IgG ELISA	➢ Congenital	92	100	Human	(Beghetto *et al.*, 2006)
	E. coli	IgG ELISA	➢ Congenital	90	100	Human	
P68	*E. coli*	IgG ELISA	➢ Acute ➢ Chronic ➢ Recent seroconversion	69.2	-	Human	(Aubert *et al.*, 2000)
	E. coli	IgM ELISA	➢ Chronic ➢ Recent seroconversion	18.3	-		
ROP1	*E. coli*	IgG ELISA	➢ Acute ➢ Chronic ➢ Recent seroconversion	59.1	-		
	E. coli	IgM ELISA	➢ Chronic ➢ Recent seroconversion	58.5	-		

	E. coli	IgG ELISA	➢ Acute ➢ Chronic	Acute: 94.6 Chronic: 15.5	-	Human	(Holec-Gasior *et al.*, 2009)
	E. coli	IgG avidity	➢ Routine screening	Acute: 85 Chronic: 25	-	Human	(Holec-Gasior *et al.*, 2010)
ROP2	*E. coli*	IgG ELISA	➢ Acute ➢ Chronic ➢ Recent seroconversion	58.3	-	Human	(Aubert *et al.*, 2000)
	E. coli	IgM ELISA	➢ Chronic ➢ Recent seroconversion	12.7	-		
	E. coli	IgG ELISA	➢ Acute ➢ Chronic	Acute: 83.3 Chronic: 54.5	-	Human	(Nigro *et al.*, 2003)
ROP5	*E. coli*	IgG ELISA	➢ Acute ➢ Chronic	23	100	Human	(Grzybowski *et al.*, 2015)
ROP8	*E. coli*	IgG Western Blot	➢ Early acute ➢ Acute ➢ Chronic	Early acute: 90 Acute : 92 Chronic: 82	94	Human	(Sonaimuthu *et al.*, 2014)
ROP18	*E. coli*	IgG ELISA	➢ Acute ➢ Chronic	38	93	Human	(Grzybowski *et al.*, 2015)
SAG1	*E. coli*	IgG ELISA	➢ Acute ➢ Chronic ➢ Recent seroconversion	74.1	-	Human	(Aubert *et al.*, 2000)
	E. coli	IgM ELISA	➢ Chronic ➢ Recent seroconversion	10.6	-		
	E. coli	IgG ELISA	➢ Acute ➢ Chronic	Acute: 98.6 Chronic: 100	-	Human	(Pietkiewicz *et al.*, 2004)
	E. coli	IgG ELISA	-	82	100	Human	(Beghetto *et al.*, 2006)
	E. coli	IgG ELISA	-	88.4	88	Human	(Jalallou *et al.*, 2010)
	E. coli	IgG ELISA	-	93	95	Human	(Selseleh *et al.*, 2012)
	E. coli	IgM ELISA	-	87	95		
	E. coli	IgG ELISA	-	100	100	Human	(Bel-Ochi *et al.*, 2013)
SAG1	*E. coli*	IgG ELISA	-	92.7	90.7	Goat	(Bachan *et al.*, 2018)
SAG2	*E. coli*	IgG ELISA	➢ Acute ➢ Chronic	Acute: 93.9 Chronic: 96.5	-	-	(Hiszczyńska-Sawicka *et al.*, 2005)
	Pichia pastoris	IgG /IgM ELISA	➢ Early acute ➢ Acute ➢ Chronic	Early acute: 80 Acute: 95 Chronic: 100	100	-	(Lau & Fong, 2008)
SAG2A		IgG ELISA IgG avidity	➢ Acute ➢ Chronic	Acute: 90 Chronic: 67	100		(Béla *et al.*, 2008)
SAG2	*E. coli*	IgG ELISA	-	81.3	85.7	Sheep	(Singh *et al.*, 2014)
SAG2	*E. coli*	IgG ELISA	-	87.1	85.7	Cattle	
SAG2	*E. coli*	IgG ELISA	-	80	88.6	Cattle	(Sudan *et al.*, 2019)
SAG2	*E. coli*	IgG ELISA	-	82.1	91.4	Cattle, Sheep & Goat	(Singh *et al.*, 2014)

LACA = Luciferase-linked antibody capture assay.

Mixture of recombinant antigens

Use of a recombinant antigen mixture in the serodiagnosis of *T. gondii* showed better performance than use of a single recombinant antigen (Jacobs *et al.,* 1999). Johnson *et al*. (1992) reported that the mixture of recombinant proteins H4/GST and H11/GST showed higher sensitivity (81.3%) than individual tests of H4/GST (54%) and H11/GST (61%) in IgM ELISA. Similarly, GRA7 and Tg34AR antigens separately had a sensitivity of 81% and 88% respectively. However, a mixture of these two antigens had a 96% sensitivity (Jacobs *et al*., 1999). At present, many recombinant protein mixtures are used in research and laboratories for the detection of *T. gondii* in humans and animals (Table 3).

