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Diagnosis of toxoplasmosis and typing of *Toxoplasma gondii*

Quan Liu[1,2*], Ze-Dong Wang[2], Si-Yang Huang[1,3] and Xing-Quan Zhu[1,3*]

Abstract

Toxoplasmosis, caused by the obligate intracellular protozoan *Toxoplasma gondii*, is an important zoonosis with medical and veterinary importance worldwide. The disease is mainly contracted by ingesting undercooked or raw meat containing viable tissue cysts, or by ingesting food or water contaminated with oocysts. The diagnosis and genetic characterization of *T. gondii* infection is crucial for the surveillance, prevention and control of toxoplasmosis. Traditional approaches for the diagnosis of toxoplasmosis include etiological, immunological and imaging techniques. Diagnosis of toxoplasmosis has been improved by the emergence of molecular technologies to amplify parasite nucleic acids. Among these, polymerase chain reaction (PCR)-based molecular techniques have been useful for the genetic characterization of *T. gondii*. Serotyping methods based on polymorphic polypeptides have the potential to become the choice for typing *T. gondii* in humans and animals. In this review, we summarize conventional non-DNA-based diagnostic methods, and the DNA-based molecular techniques for the diagnosis and genetic characterization of *T. gondii*. These techniques have provided foundations for further development of more effective and accurate detection of *T. gondii* infection. These advances will contribute to an improved understanding of the epidemiology, prevention and control of toxoplasmosis.

Keywords: *Toxoplasma gondii*, Toxoplasmosis, Diagnosis, Genetic characterization, Genotyping, Serotyping

Review

Toxoplasma gondii is a protozoan parasite that infects almost all warm-blooded animals, including humans, and is considered one of the most successful eukaryotic pathogens [1]. Approximately 30 % of human population worldwide is chronically infected with *T. gondii* [2]. Human infections are primarily obtained by ingesting undercooked or raw meat containing viable tissue cysts, or by ingesting food or water contaminated with *T. gondii* oocysts [3, 4]. Primary infections in adults are mostly asymptomatic, but lymphadenopathy or ocular toxoplasmosis can present in some patients [5]. Severe acute, disseminated toxoplasmosis may occur in immunocompetent individuals when infected with some isolates [6–10]. Reactivation of a latent infection in immunocompromised individuals can cause fatal toxoplasmatic encephalitis, myocarditis and pneumonitis [11, 12]. The immunocompromised patients are also at risk of severe disease following primary infection or reactivation of chronic infection [13, 14]. Infection acquired during pregnancy can cause severe damage to the fetus, such as long-term disabling sequelae, stillbirths or fetal death [15].

Toxoplasma gondii has been considered a single species in the genus *Toxoplasma*. Early studies on the parasite strains from North America and Europe identified limited genetic diversity, which were classified into genetic types I, II, and III [16]. Recent multilocus polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) genotyping of approximately 1500 samples worldwide has revealed 189 different genotypes, with the *Toxoplasma* genome database (ToxoDB) PCR-RFLP (http://www.toxodb.org/toxo/) genotypes #1 (type II), #2 (type III) and #3 (type II variant) found worldwide, and highly prevalent in Europe, genotypes #1, #2, #3, #4 and #5 prevalent in North America, genotypes #2 and #3 (type III and type II variant)

* Correspondence: liuquan1973@hotmail.com; xingquanzhu1@hotmail.com
[1]State Key Laboratory of Veterinary Etiological Biology, Key Laboratory of Veterinary Parasitology of Gansu Province, Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Lanzhou, Gansu Province 730046, People's Republic of China
[3]Jiangsu Co-innovation Center for the Prevention and Control of Important Animal Infectious Diseases and Zoonoses, Yangzhou University College of Veterinary Medicine, Yangzhou, Jiangsu Province 225009, People's Republic of China
Full list of author information is available at the end of the article

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prevalent in Africa, and genotypes #9 (Chinese 1) and #10 (type I) prevalent in East Asia [17]. The consequences of infection with *T. gondii* may depend on parasite genotypes and host species [18]. In humans, disease manifestations range from asymptomatic to severe acute toxoplasmosis [4, 19]. Type I or type I variants are more likely to be associated with severe toxoplasmic retinochoroiditis [20], and the atypical isolates often cause severe acute or disseminated toxoplasmosis in immunocompetent individuals [19]. Type I isolates are uniformly lethal to out-bred mice, while type II and III isolates are significantly less virulent [21].