Chimeric epitope and multiepitope peptide

Recently, chimeric antigens have been considered as a new diagnostic strategy for the detection of *T. gondii* in humans and animals. These chimeric antigens are proteins with immunoreactive epitopes from selected antigens of *T. gondii*. These immunoreactive epitopes have highly exposed protein surfaces which are readily recognized by the receptors of T cells and B cells (Saha & Raghava, 2006). Researchers developed the predictive chimeric epitope based on computer modeling of *T. gondii*. Modeling depends on the physio-chemical properties of amino acids and predicts the location and arrangement of peptide epitopes. Predictive software techniques were also used to evaluate the antigenic properties of chimeric epitopes from different *T. gondii* antigens (Dai *et al*., 2012; Wang *et al*., 2014b). Several research groups applied advanced techniques to recognize epitopes, such as phage display of cDNA libraries, epitope mapping, and monoclonal antibodies reactions (Cardona *et al*., 2009). Beghetto *et al*. (2006) used IgM Rec-ELISAs based on GST-EC2 and GST-EC3 chimeric antigens to diagnose toxoplasmosis in both acquired and congenital patients and reported 70% positive results with IgM Rec-ELISA and 35% positive results with commercially available assays (ELFA-IgM bioMérieux, France or ETI-TOXOK-M Diasorin, Italy). This provides strong evidence for the efficacy of this type of antigen. In another study, Holec-Gasior *et al*. (2012) found a higher sensitivity of IgG-ELISA for MIC1-MAG1 recombinant chimeric antigen (90.8%) than individual recombinant antigens rMAG1 (60%), rMIC1ex2 (75.5%), or their mixture (69.1%). It was also found that trivalent recombinant MIC1-MAG1-SAG1 chimeric antigen had a more satisfactory result than MIC1, MAG1, or bivalent recombinant MIC1-MAG1 chimeric antigen. These findings suggest that chimeric antigens formed with more protein units may be more effective (Holec-Gasior *et al*., 2012a). Ferra *et al*. (2015a) used five trivalent recombinant chimeric antigens (MIC1-MAG1-SAG1S, SAG1L-MIC1-MAG1, SAG2-GRA1-ROP1S, SAG2-GRA1-ROP1L, and GRA1-GRA2-GRA6) in IgG-ELISA to detect *T. gondii* in horses, pigs, and sheep. Their team found a sensitivity and specificity ranging from 90% to 100% (Ferra *et al*., 2015a). Recently, a group of researchers developed tetravalent chimeric antigens from SAG2, GRA1, ROP1, and AMA1 antigen precursors (SAG2-GRA1-ROP1-AMA1N, AMA1N-SAG2-GRA1-ROP1, AMA1C-SAG2-GRA1-ROP1, and AMA1-SAG2-GRA1-ROP1) which have 100% sensitivity and specificity in IgG-ELISAs (Tomasz *et al*., 2019). Moreover, combination of the peptide microarray analysis and predictive computer programing is an advanced and robust tool for the diagnosis of *T. gondii*. In this combined technology B cell epitopes, such as SAG1, SAG2, SAG3, GRA5, GRA6 and P35 are frequently used (Dai *et al*., 2012). Three recombinant epitopes (SAG1-EP2, SAG2-EP1, and SAG3-EP2, derived from SAG1, SAG2 and SAG3, respectively) were used for the development of a recombinant multiepitope fusion peptide (rMEP), which is more effective at distinguishing between recent and past *T. gondii* infection (Dai *et al*., 2012, 2013). In addition, two recombinant multiepitope based antigens, $ROP1_L$ and USM.TOXO1 were derived from SAG2, GRA1, ROP1 and SAG1, GRA2, GRA7, respectively. The recombinant antigens had 100% sensitivity and specificity in IgG-ELISA and Western blot (Ferra *et al*., 2015b; Hajissa *et al*., 2015). In an additional validation study with 157 human serum samples, USM.TOXO1 had a sensitivity of 85.43% and specificity of 81.25% (Hajissa *et al*., 2017). The chimeric epitopes and multiepitope peptides used for the diagnosis of *T. gondii* infection in humans and animals are listed in Table 4.

Molecular detection and genotyping techniques

Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) is widely used for the identification of *T. gondii* infection since it was first used by Burg *et al*. (1989) to target the B1 gene (Marques *et al*., 2020; Ngui *et al*., 2020; Tehrani *et al*., 2020). It is also accounted for higher target gene fragments amplifications, sensitivity and specificity. However, sensitivity and specificity are also affected by other factors such as DNA extraction technique, the primers, and the parameters of the amplification reaction (Edvinsson *et al*., 2004). There are a variety of PCR techniques, including conventional, nested, and real-time PCRs (Fallahi *et al*., 2014a; Marques *et al*., 2020). In PCR analysis of *T. gondii* infections, various body fluids, placenta, brain, blood, faeces, and urine can be used as samples (Schares *et al*., 2008).

In conventional PCR, single-copy targeting genes, such as SAG1, SAG2, SAG3, GRA1, SAG4, and GRA4, are used to detect *T. gondii* in humans and animals (Switaj *et al*., 2005). However, the sensitivity and specificity is low, especially in blood samples (Liu *et al*., 2015). Therefore, PCR targeting multi-copy genes, including B1 gene, 529 bp repeat element, and 18S rDNA gene, have been developed to improve sensitivity and specificity (Chabbert *et al*., 2004; Liu *et al*., 2015). Athough, the B1 gene with 35 repeats is considered the gold standard target for PCR detection of *T. gondii* in humans and animals (Chabbert *et al*., 2004), studies has shown that PCR with the 529 bp repeat element is ten to hundred times more sensitive than that of B1 gene (Reischl *et al*., 2003). Furthermore, similar sensitivity to the B1 gene has been documented for the internal transcribed spacer (ITS-1) and 18S rDNA multi-copy genes (Calderaro *et al*., 2006). Functionally, conventional PCR is effective for the diagnosis of toxoplasmosis in congenitally infected prenatal babies (sensitivity 64.0 to 97.4%) as well as in immuno-deficient patients (sensitivity 13.0 to 87.5%) (Switaj *et al*., 2005).

With the advancement of technology, a hybrid diagnostic technique has been developed by combining conventional PCR and Southern blotting with a specific probe. This technique requires an extra 12–24 hours to complete following conventional PCR (Liu *et al*., 2015). Another PCR technique is the PCR-ELISA, a sero-molecular diagnostic assay developed by Emrich and Karl (2020). This assay uses hybridization with an immobilized capture probe to detect sequences within the PCR product. The PCR-ELISA assay has been adapted to use polystyrene beads which decreases the time requirement. The sensitivity of this method is similar to Southern blotting (Martinez *et al*., 2003). In addition, a magnetic capture method combined with conventional PCR has been developed, which can detect and genotype *T. gondii* in meat samples (Opsteegh *et al*., 2010).

Table 3. Recombinant antigenic mixtures used for the diagnosis of *T. gondii* infection in humans and animals

Antigen	Expression system	Assay	Type of infection	Sensitivity (%)	Specificity (%)	Host	References
GRA7 Tg34AR	*E. coli*	IgG/IgM ELISA	➢ Acute ➢ Chronic	Chronic: 96	-	Human	(Jacobs *et al*., 1998)
GRA7 SAG1 GRA8	*E. coli*	IgG ELISA	➢ Acute ➢ Chronic ➢ Recent seroconversion	93.1	95.7	Human	(Aubert *et al*., 2000)
GRA7 GRA8 ROP1	*E. coli*	IgM ELISA	➢ Acute ➢ Recent seroconversion	74.6	95.7		
SAG2 H4 GRA7 GRA8	*E. coli*	IgG ELISA	➢ Acute ➢ Chronic	Acute: 90 Chronic: 98.6	Acute: 97	Human	(Li *et al*., 2000)
SAG1 GRA1 GRA7	*E. coli*	IgG ELISA	➢ Acute ➢ Chronic	Acute: 100 Chronic: 91.1	-	Human	(Pietkiewicz *et al*., 2004)
MICex2 MAG1 MIC3	*E. coli*	IgG ELISA	➢ Chronic	88.9	100	Human	(Holec *et al*., 2008)
GRA8 SAG2 GRA6	*E. coli*	IgG ELISA	➢ Chronic	94.4	100		
MAG1 SAG1 GRA5	*E. coli*	IgG ELISA	➢ Acute ➢ Post-acute ➢ Chronic	92.6	100	Human	(Holec-Gasior & Kur, 2010)
GRA2 SAG1 GRA5	*E. coli*	IgG ELISA	➢ Acute ➢ Post-acute ➢ Chronic	93.1	100		
ROP1 SAG1 GRA5	*E. coli*	IgG ELISA	➢ Acute ➢ Post-acute ➢ Chronic	94.2	100		
H4 H11	*E. coli*	IgG ELISA	-	H4-ELISA: 100 H11-ELISA: 50 M-ELISA: 100 TEA-ELISA: 100	H4-ELISA: 100 H11-ELISA: 100 M-ELISA: 100 TEA-ELISA: 100	Sheep & Cat	(Tenter *et al*., 1992)
H4 H11	*E. coli*	IgG ELISA	-	H4-ELISA: 93 H11-ELISA: 64 M-ELISA: 95 TEA-ELISA: 98 IFAT: 94	H4-ELISA: 100 H11-ELISA: 100 M-ELISA: 100 TEA-ELISA: 99 IFAT: 92	Cat	(Tenter *et al*., 1994)
SAG1 SAG2 GRA6	*E. coli*	IgG RDT	-	100	99.2	Cat	(Chong *et al*., 2011)
GRA1 GRA7	*E. coli*	IgG ELISA	-	GRA1-ELISA: 81 GRA7-ELISA: 91 TLA-ELISA: 88.1	GRA1-ELISA: 95.4 GRA7-ELISA: 97.7 TLA-ELISA: 96.8	Dog	(Wang *et al*., 2014a)
SAG1 SAG2	*E. coli*	DFICT	-	92	93.1	Dog & Cat	(Jiang *et al*., 2015)

SAG1 MIC1 MAG1	*E. coli*	IgG ELISA	-	77.9	92.2	Horse, Pig & Sheep	(Ferra *et al.*, 2015a)
SAG2 GRA1 ROP1	*E. coli*	IgG ELISA	-	100	100		
GRA1 GRA2 GRA6	*E. coli*	IgG ELISA	-	92.1	100		
GRA1 ROP1	*E. coli*	IgG ELISA	-	100	100	Sheep	(Holec-Gasior *et al.*, 2014)
GRA1 SAG2	*E. coli*	IgG ELISA	-	100	95		
SAG2 ROP1	*E. coli*	IgG ELISA	-	100	95		
GRA1 SAG2 ROP1	*E. coli*	IgG ELISA	-	100	100		
MAG1 SAG1	*E. coli*	IgG ELISA	-	86.7	100	Pig	(Holec-Gasior *et al.*, 2010)
MAG1 GRA7	*E. coli*	IgG ELISA	-	89.2	100		
SAG1 GRA7	*E. coli*	IgG ELISA	-	92	100		
MAG1 SAG1 GRA7	*E. coli*	IgG ELISA	-	97.3	100		
SAG2 GRA7 GRA14	*E. coli*	IgG ELISA IgM ELISA IgG ICT	-	GRA7-ELISA: 90.6 GRA7-ICT: 71.9	GRA7-ELISA: 85.2 GRA7-ICT: 96.3	Pig	(Terkawi *et al.*, 2013)
SAG1 MIC1 MAG1	*E. coli*	IgG ELISA	-	88.9	100	Pig	(Ferra *et al.*, 2015a)
SAG2 GRA1 ROP1	*E. coli*	IgG ELISA	-	81.5	100		
GRA1 GRA2 GRA6	*E. coli*	IgG ELISA	-	54.3	100		
CCp5A OWP1	*E. coli*	IgG WB IgG ELISA	-	100	-	Pig	(Santana *et al.*, 2015)
SAG1 MIC1 MAG1	*E. coli*	IgG ELISA	-	88.9	100	Horse	(Ferra *et al.*, 2015a)
SAG2 GRA1 ROP1	*E. coli*	IgG ELISA	-	77.8	100	Horse	
GRA1 GRA2 GRA6	*E. coli*	IgG ELISA	-	66.7	100	Horse	
GRA1 GRA7	*E. coli*	IgY ELISA	-	GRA1-ELISA: 81.3 GRA7-ELISA: 100 TSA-ELISA: 93.8	GRA1-ELISA: 94.7 GRA7-ELISA: 98.9 TSA-ELISA: 97.9	Chicken	(Sun *et al.*, 2015)

Table 4. Chimeric epitopes and multiepitope peptides used for the diagnosis of *T. gondii* infection in humans and animals