Clinical symptoms of *T. gondii* infection are non-specific and unreliable for diagnosis [4, 22]. The traditional diagnosis of *T. gondii* infection usually depends on bioassays and serological tests, with the limitations in detection or differentiating parasite strains [23, 24]. The detection of *T. gondii* infection by molecular methods is appealing, due to their high sensitivity and specificity [25]. Moreover, abundant *T. gondii* genotypes have been identified from various mammals and birds using PCR-based molecular methods [17, 18]. In this review, we conducted English literature searches in PubMed from 1948 to 2014 using the key words *Toxoplasma gondii*, toxoplasmosis, diagnosis, genetic characterization, genotyping and serotyping, and summarize the biotechnological advances in diagnosis of toxoplasmosis and typing of *T. gondii.*

Traditional, non-DNA-based diagnostic methods

Microscopic diagnosis

The detection of *T. gondii* in fecal, water, environmental and tissue samples has traditionally relied on microscope examination. However, identification based on light microscopy alone is less sensitive and unreliable. The oocysts in fecal, water and environment can be enriched from large volumes of samples by filtration or centrifugation for examination, and the tissue cysts can be stained, which helps to distinguish the parasites from host cells. Giemsa and Haematoxylin and Eosin (HE) staining is simple and cost-effective, and commonly used for this purpose [26–28]. Periodic acid schiff (PAS) can stain amylopection granules in bradyzoites [26]. These methods are relatively time consuming and require considerable skill to obtain reliable detection results. Electron microscope is also employed to detect tissue cysts in mouse brain and oocysts in the small intestine of infected cats, but it is difficult to be applicable for routine use [29, 30].

Bioassay

The isolation of *T. gondii* by bioassay using laboratory animals is generally considered as the gold standard for detection of *T. gondii* infection. Secretions, excretions, body fluids, lymph nodes, muscle and brain tissues are possible specimens used for the isolation [31, 32]. Mice and cats are commonly used for bioassay of *T. gondii.* To achieve higher success rate in *T. gondii* isolation, INF-gamma knockout mice are preferred, due to high sensitivity of these mice to *T. gondii* infection. Alternatively, normal mice may be immune suppressed by administrating dexamethason (10–15 μg/ml) in drinking water during the course of bioassay to increase success rate. Cats can be used to detect small number of viable *T. gondi* in meat because larger volumes of tissues can be fed to cats, therefore increasing the sensitivity. Overall, the bioassay is expensive and time-consuming (usually requires 6 weeks). Thus, it cannot be used for large-scale screening.

Serological assays

T. gondii infection usually shows no or non-specific clinical symptoms in most individuals, whose diagnosis mainly relies on serological tests. A variety of serological tests, such as dye test (DT), modified agglutination test (MAT), enzyme-linked immunosorbent assays (ELISA), immunosorbent agglutination assay (ISAGA), indirect fluorescent antibody test (IFAT) and indirect haemagglutination assays (IHA), have been developed to detect different antibody classes or antigens (Table 1). IgM antibodies are detectable about 1 week after the infection and remain for several months or years. So the detection of IgM antibodies alone is insufficient for the establishment of acute infection. IgA antibodies are considered to be a marker of acute infection, which are produced earlier than IgM, and may persist for several months. The shorter period of IgE may give a greater indication of current infection. The presence of IgG antibodies suggestions the occurrence of infection, but does not provide any information about the timing of infection.

Dye test (DT)

DT, first developed by Sabin and Feldman in 1948, has been considered as gold standard for the detection of anti-*T. gondii* antibodies in humans [33, 34]. DT is both specific and sensitive in humans, but may be unreliable in cattle and avian species [35, 36]. The major disadvantage of DT requires live parasites and healthy human serum as an accessory factor, severely limiting the availability of the DT [37]. The test is potentially hazardous, and requires a high degree of technical expertise, thus only performed in reference laboratories. Though tachyzoites prepared from cell culture can be routinely used in DT, the false negative results may occur in some cases. Therefore, tachyzoites prepared from mice are preferred for DT [38].

Table 1 Summary of serological methods for detection of *T. gondii* infection

Serological methods	Antigens or antibodies used	Antibody/antigen type tested	References
DT	Live tachyzoite	IgG, IgM, IgA	[33]
MAT	Formalin-fixed tachyzoite	IgG	[39]
IFAT	Killed whole tachyzoite	IgG, IgM	[55, 76]
IHA	Tanned red blood cells sensitized with soluble antigens	IgG	[50]
ELISA	Tachyzoite lysate antigen, recombinant antigens, specific antibodies	IgG, IgM, IgA, antigens	[192, 193]
ISAGA	anti-human IgM antibodies	IgM	[79]
LAT	Soluble antigen coated latex particles	IgG, IgM	[194, 195]
PIA	Antigen coated gold nanoparticles	IgG	[90]
WB	Tachyzoite lysate antigen, recombinant antigens	IgG, IgM	[196]
ICT	Antigens or antibodies labeled with colloidal gold	IgG, ESA	[83, 84]
Avidity test	tachyzoite lysate antigen, recombinant antigens	IgG, IgA, IgE	[100]

Modified agglutination test (MAT)

For MAT test, formalin-fixed *T. gondii* tachyzoites are added to U-shaped microtiter plates and diluted test sera are then added. Positive serum samples will produce a thin mat of agglutination, while negative samples will produce a compact pellet of precipitated tachyzoites at the bottom of the well [39]. This test was first described by Fulton and Turk [40] with low specificity and sensitivity, due to the binding of normal IgM to the surface of the parasite, and improved by preparing the antigen using a buffer containing 2-mercaptoethanol to remove non-specific IgM. This test detects IgG antibodies, without limitation of host species, but the false negative results may occur during early stages of acute infection. The specificity and sensitivity of MAT are comparable to the DT in most species, but it can produce high false negative results in dogs [41, 42].