Multiepitope peptide	Expression system	Assay	Type of infection	Sensitivity (%)	Specificity (%)	Host	References
EC2 GST	Recombinant protein + GST domain	IgM ELISA IgG ELISA	➢ Primary	Infants: 70	Infants: 100	Human	(Beghetto *et al.*, 2006)
			➢ Acquired	Adults: 98	Adults: 100		
EC3 GST	Recombinant protein + GST domain	IgM ELISA	➢ Primary	Infants:50	Infants: 100		
			➢ Acquired	Adults: 84	Adults: 100		
SAG1 SAG2	Recombinant protein + His-Tag domain	Western blot (IgG/IgM)	➢ Early acute ➢ Acute ➢ Chronic	100	100	Human	(Lau *et al.*, 2011)
MIC1 MAG1	Recombinant protein + His-Tag domain	IgG ELISA	➢ Acute ➢ Post-acute ➢ Chronic	Acute and Post-acute:100 Chronic: 85.1	100	Human	(Holec-Gasior *et al.*, 2012b)
MIC1 MAG1 SAG1	Recombinant protein + His-Tag domain	IgG ELISA	➢ Acute ➢ Post acute ➢ Chronic	Acute and Post-acute: 100 Chronic: 96.9	100		
rMEP	Recombinant protein + His tag and Trx tag domains	IgG ELISA	➢ Acute ➢ Chronic	Acute: 87.5 Chronic: 97.4	100	Human	(Dai *et al.*, 2012)
		IgM ELISA	-	96.9	100		
rMEP	Recombinant protein + His tag and Trx tag domains	IgG ELISA	➢ Acute ➢ Chronic	Acute: 25.9 Chronic: 97.1	98.7	Human	(Dai *et al.*, 2013)
		IgM ELISA	-	96.6	100		
SAG2 GRA1 $ROP1_L$	Recombinant protein + His tag domain	IgG ELISA	➢ Acute ➢ Post acute ➢ Chronic	100	100	Human	(Ferra *et al.*, 2015a)
GRA1 GRA2 GRA6	*E. coli*	IgG ELISA	➢ Naturally infected ➢ Healthy animals	66.1	100	Horse	
MIC1 MAG1 $SAG1_S$	*E. coli*	IgG ELISA	➢ Naturally infected ➢ Healthy animals	75	100	Horse	
$SAG1_L$ MIC1 MAG1	*E. coli*	IgG ELISA	➢ Naturally infected ➢ Healthy animals	77.8	100	Horse	
SAG2 GRA1 $ROP1_S$	*E. coli*	IgG ELISA	➢ Naturally infected ➢ Healthy animals	50	100	Horse	
SAG2 GRA1 $ROP1_L$	*E. coli*	IgG ELISA	➢ Naturally infected ➢ Healthy animals	100	100	Horse	
SAG1 GRA2 GRA7	Recombinant protein + His tag domain	IgG ELISA	➢ Acute ➢ Chronic	85.43	81.25	Human	(Hajissa *et al.*, 2015, 2017)

CCp5A OWP8 GRA7	*E. coli*	IgY ELISA	➢ Healthy free-range chickens	CCp5A: 9.4% OWP8: 13,5% GRA7: 10.4%	Undefined	Chicken	(Liu *et al.*, 2019)
GRA7 OWP8 CCp5A	*E. coli*	IgG ELISA	➢ Healthy animals	GRA7: 16.7% OWP8: 12.2% CCp5A:12.2%	Undefined	Pig	
SAG2 GRA7 GRA14	*E. coli*	IgG ELISA IgM ELISA IgG ICT	-	GRA7-ELISA: 90.6% GRA7-ICT: 71.9%	GRA7-ELISA: 85.2% GRA7-ICT: 96.3%	Pig	(Terkawi *et al.*, 2013)
GRA2 GRA6 GRA7 GRA15	*E. coli*	IgG ELISA	-	70.3%	86.1%	Cat	(Abdelbaset *et al.*, 2017)
SAG2 GRA2 GRA6 GRA7 GRA15	*E. coli*	IgG ELISA	-	89.2%	95.4%	Cat	
MIC1 MAG1 SAG1S	*E. coli*	IgG ELISA	➢ Naturally infected ➢ Healthy animals	97.9%	100%	Sheep	(Ferra *et al.*, 2015a)
SAG1L MIC1 MAG1	*E. coli*	IgG ELISA	➢ Naturally infected ➢ Healthy animals	100%	100%	Sheep	
SAG2 GRA1 ROP1S	*E. coli*	IgG ELISA	➢ Naturally infected ➢ Healthy animals	100%	100%	Sheep	
SAG2 GRA1 ROP1L	*E. coli*	IgG ELISA	➢ Naturally infected ➢ Healthy animals	100%	100%	Sheep	
GRA1 GRA2 GRA6	*E. coli*	IgG ELISA	➢ Naturally infected ➢ Healthy animals	92.1%	100%	Sheep	
AMA1N SAG2 GRA1 ROP1	*E. coli*	IgG ELISA	➢ Naturally infected ➢ Healthy animals	88.9%	100%	Goat	(Holec-Gasior *et al.*, 2019)
AMA1C SAG2 GRA1 ROP1	*E. coli*	IgG ELISA	➢ Naturally infected ➢ Healthy animals	95.6%	97.6%	Goat	
AMA1 SAG2 GRA1 ROP1	*E. coli*	IgG ELISA	➢ Naturally infected ➢ Healthy animals	95.6%	100%	Goat	
SAG2 GRA1 ROP1 GRA2	*E. coli*	IgG ELISA	➢ Naturally infected ➢ Healthy animals	57.8%	95.1%	Goat	

Nested PCR is another PCR technique used to identify *T. gondii*. In this technique, two sets of primers are allowed to run consecutively and the products of the first reaction becme templates for the second reaction. This technique was developed to increase sensitivity and specificity (Jones *et al*., 2000; Fallahi *et al*., 2014a) (Table 5). The B1 gene is primarily used for the identification of *T. gondii* with this type of PCR (Botein *et al*., 2019; Nakashima *et al*., 2020; Ngui *et al*., 2020). However, the Me49, GRA6, and Rep529 genes have also been used as targets (Mousavi *et al*., 2016; Bahadori *et al*., 2019; Shahbazi *et al*., 2019). It is noteworthy that both the B1 and REP 529 genes of *T. gondii* show more sensitivity and specificity in nested PCR than conventional PCR (Jones *et al*., 2000; Fallahi *et al*., 2014b).

Unlike conventional and nested PCRs, real-time PCR is capable of identifying a very low amount of target genes. The amplified product can then be measured in standard concentration during every cycle using probes (Liu *et al*., 2015) (Table 6). In addition, the elimination of contaminants through a rapid closed tube system has allowed for standardization of real-time PCR. Aside from detecting *T. gondii* infection, real-time PCR can also measure the magnitude of infection. Thus, this technique could be used evaluate disease progression and the effectiveness of treatment. Teixeira *et al*. (2013) showed that real-time PCR targeting the B1 gene is more effective at diagnosing congenital toxoplasmosis than conventional or nested PCRs. Me49, Rep529, ITS-1, and P30 genes are also used as targets in real-time PCR for the diagnosis of toxoplasmosis (Marques *et al*., 2020). A combination of magnetic capture and real-time PCR can identify predilection sites of *T. gondii* and its intensity in domestic animals.