The results of MAT differ, depending on the preservative used to prepare the antigen. MAT using acetone in place of formalin can detect IgG antibodies in acute infection, which is very useful in diagnosis of toxoplasmosis in AIDS patients, and acute glandular toxoplasmosis [43]. In addition, MAT can also be used to detect cardiac fluids for the survey of *T. gondii* infection in slaughtered sheep for human consumption, with higher sensitivity than other serological tests [44]. MAT is so simple and accurate that, it is convenient both for laboratory diagnosis and for epidemiological survey.

Latex agglutination test (LAT)

In this test, soluble antigen is coated on latex particles, and agglutination is observed when the positive serum is added. LAT is rapid and easy to perform to detect anti-*T. gondii* IgG antibodies. LAT has a sensitivity of 86–94 % and specificity of 100 % in humans, a low sensitivity of 78.6 % and specificity of 61.9 % in sheep [45, 46]. Thus, LAT is often used as a screening tool in epidemiologic survey due to the simplicity of performance, but the positive result requires further examination using other serological tests [47].

LAT has also been modified to detect anti-*T. gondii* IgM antibodies in humans for diagnosis of recent infection. Sato *et al.* [48] isolated microsomal antigen Sp-2 reactive with anti-*T. gondii* antibodes, whose reactivity with IgM and IgG antibodies varies with the concentration. Sp-2 antigen only reacts with IgM when latex particles are sensitized with less than or equal to 100 mg of this antigen/mg of particles. Based on this unique reaction of the antigen, a passive latex agglutination reaction to detect IgM antibodies has developed. Cambiaso *et al.* [49] utilized proteinase K-treated antigen-coated particles to establish LAT for the detection of IgM antibodies in humans, with an advantage of no significant interferences by IgG antibodies, or by rheumatoid factor or antinuclear antibodies.

Indirect hemagglutination test (IHA)

The principle of IHA is that the tanned red blood cells sensitized with *T. gondii* soluble antigen can be agglutinated by the positive serum [50]. However, detectable IHA IgG antibodies are later than DT, so acute and congenital infections are likely to be missed by this test [51, 52]. In animals, the detected antibodies with lower titers may be non-specific [50]. The IgG-IHA test is simple and rapid, thus recommended for mass screening in epidemiologic surveys [53]. Yamamoto *et al.* [54] described a modified IgM-IHA test by stabilized human red cells coated with a *T. gondii* heat-stable alkaline-solubilized extract, which can be used for the serodiagnosis of acute toxoplasmosis in humans, with a sensitivity of 100 % and specificity of 98.5 %.

Indirect fluorescent antibody test (IFAT)

IFAT is a simple test detecting both IgG and IgM antibodies, and has been widely used in detection of *T. gondii* antibodies in humans and animals [55–58]. Killed

T. gondii tachyzoites are incubated with test serum, the fluorescent anti-species antibodies are added, and the result is read under a fluorescence microscope. The test shows sensitivities of 80.4–100 % and specificities of 91.4–95.8 % [59, 60]. Fluorescent-labeled antibodies for a variety of species are commercially available, and the method is relatively inexpensive. However, a fluorescence microscope is necessary for the test, and the results are read by eye, so individual variation may occur. It may be difficult to find some species-specific conjugates, and there is a risk of possible cross-reactivity with rheumatoid factor and anti-nuclear antibodies [61].

Enzyme-linked immunosbsorbent assay (ELISA)

The ELISA system usually includes the solid phase antigen or antibody, enzyme labeled antigen or antibody, and the substrate of the enzyme reaction, which can be modified to test both antibodies and antigens (Fig. 1). ELISA can be automated so that a large number of samples can be simultaneously tested. There have been different types of ELISA developed to detect *T. gondii* antibodies or antigens, such as indirect ELISA, and sandwich ELISA.