Loop mediated isothermal amplification (LAMP)

Loop mediated isothermal amplification is a modified nucleic acid amplification assay under isothermal conditions (60-65°C). It uses 4-6 specific primers, which can identify 6-8 distinct regions within the target DNA with high sensitivity, specificity, and speed (Alhassan *et al*., 2007; Abbasi *et al*., 2010; Gallas-Lindemann *et al*., 2013). LAMP is easy, reliable, inexpensive, and does not require a highly equipped reference laboratory (Mori & Notomi, 2009). These advantages have inspired many researchers to use this test in a variety of animal samples (Hu *et al*., 2012; Dai *et al*., 2013; Wang *et al*., 2013; Fallahi *et al*., 2015). This molecular technique is able to detect *T. gondii* in a variety of samples including blood (Mikita *et al*., 2013; Sun *et al*., 2017), water (Gallas-Lindemann *et al*., 2013) and environmental air (Lass *et al*., 2017). LAMP's sensitivity is higher than conventional PCR and lower than real time PCR (Zhang *et al*., 2014). However, a recent study suggested that LAMP is more satisfactory than both conventional and real time PCR for the detection of *T. gondii* in congenital and immunocompromised patients (Rostami *et al*., 2018). Different target genes, incuding SAG1, SAG2, GRA1, B1, 18S rRNA, 529-bp REP and oocyst wall protein (OWP) can be identified by LAMP (Lau *et al*., 2010b; Hu *et al*., 2012; Qu *et al*., 2013). In LAMP assay, SAG1 and SAG2 target genes are most useful for the diagnosis of early and active toxoplasmosis (Wang *et al*., 2013). SAG1, SAG2 and B1 target genes are typically used to identify *T. gondii* in human blood samples (Lau *et al*., 2010b; Hu *et al*., 2012; Mikita *et al*., 2013). 18s rRNA and REP 529 target genes are used in a modified LAMP assay and reverse transcription LAMP (RT-LAMP) to detect *T. gondii* in mice and meat samples (Qu *et al*., 2013).

Microsatellite analysis (MSA)

Microsatellite analysis (MSA) is based on the identification of short tandem repeats (2–6 nucleotides) in DNA. Markers generated from repeated nucleotides are highly polymorphic because of length variations and they have multiple alleles, which make them reliable for genotypic investigations. Various MS marker sets (with 515 markers) have been used in research studies (Ajzenberg *et al*., 2010). A multiplex PCR technique for 15 MSs allowing multilocus analysis of isolates by a single PCR amplification has been developed for the high resolution genotyping of *T. gondii* strains (Ajzenberg *et al*., 2010). The mutation rate for MSs is 10^{-22}-10^{-25} per locus per replication, which is faster than single nucleotide polymorphisms (SNPs) (Bond, 2000). However, a high rate of mutation is a limits the use of these makers due to homoplasy. In 15 MS multiplex PCR, homoplasy has been diminished by the selection of variable and numerous MS markers located on 11 different chromosomes. The eight MS markers ([TG/AC]n, or [TC/AG]n: TUB2, W35, TgM-A, B18, B17, M33, IV.1 and XI.1) showed lower mutability but were able to differentiate major clonal lineages from atypical strains. Seven MS markers ([TA/ AT]n: M48, M102, N60, N82, AA, N61 and N83) are standard for distinguishing closely related isolates or analyzing the intratype population structure. The Simpson's index of diversity, tested on 369 type II isolates, was found to be 0.999, which almost reaches the maximum of 1.0 (Ajzenberg *et al.,* 2010). The degree of polymorphism is enhanced by MS markers and the rapid mutation rate makes microsatellite analysis techniques important for molecular epidemiology, forrecent evolution, and for individual identification of *T. gondii* isolates (Vielmo *et al*., 2019).

Multilocus sequence typing (MLST)

The Multilocus sequence typing technique (MLST) efficiently identifies DNA sequence polymorphisms, such as single nucleotide polymorphisms (SNPs). SNPs occur due to the insertion and deletion of nucleotides at different loci in the genome. Thus, the MLST technique has a higher resolution than techniques using single locus typing. However, MLST does require a large amount of genomic DNA. The MLST of *T. gondii* is usually based on the amplification and sequencing of Apico, B1, BTUB, c22-8, c29-2, L358, PK1, SAG1, SAG2, SAG3, and SAG4 gene loci (Su *et al*., 2006; Dubey *et al*., 2008). The requirement for a large quantity of genomic DNA limits the use of MLST for identification of *T. gondii* in clinical samples (Liu *et al*., 2015).

PCR-restriction fragment length polymorphisms (PCR-RFLPs)

The PCR-restriction fragment length polymorphisms (PCR-RFLPs) is based on the ability of restriction endonucleases to identify single nucleotide polymorphisms (SNPs). After the PCR products are treated with restriction enzymes, they are visualized via electrophoresis in agarose or polyacrylamide gels. Each SNPs has a variable length band pattern (Howe & Sibley, 1995). Type I, II, and III linages were identified from 106 *T. gondii* strains isolated from both humans and animals. Isolation was done by PCR-RFLP using six markers (Howe & Sibley, 1995). Conventional PCR-RFLPs depends on single-copy polymorphic DNA sequences, and always requires a large amount of DNA. This is a major limitation of conventional PCR-RFLPs when attempting to genotype *T. gondii* isolated from biological samples (Liu *et al*., 2015). In order to overcome the shortcomings of conventional PCR-RFLPs, a new approach was developed:

Table 5. Target genes and primers of nested PCR used for the detection of *T. gondii* in humans and animals