In the indirect ELISA, the antigen is coated onto the solid phase and the sample containing antibodies are added, the antigen-antibody reaction is enhanced by the addition of a secondary enzyme-linked antibody, and the reaction can be evaluated by quantification of the color that develops (Fig. 1a). The tests are almost all used to detect anti-*T. gondii* IgG, IgM, and IgA antibodies rather than antigens, depending on the enzyme-linked antibody type [62]. The conventional indirect ELISAs using tachyzoite lysate antigen (TLA) as coating antigen show a high degree of agreement with DT, MAT or IFAT detecting IgG or IgM antibodies in humans and animals [61–63]. Despite the satisfactory results, TLA-based ELISA may vary significantly between laboratories, or between batches, thus difficult to standardize, and the test results are difficult to evaluate. An alternative approach is to use recombinant proteins, with an advantage of the precise antigen and easy standardization. In the past 20 years, numerous recombinant antigens, including granule antigens GRA1, GRA2, GRA4, GRA6, GRA7, and GRA8, rhoptry proteins ROP1 and ROP2, matrix protein MAG1, microneme proteins MIC2, MIC3, MIC4, and MIC5, and surface antigens SAG1 and SAG2, have been expressed in *Escherichia coli* or yeast, and their potential diagnostic value was evaluated in humans or animals by ELISA to detect specific IgG and IgM antibodies [23, 64–68]. Combinations of recombinant antigens have been shown more sensitive and specific than using single antigen. For example, combinations of SAG2A, GRA2, GRA4, ROP2, GRA8 and GRA7 are potentially useful to detect IgG antibodies in humans with recently acquired infection [69], ROP1, SAG1, GRA7, GRA8, and GRA6 are promising to detect specific IgM antibodies [70], while GRA7 and GRA8 are used to detect specific IgA antibodies [23, 71]. Hill *et al.* [72] identified a sporozoite-specific embryogenesis-related protein (ERP), which can react with oocyst-specific antibodies, and be used to differentiate oocyst-induced infection from tissue cyst-induced infection.

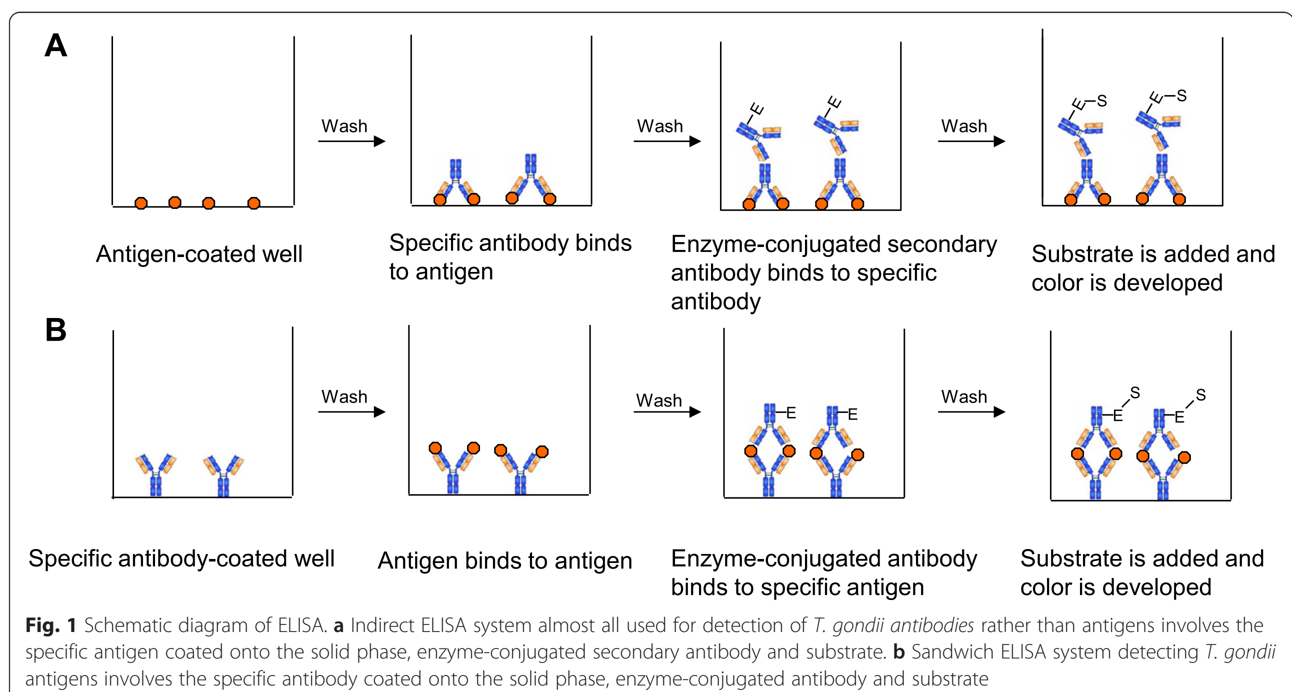

Fig. 1 Schematic diagram of ELISA. **a** Indirect ELISA system almost all used for detection of *T. gondii antibodies* rather than antigens involves the specific antigen coated onto the solid phase, enzyme-conjugated secondary antibody and substrate. **b** Sandwich ELISA system detecting *T. gondii* antigens involves the specific antibody coated onto the solid phase, enzyme-conjugated antibody and substrate

In the sandwich ELISA, the antigens or antibodies are coated onto the solid phase, and the sample containing antibodies or antigens are added. After incubation and washing, the antibody-antigen complex is attached to the solid phase. The captured antibodies or antigens are detected by the addition of enzyme-labeled specific antigens or antibodies (Fig. 1b). The sandwich ELISA has been developed to detect *T. gondii* antibodies and antigens. The sandwich ELISA with TLA is more sensitive and more specific to detect human IgM antibodies than IFAT [62], and the sandwich ELISA with recombinant P35 is more specific for the acute infection than IgM-ELISA using TLA [73, 74]. Another sandwich ELISA with anti-MIC10 antibody prepared from two different species can be used to detect circulating antigen MIC10 for early diagnosis of toxoplasmosis [75]. ELISA is simple, economical and easily adoptable for field use. Using an improved ELISA format, it is possible to detect *T. gondii* specific IgM, IgG and IgA antibodies, and circulating antigens. However, development of an ELISA test is labor-intensive and time-consuming, especially when evaluating its sensitivity and specificity.