Target gene	PCR round	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	Fragment size (bp)	Host	References
B1	1st	CCGTTG GTTCCGCCTCCTTC	GCAAAACAGCGGCAG CGTCT	-	Human	(Ngui *et al*., 2020)
	2nd	CCGCCTCCT TCGTCCGTCGT	GTGGGGGCGGACCTC TCTTG	-		
B1	1st	TGT TCTGTCCTATCGCAACG	TCTTCCCAGACGTGGATTTC	-	Goat	(Sroka *et al*., 2017)
	2nd	ACGGATGCAGTTCCTTTCTG	CTCGACAATACGCTGCTTGA	-		
529 bp	1st	TGACTCGGGCCCAGCTGCGT	CTCCTCCCTTCGTCCAAGCCTCC	-	Chicken	(Mose *et al*., 2016)
	2nd	AGGGACAGAAGTCGAAGGGG	GCAGCCAAGCCGGAAACATC	-		
Me 49	1st	TGCGGAAGGATCATTCACACG	CCGTTACTAAGGGAATCATAGTT	1000	Pig	(Vitale *et al*., 2013)
	2nd	GATTTGCATTCAAGAAGC(G)TGATAGTAT	AGTT(T)AGGAAGCAATCTGAAAGCACATC	500		
B1	1st	GGAACTGCATCCGTTCATGAG	TCTTTAAAGCGTTCGTGGTC	193	Human	(Botein *et al*., 2019)
	2nd	TGCATAG GTTGCAGTCACTG	GGCGACCAATGTGCGAATAGACC	96		
B1	1st	TTCTCGCCTCATTTCTGGGTCTAC	GCACCTTTCGGACCTCAACAACCG	288	Human	(Nakashima *et al*., 2020)
	2nd	AACGGGCGAGTAGCACCTGAGGAGA	TGGGTCTACGTCGATGGCATGACAACT	115		
B1	1st	TGCACCTTTCGGACCTCAACAAC	TCGCCTCATTTCTGGGTCTACGT	284	Human	(Teixeira *et al*., 2013)
	2nd	AACGGGCGAGTAGCACCTGAGGAGA	TGGGTCTACGTCGATGGCATGACAACT	115		
GRA6	1st	ATTTGTGTTTCCGAGCAGGT	GCACCTTCGCTTGTGGTT	546	Sheep	(Shahbazi *et al*., 2019)
	2nd	TTTCCGAGCAGGTGACCT	TCGCCGAAGAGTTGACATAG	351		
B1	1st	GGAACTGCATCCGTTCATGAG	TCTTTAAAGCGTTCG TGGTC	193	Human	(Mahalakshmi *et al*., 2006)
	2nd	TGCATAGGTTGCAGTCACTG	GGCGACCAATCTGCG AATACACC	96		
GRA6	1st	ATTTGTGTTTCCGAGCAGGT	GCACCTTCGCTTGTGGTT	546	Cat	(Bahadori *et al*., 2019)
	2nd	TTTCCGAGCAGGTGACCT	TCGCCGAAGAGTTGACATAG	344		
B1	1st	GGAACTGCATCCGTTCATGAG	TCTTTAAAGCGTTCGTGGTC	193	Cat	(Zamora-Vélez *et al*., 2020)
	2nd	TGCATAGGTTGCCAGTCACTG	GGCGACCAATCTGCGAATACACC	96		
B1	1st	TGTTCTGTCCTATCGCAACG	ACGGATGCAGTTCCTTTCTG	580	Tick	(Kim *et al*., 2020)
	2nd	TCTTCCCAGACGTGGATTTC	CTCGACAATACGCTGCTTGA	530		
B1	1st	GGAACTGCATCCGTTCATGAG	GGCGACCAATCTGCGAATACACC	-	Human	(Halleyantoro *et al*., 2019)
	2nd	TGCATAGGTTGCAGTCACTG	TCTTTAAAGCGTTCGTGGTC	-		
529 bp	1st	CTGCAGGGAGGAAGACGAAAGTTG	CAGTGCATCTGGATTCCTCTCC	529	Human	(Mousavi *et al*., 2016)
	2nd	GTGCTTGGAGCCACAGAAGGGAC	GAGGAAAGCGTCGTCTCGTCTGG	-		
B1	1st	TCAAGCAGCGTATTGTCGAG	CCGCAGCGACTTCTATCTCT	194	Human	
	2nd	GGAACTGCATCCGTTCATGAG	TCTTTAAAGCGTTCGTGGTC	-		
B1	1st	TGTTCTGTCCTATCGCAACG	ACGGATG CAGTTCCTTTCTG	580	Human	(Alfonso *et al*., 2009)
	2nd	TCTTCCCA GACGTGGATTTC	CTCGACAATACGCTGCTTGA	530		
GRA6	1st	GCACCTTCGCTTGTGGTT	ATTTGTGTTTCCGAGCAGGT	546	Cattle & Sheep	(Azizi *et al*., 2014)
	2nd	TCGCCGAAGAGTTGACATAG	TTTCCGAGCAGGTGACCT	351		

B1	1st	GGAACTGCATCCGTTCATGAG	TCTTTAAAGCGTTCGTGGTC	-	Human & Goat	(Jones *et al.*, 2000)
	2nd	TGCATAGGTTGCAGTCACTG	GGCGACCAATCTGCGAATACACC	193		
P30	1st	TTGCCGCGCCCACACTGATG	CGCGACACAAGCTGCGATAG	-		
	2nd	CGACAGCCGCGGTCATTCTC	GCAACCAGTCAGCGTCGTCC			
529 bp	1st	TGACTCGGGCCCAGCTGCGT	CTCCTCCCTTCGTCCAAGCCTCC	-	Human	(Thiongo *et al.*, 2016)
	2nd	AGGGACAGAAGTCGAAGGGG	GCAGCCAAGCCGGAAACATC	-		
B	1st	TGTTCTGTCCTATCGCAACG	ACGGATGCAGTTCCTTTCTG	580	Sheep	(Habibi *et al.*, 2012)
	2nd	TCTTCCCAGACGTGGATT TC	CTCGACAATACGCTGCTTGA	531		
B1	1st	TCAAGCAGCGTATTGTCGAG	CCGCAGCGACTTCTATCTCT	-	Camel	(Kareshk *et al.*, 2018)
	2nd	GGAACTGCATCCGTTCATGAG	TCTTTAAAGCGTTCGTGGTC	-		
GRA6	1st	GGCAAACAAAACGAAGTG	CGACTACAAGACATAGAGTG	-		
	2nd	GTAGCGTGCTTGTTGGCGAC	TACAAGACATAGAGTGCCCC	-		
B1	1st	GGAACTGCATCCGTTCATGAG	TCTTTAAAGCGTTCGTGGTC	191	domestic ducks	(Lv *et al.*, 2021)
	2nd	TGCATAGGTTGCAGTCACTG	GGCGACCAATCTGCGAATACACC	134		
B1	1st	ACA GAA AGG GAG CAA GAG TT	CTG GGT TGG CTG AAA GAT A	1484	Sheep, Goat, Fish & Chicken	(Rajendran *et al.*, 2018)
	2nd	AGG GTA CGT GTT GCA TCAT	TCT CCG CAG CGA CTT CTA	499		
B1	1st	GGAACTGCATCCGTTCATGAG	TCTTTAAAGCGTTCGTGGTC	-	Cattle	(Ge *et al.*, 2014)
	2nd	TGCATAGGTTGCAGTCACTG	GGCGACCAATCTGCGAATACACC	193		
ITS1	1st	TGCGGAAGGATCATTCACACG-	CCGTTACTAAGGGAATCATAGTT	1000	Water buffaloes	(Bărburaș *et al.*, 2019)
	2nd	GATTTGCATTCAAGAAGC(G)TGATAGTAT	AGTT(T)AGGAAGCAATCTGA AAGCACATC	313		
B1	1st	GGAACTGCATCCGTTCATGAG	TCTTTAAAGCGTTCGTGGTC	-		
	2nd	TGCATAGGTTGCAGTCACTG	GGCGACCAATCTGCGAATACACC	193		
529 bp	1st	TGACTCGGGCCCAGCTGCGT	CTCCTCCCTTCGTCCAAGCCTCC	-		
	2nd	AGGGACAGAAGTCGAAGGGG	GCAGCCAAGCCGGAAACATC	164		