A modified ELISA technique, dot-ELISA, in which the antigen-antibody reaction is performed on nitrocellulose in place of the polystyrene plate, has been established to detect *T. gondii* antigens and antibodies [76, 77]. This test is sensitive, and easy to perform in comparison with standard ELISA and no special equipment is required [76, 78].

Immunosorbent agglutination assay (ISAGA)

In this test, microtiter plates are coated with anti-human IgM antibodies, and the serum sample is added to the wells for 2 h at 37 °C to allow the binding of IgM. The plates are washed and the suspension of fixed tachyzoites is added to the wells, which are incubated in moist chamber overnight at 37 °C. The specific IgM in serum sample will bind to the anti-species IgM and agglutinate fixed parasite antigens, which is observed as that of MAT [79]. This test is simpler and easier to perform than the IgM-ELISA, but it requires large numbers of *T. gondii* tachyzoites. Thereafter, the IgM-ISAGA is modified by replacing *T. gondii* tacyzoites with latex beads coated with soluble antigens [80]. IgM-ISAGA can be used for the diagnosis of acute acquired and congenital *T. gondii* infection.

Immunochromatographic test (ICT)

The immunochromatographic test is a rapid detection technique in which the colloidal gold-labeled antigen or antibody is used as the tracer, and the cellulose membrane is used as the solid support (Fig. 2) [81, 82], and the detection antibodies or antigens are dropped at the sample pad on the nitrocellulose membrane, which will slowly infiltrate the conjugated pad through capillary action, and antibody-antigen complexes show colloidal gold color reaction [83]. A rapid immunochromatographic strip using colloid gold conjugated anti-excretory/secretory antigens (ESA) IgG antibodies was developed to detect ESA in acute infection of *T. gondii* as early as 2–4 days post-infection, showing high agreement with ELISA in sensitivity and specificity [83]. The antibody detection results of GRA7-, SAG2-based ICT are consistent with those of LAT and ELISA [84, 85]. As ICT is easy, rapid, and convenient to perform, and no special equipment is required, it is suitable for field application.

Piezoelectric immunoagglutination assay (PIA)

The agglutination of antigen-coated gold nanoparticles in the presence of the specific antibodies can be detected by a piezoelectric device, which has been used for the detection of parasite infection [86–89]. Wang *et al.* [90] developed a piezoelectric immunoagglutination assay for *T. gondii* antibodies, whose detection results were in satisfactory agreement with those of ELISA. In contrast to the conventional piezoelectric assays, the immobilization of antibody or antigen on a piezoelectric crystal is not necessary.

Western blotting (WB)

WB can be uses as an aid to conventional serological test described previously. In this test, sera are reacted with *T. gondii* antigen on a membrane transferred from a polyacrylamide gel, and the resulting banding patterns are matched with known molecular weight.

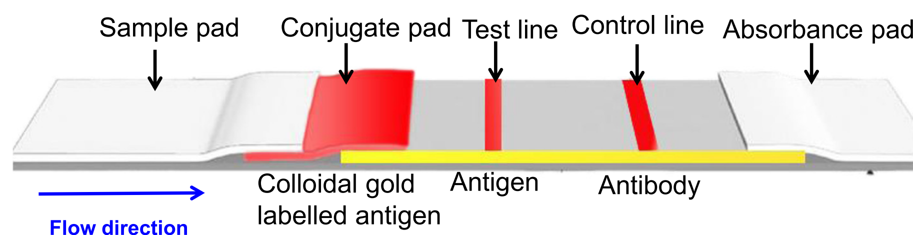

Fig. 2 Schematic diagram of the immunochromatographic test for detection of *T. gondii*-specific antibody. The colloidal gold-labeled antigen or antibody is used as the tracer and the cellulose membrane is used as the solid support

An immunoblot test exhibited a specificity of 100 % and a sensitivity of 98.5 % to detect specific anti-*T. gondii* IgG antibodies in human saliva [91], but showed a lower specificity of 83 % for toxoplasmic chorioretinitis [92]. WB is a useful complementary tool for the early postnatal diagnosis of congenital toxoplasmosis, as the combination of IgA- and IgM-ELISAs, IgG and IgM WB, and the combination of both techniques shows a sensitivity of 94 %, 94 %, and 100 % during the first 3 months of life, respectively [93].

Avidity test

The presence of anti-*T. gondii* IgG antibodies implies the parasite infection, but gives no information on infection time; anti-*T. gondii* IgM is not an accurate marker of acute infection [94–96], nor is IgA a specific marker of the acute phase [97]. The IgG avidity test, first described by Hedman *et al.* [98], is now widely used to differentiate between acute and chronic *T. gondii* infections [99].

The avidity of *T. gondii* antigen to specific antibodies can vary during the course of infection. During the early stage of infection, avidity values are low, and increase with duration of infection [97, 98]. Thus, the avidity test can distinguish acute and chronic infection of *T. gondii*. In the test, sera are run with or without treatment with urea, or other protein denaturing agents, and the difference in titers can be used to determine recent infection. The test is applicable in IgG, IgA, and IgE by different serological procedures, such as ELISA and WB [100–103]. However, there are limitations to the test. *T. gondii*-specific low-avidity IgG antibodies in pregnant women may persist for months [104, 105], and treatment of *T. gondii* may delay the avidity maturation during pregnancy [106–108]. High concentration of antibodies in serum sample may affect the results of avidity test, making it necessary to improve detection methods of antibody avidity [109].

Imaging techniques

Imaging techniques, such as computed tomography (CT), magnetic resonance imaging (MRI), and ultrasonography (US), are not specific, but can facilitate the diagnosis of toxoplasmosis and monitor the therapeutic effect [110–115]. As immuno-deficient patients often develop encephalitis and brain abscesses when infected with *T. gondii*, CT and MRI can be used to locate the lesions. CT is often used as an initial screening test, and MRI is more suitable for the determination of the damage extent [113]. For congenital toxoplasmosis, US is recommended for prenatal diagnosis [116, 117], and CT can detect diffuse hydrocephalus and brain calcifications of toxoplasmosis in infants [115].

Molecular methods based on detection of parasite nucleic acids

Molecular methods are used in addition to conventional serological methods for the diagnosis of toxoplasmosis. Conventional methods are usually not misleading, but are limited in prenatal cases or in immunocompromised patients. For example, a mother may be diagnosed accurately by serology that she has had a current infection during pregnancy and so her baby is potentially at risk of congenital infection but the serology results cannot confirm whether the parasite has been transferred to the baby. However, the molecular diagnostic techniques may do so.

Conventional PCR

Due to inherent limitations of traditional diagnostic methods, PCR can be used in addition to serology to diagnose *T. gondii* infection. PCR is an efficient *in vitro* enzymatic amplification method that allows specific amplification of DNA from minute amounts of starting material in a short time [118]. To achieve high sensitivity, several multicopy targeting genes are usually used for the detection of *T. gondii* in biological samples, including the B1 gene, the 529 bp repeat element and the internal transcribed spacer (ITS-1) or 18S rDNA sequences (Table 2). The presence of a parasitaemia is seldom detected therefore PCR of blood has a low negative predictive value. Several other single-copy genes, such as SAG1, SAG2, and GRA1, have also been used as PCR targets in some laboratories.

The first PCR method for *T. gondii* detection, targeting the B1 gene, was established in 1989 [119]. This method has widely been used in prenatal diagnosis of congenital toxoplasmosis and *T. gondii* infection in immunocompromised patients [120–124]. PCR with the 529 bp repeat element was reported to be 10- to 100-times more sensitive than the B1 gene [125, 126]. The multicopy ITS-1 and 18S rDNA have also been used as the targets in a few studies, showing a similar sensitivity of the B1 gene [127–129].

To further improve the sensitivity and specificity, nested PCRs based on the B1 gene, the 529 bp repeat element, and ITS-1 sequences have been developed [130, 131]. In the nested PCR, two sets of primers are used in two successive PCRs. The products of the first reaction are used as templates for the second PCR. For a given targeting gene, nested PCR is more sensitive than the conventional PCR. The detection limit of the 529 bp repeat element-nested PCR is 640 fg of parasite DNA, while the rate for B1-nested PCR is 5.12 pg [130], and the nested PCR targeting the B1 gene is more sensitive than targeting ITS-1 sequence [131].

The sequence of the PCR product must be verified to provide adequate diagnostic specificity. The conventional

Table 2 Summary of the molecular approaches used for detection and genetic characterization of *T. gondii*

Molecular methods	Main purposes	DNA target regions	References
Conventional PCR	Species detection	B1 gene, 529 bp repeat element, 18S rDNA gene, SAG1, SAG2, and GRA1	[18, 147, 148, 197]
Real-time PCR	Species detection	B1 gene, 529 bp repeat element, 18S rDNA gene, SAG1	[198, 199]
LAMP	Species detection	529-bp repetitive element, B1, SAG1, SAG2, GRA1, oocyst wall protein genes	[145, 200]
Microsatellite analysis	Genotyping	TUB2, W35, TgM-A, B18, B17; M33, IV.1, XI.1, M48, M102, N60, N82, AA, N61, and N83	[156]
Multilocus sequence typing	Genotyping	BTUB, SAG2,, GRA6, and SAG3	[162, 163]
PCR-RFLP	Genotyping	SAG1, SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1 and Apico	[18]
RAPD-PCR	Genotyping	Genomic DNA	[177]
High-resolution melting (HRM) analysis	Genotyping	B1 gene	[182]

technique is hybridization with a specific probe by Southern blotting, which requires an additional 12–24 h to complete. The PCR-ELISA is an alternative technique, in which PCR products hybridize to an immobilized capture probe. The assay thus measures sequences internal to the PCR product [132]. Martinez *et al.* [133] developed a rapid PCR-ELISA assay using polystyrene beads for the detection of *T. gondii* DNA, whose detection threshold is equivalent to Southern blotting.