Table 6. Target genes, primers and reaction conditions of real-time PCR used for the detection of *T. gondii* in humans and animals

Target gene	Forward primer sequence (5′-3′)	Reverse primer sequence (5′-3′)	Fragment size (bp)	Denaturation	Annealing	Extension	Host	References
				Temperature (°C)				
529 bp	CACAGAAGGGACAGAAGT CGAA	CAGTCCTGATATCTCTCCTCC AAGA	529	95	62	68	Human	(Tehrani *et al*., 2020)
529 bp	TGGTTGGGAAGCGACGAGAG	5CATCACCACGAGGAAAGCGTC	529	95	95	60	Human	(Talabani *et al*., 2009)
B1	GGAACTGCATCCGTTCATGAG	TCTTTAAAGCGTTCGTGGTC	-	94	55	72	Human	(Burg *et al*., 1989)
B1	GAAAGCCATGAGGCACTCCA	TTCACCCGGACCGTTTAGC	71	94	62	72	Human	(Ghazzay *et al*., 2020)
RE	AGAGACACCGGAATGCGATCT	TTCGTCCAAGCCTCCGACT	112	95	72	72		
B1	AGAGACACCGGAATG CGATCT	TTCGTC CAAGCC TCCGACT	-	-	-	-	Pig	(Paraboni *et al*., 2020)
529 bp	GAA AGCCAT GAG GCA CTC CA	TTCACC CGGACC GTT TAGC	-	-	-	-		
B1	TCCCCTCTGCTGGCGAAAAGT	AGCGTTCGTGGTCAACTATCGATTG	97	95	95	60	Mussel	(Santoro *et al*., 2020)
ITS-1	AGTTTAGGAAGCAATCTGAAAGCACATC	GATTTGCATTCAAGAAGCGTGATAGTAT	100	94	55	72	Chicken	(Hamidinejat *et al*., 2014)
B1	CCGCCTCCTTCGTCCGTCGT	GTGGGGGCGGACCTCTCTTG	213	94	58	72	Turkey	(Sarkari *et al*., 2014)
B1	CCGTTGGTTCCGCCTCCTTC	GCAAAACAGCGGCAGCGTCT	432	94	57	72		
B1	GGAACTGCATCCGTTCATGAG	TCTTTAAAGCGTTCGTGGTC	-	94	55	72	Goat & Pig	(Hill *et al*., 2006)
B1	AACGGGCGAGTAGCACCTGAGGAGA	TGGGTCTACGTCGATGGCATGACAAC	-	94	57	72	Sheep	(Boughattas *et al*., 2014)
B1	GGAGGACTGGCAACCTGGTGTCG	TTGTTTCACCCGGACCGTTTAGCAG	2214	-	60	-	Cat, sheep, cattle & pig	(Berger-Schoch *et al*., 2011)
RE	TGGTTGGGAAGCGACGAGAG	CATCACCACGAGGAAAGCGTC	529	95	55	60	Human	(Talabani *et al*., 2009)
B1	TGCATCCAACGAGTTTATAA	GGCATTCCTCGTTGAAGATT	400	94	50	60	Human	(Murata *et al*., 2020)
B1	TCGAAGCTGAGATGCTCAAAGTC	AATCCACGTCTGGGAAGAACTC	129	95	95	60	Wild birds	(Gazzonis *et al*., 2021)
529 bp	CTGCAGGGAGGAAGACGAAAGTTG	CTGCAGACACAGTGCATCTGG ATT	529	94	50	72	Sheep	(Gutierrez *et al*., 2010)
529 bp	AGAGACACCGGAATGCGATCT	CCCTCTTCTCCACTCTTCAATTCT	529	95	95	60	Cat	(Adriaanse *et al*., 2020)
529 bp	CACAGAAGGGACAGAAGT	TCGCCTTCATCTACAGTC	529	95	95	60	Cat	(Pepe *et al*., 2021)
RE	TCGTCTCGTCTGGATCGCAT	GCCGGAAACATCTTCTCCCTCTCC	134	95	95	60	Cat	(Karakavuk *et al*., 2021)
B1	TCCCCTCTGCTGGCGAAAAGT	AGCGTTCGTGGTCAACTATCGATTG	-	95	95	59	Chicken	(Al-Hadad & Al-Rubaie, 2021)
B1	TCCCCTCTGCTGGCGAAAAGT	AGCGTTCGTGGTCAACTATCGATTG	-	95	95	60	Human & Chicken	(Al-nasrawi *et al*., 2014)

multiplex multilocus nested PCR-RFLPs (Mn-PCR-RFLPs). This technique uses 10 genetic markers (SAG1, SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1 and Apico) and has been shown to have satisfactory sensitivity (Dubey *et al*., 2007). Mn-PCR-RFLP has been widely used to type clinically isolated positive samples. It has also been used to determine the genetic diversity and population structure of *T. gondii* (Zhou *et al*., 2013). One concern with this test is that incorrect findings may be produced if contamination occurs in the early cycles of PCR. In order to prevent this error, a negative control must be used in each experiment (Liu *et al*., 2015).