Real-time PCR

Real-time PCR can detect low concentrations of target DNA and quantify starting copies of specific template DNA. The amplification product is measured during each cycle using probes or intercalating dyes, and can be quantified by a standard of known concentration. Real-time PCR has been successfully used to detect *T. gondii* DNA in human blood, cerebrospinal fluid, aqueous humor, amniotic fluid, and other samples [134–137]. The real-time PCR is also used to evaluate toxoplasmosis progression and treatment efficacy, since it can estimate the intensity of *T. gondii* infection [138]. The real-time PCR assay with the B1 gene is considered as the best-performing technique for diagnosis of congenital toxoplasmosis, compared with conventional PCR and nested-PCR [139]. As a rapid closed-tube system, real-time PCR eliminates the possible risk of contamination and produces reproducible quantitative results. Thus it is suitable for standardization [140].

Opsteegh *et al.* [141] described a sequence-specific magnetic capture method for the isolation of *T. gondii* DNA from large samples of tissue, which can overcome the heterogenous distribution of *T. gondii* tissue cysts, and the small size of the sample. This technique combined with real time PCR can be used in meat samples, and provide an alternative for bioassays to evaluate the burden of *T. gondii* in various tissues of food-borne animals [142, 143].

Loop-mediated isothermal amplification (LAMP)

LAMP is a unique DNA amplification technique under isothermal conditions using four primers that recognize six regions on the target DNA [144]. This method is slightly more sensitive than conventional PCR, but slightly lower than real time PCR [145, 146]. LAMP assays targeting the *T. gondii* SAG1, 529-bp repetitive element, B1, SAG2, GRA1, oocyst wall protein (OWP) genes, and 18S rRNA were developed for the veterinary and medical samples, and water samples [145, 147–152]. The LAMP based on SAG1 can detect *T. gondii* in the blood of experimentally infected pigs as early as 2 days post-infection, suggesting that this approach can be used for early diagnosis of toxoplasmosis [145]. The detection limit of both the B1- and OWP-LAMP assays is 0.1 tachyzoites DNA [151]. LAMP assays targeting SAG1, SAG2, and B1 are useful to detect *T. gondii* in blood samples of humans [147, 150, 153, 154], as well as in water resources [155]. As LAMP requires only a water bath or heat block, and allows visual detection of amplification products, it may be an alternative diagnostic method in the field, where sophisticated and expensive equipment may not be available [144]. However, in our hands, the LAMP seems extremely sensitive to contamination; therefore a rigorous quality control is essential to rule out false positives.

Genotyping methods based on molecular technologies

For epidemiological studies, it is important to identify genotypes of *T. gondii* infection, and some molecular technologies, including microsatellite analysis, multilocus sequence typing, PCR-RFLP, RAPD-PCR, and high-resolution melting (HRM) analysis, have been developed.

Microsatellite analysis

Microsatellite (MS) sequences are tandem short DNA motif repeats that are widespread in eukaryotic genomes and the sequences usually change due to insertion or deletion of repeat units. The numbers of repeat units differ in a population, thus producing multiple alleles at an MS locus. The tandem repeats in *T. gondii* are often simple, and composed with as few as 2 nucleotides, and occur 2–20 times [156–159]. A total of 15 MS markers, including TUB2, W35, TgM-A, B18, B17; M33, IV.1, XI.1, M48, M102, N60, N82, AA, N61, and N83, have been used to genotype *T. gondii* in different laboratories [156, 157, 160, 161]. Ajzenberg *et al.* [156] developed an easy-to-use method for genotyping *T. gondii* in a single multiplex PCR assay using 15 microsatellite markers, in which the 8 MS markers (TUB2, W35, TgM-A, B18, B17, M33, IV.1, and XI.1) could differentiate types I, II, and III from all the atypical genotypes, and the other 7 markers (M48, M102, N60, N82, AA, N61, and N83) could enhance genetic resolution in differentiating closely related isolates within one haplogroup or clonal lineage [158]. The 15-MS multiplex assay is the best tool available to identify *T. gondii* isolates genetically different or identical, i.e., to identify the infection source in an outbreak, laboratory contamination and mixed infections [158]. The limitation of this assay is the requirement for an automated sequencer. In addition, small amount of DNA from biological samples could cause the absence of detectable peaks or peaks of low intensity, which is undistinghishable from nonspecific PCR products [156].