Random amplified polymorphic DNA-PCR (RAPD-PCR)

The Random amplified polymorphic DNA-PCR (RAPD-PCR) is used to identify DNA polymorphisms based on the amplification of genomic DNA with single short arbitrary primers under low stringency conditions (Liu *et al*., 2015). RAPD-PCR is excellent at identifying genetic differentiation among closely related species and has been used to classify the genotype of *T. gondii* strains (Ferreira *et al*., 2004). *T. gondii* can be categorized into virulent and avirulent strains based on murine virulence by RAPD-PCR. Certain primers are useful for detecting virulence markers. Although it is a fast, easy, and effective technique, RAPD-PCR band profiles can be difficult to replicate, even within individual laboratories, if the staff, facilities or conditions are altered. For this assay, only a small amount of DNA is needed, but it must be very pure (Arif *et al*., 2010). Thus, for clinical samples, RAPD-PCR is not useful (Liu *et al*., 2015).

High-resolution melting (HRM)

The high-resolution melting (HRM) is a homogeneous, close-tube and post-PCR tool for the study of genetic variants. It characterizes polymorphisms based on their melting temperature, which is associated with the polymorphism sequence, length, and GC content (Jex *et al*., 2008). HRM analysis can accurately distinguish *T. gondii* strains into three distinct groups based on a single SNP of the multicopy B1 gene (Costa *et al*., 2011). HRM is more descriptive than the study of microsatellites, thus it provides an additional test for multilocus microsatellite analysis (Costa *et al*., 2011, 2013). This method was developed to directly genotype *T. gondii* from biological samples. Its genotyping ability is better with multi-copy genes rather than single-copy gene, thereby avoiding cell culture or bioassay (Costa *et al*., 2013). HRM may provide an easy solution for genotyping, mutation scanning, and sequence matching (Liu *et al*., 2015).

Metagenomics analysis (MGA)

Metagenomics analysis (MGA) is based on the identification of causal agents from unknown independent environmental samples (Greninger, 2018). No prerequisite information about specific infectious agents is required for meta-genomics. Metagenomics can detect every infectious and non-infectious pathogen in a single sample using bio-informatics and computational strategy (Hu *et al*., 2018). A group of researchers applied metagenomics to identify unknown causal agents in the brain lesions of a human immunodeficiency virus (HIV) infected patient. They identified *T. gondii* by analyzing a genomic sequence found in the cerebrospinal fluid (CSF) of the patient (Hu *et al*., 2018). Thus, metagenomics analysis may be an important diagnostic tool for the identification of toxoplasmic encephalitis. However, the method needs more assessment as a diagnostic tool for toxoplasmosis.

Whole-genome sequencing (WGS)

Whole-genome sequencing (WGS) involves the identification of the complete DNA sequence of an organism's genome in a single shot. At the present time, sixteen haplogroups of *T. gondii* containing over one hundred whole-genome sequences are stored in the Sequence Read Archive (SRA) (https://www.ncbi.nlm.nih.gov/sra). A total of 14 chromo-somes were identified from the whole-genome shotgun sequences (www.toxodb.org) (Kissinger *et al*., 2003) and composite genome map (types III strains) of *T. gondii* (Khan *et al*., 2005; Sibley, 2009). These sequences were obtained using next-generation sequencing (NGS) platforms, such as Illumina HiSeq or MiSeq (Lorenzi *et al*., 2016). NGS enhances variations in structure and copy number, to identify recombinant strains of *T. gondii* at the whole-genome level. Currently, 62 strains with 138 unique genotypes from 15 different haplogroups of *T. gondii* have been submitted to WGS and ref-mapped against ME49 (Lorenzi *et al*., 2016). A total of 802,764 SNPs have been identified in the *T. gondii* genome using whole genome comparison analysis. It is providing new insignt into the genomics of *T. gondii*. This helps researchers identify the ancestral origin, evolution, hybridization, distribution, and transmission of different *T. gondii* strains worldwide. However, this method has several challenges, such as parasite isolation, parasite purificant in cell culture, DNA extraction for sequencing, expense, a technically laborious and challenging procedure (Lorenzi *et al*., 2016).

CONCLUSIONS

In the current era, accurate diagnosis of *T. gondii* infection is imperative in humans and animals due to its public health importance. This review has attempted to review all advanced and contemporary diagnostic techniques for the detection and genetic characterization of *T. gondii*. Over the previous three decades, direct microscopic and bioassays methods were commonly applied to identify *T. gondii.* However, some unavoidable limitations and difficulties of the methods drove researchers to develop effective serological and molecular techniques for the efficient identification and interpretation of *T. gondii* infection in humans and animals. In this process, a number of molecular and serological methods were developed. Currently, molecular and serological diagnosis plays an important role in the identification of *T. gondii* infection. The advantages, disadvantages, sensitivity, specificity and limitations of different serological tests are having been addressed in a wide variety of studies and methods are revised on a continuous basis to enhance their efficacy. This continuous research and revision led to the identification of recombinant proteins (single and combination) and chimeric antigens that can be used for the serological detection of *T. gondii* infections. These recombinant proteins and chimeric antifens can overcome some of the limitations of serological tests as well as improve their sensitivity and specificity. The key success of recombinant proteins and chimeric antigens is the identification of different phases of *T. gondii* infections. Similarly, numerous molecular techniques, including PCR, LAMP, microsatellite analysis, MLST, PCR-RFLP, RAPD-PCR, HRM, metagenomics analysis and WGS, have been applied for the detection and genetic characterization of *T. gondii*. After extracting findings from a wide variety of studies, we concluded that recombinant proteins in single and/or

combination forms and chimeric epitope are highly recommended for the diagnosis of *T. gondii* infections. Diagnostic methods based on recombinant proteins based have satisfactory sensitivity and specificity and are standardized and cost effective.

In the process of toxoplasmosis diagnostic advancement, researchers should focus on integrating genomic technologies, including genomics, transcriptomics, proteomics, and metabolomics. This research would help obtain a more comprehensive understanding of the pathogenesis and immune mechanisms of the parasite. It would also aid in the development of vaccines. Additionally, advancements in bioinformatics technologies would help with further genetic characterization of *T. gondii* and potentially provide new options for the diagnosis of toxoplasmosis.

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Conflict of Interest

The authors have declared that no competing interests exist.

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