Multilocus sequence typing (MLST)

Multilocus sequence typing (MLST) is based on DNA sequence polymorphisms, including the single nucleotide polymorphisms (SNPs), and deletion and insertion of nucleotides, which has the highest resolution among all typing methods when enough genomic DNA is available [18]. Several studies have revealed some alleles unique to the Brazil isolates, including 5′-SAG2, 3′-SAG2, BTUB, GRA6, and SAG3 [162, 163]. However, this approach is not a good choice for clinical samples, as a large quantity of genomic DNA is required for this assay.

PCR-RFLP

The PCR-RFLP is based on the ability of restriction endonucleases to recognize SNPs, digest PCR products and subsequently display distinct DNA banding patterns on agarose gels electrophoresis [16]. How and Sibley [16] identified 3 predominant lineages (types I, II and III) from 106 *T. gondii* isolates from humans and animals by PCR-RFLP using 6 markers. Since then, several different sets of multilocus PCR-RFLP markers have been employed to characterize individual *T. gondii* isolates in different laboratories [164–169]. The conventional multilocus PCR-RFLP relies on single-copy polymorphic DNA sequences, and usually requires a relatively large amount of parasite DNA. Thus, it is difficult to genotype *T. gondii* in biological samples, due to the limited parasite DNA available.

To alleviate this problem, a multiplex multilocus nested PCR-RFLP (Mn-PCR-RFLP) was developed, using 10 genetic markers, including SAG1, SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1 and Apico [170]. The sensitivity of this method is increased by at least 10 times, comparing with conventional PCR-RFLP [18]. The advantage of this method is that only a limited amount of DNA sample is needed, and it is very useful when only small amounts of ‘precious’ samples are available. Mn-PCR-RFLP has widely been applied to the genetic typing of clinically positive samples, and a large amount of data regarding genetic diversity and population structure of the parasite were generated [171–176]. The major precaution for this assay is that, if the contamination occurs in the early cycles of PCR, erroneous results may be generated. To avoid error results of PCR amplification in Mn-PCR-RFLP, the negative control has to be included in each experiment. In addition, several reference *T. gondii* isolates should be included to monitor the efficiency of PCR amplification and restriction enzyme digestion [18].

Random amplified polymorphic DNA-PCR (RAPD-PCR)

RAPD-PCR is a PCR-based technique that can be used to identify DNA polymorphisms without predetermined genetic data. It is based on the amplification of genomic DNA using single short arbitrary primers under low stringency conditions. RAPD-PCR is good for detecting genetic differentiation of closely related organisms, and has been employed to identify the genotype of *T. gondii* strains [177–179]. *T. gondii* could be classified into virulent and avirulent strains based on the murine virulence by RAPD-PCR using arbitrary primers, and some primers are useful to identify the virulence markers [177]. This technique is quick, simple and efficient. However, RAPD band profiles may be difficult to reproduce between, even within laboratories, if personnel, equipment or conditions are changed. Only a small amount of DNA is required for this assay, but it must be highly pure [180]. Thus, RAPD-PCR cannot be directly used for the clinical samples.

High-resolution melting (HRM)

HRM is a homogeneous, close-tube and post-PCR method to analyze genetic variations, which can characterize polymorphisms based on their melting temperatures related to their sequences, lengths and GC contents [181]. Based on a single SNP of the multicopy B1 gene, HRM analysis can correctly classify *T.*

gondii strains into three distinct types [182]. HRM is more informative than the microsatellite analysis, therefore, becoming a supplementary test for multi-locus microsatellite analysis [182, 183]. This assay was developed to directly genotype *T. gondii* infection from biological samples, with a higher genotyping capacity using multi-copy gene than single-copy gene, thus avoiding cell culture or bioassay [183]. HRM is a potentially simple solution for genotyping, mutation scanning, and sequence matching.

Serotyping methods based on polymorphic polypeptides

T. gondii infection induces a strong and persistent humoral immune response in the hosts. Some *T. gondii* antigenic proteins present sequence polymorphisms in different clonal types. The polymorphic peptides from the *T. gondii* antigens SAG2A, GRA3, GRA6, and GRA7 can accurately recognize the type I, II, and III in mice, and peptides from GRA6 can distinguish type II from non–type II infection [184]. Xiao *et al.* [185] developed ELISAs based on polymorphic peptides derived from three dense granule antigens GRA5, GRA6 and GRA7, which can distinguish type III- from type I-infections in humans. Several trials have been made to type *T. gondii* infections using ELISA formats, in which synthetic peptides are coupled via keyhole limpet hemocyanin, or directly to the solid phase [185–188]. The recombinant antigens can also be used for serotyping [189]. The peptide-microarray tests for *T. gondii* serotyping in humans and cats have been established, which are more sensitive than peptide-ELISAs [190, 191]. As serotyping is fast, inexpensive, relatively noninvasive, and there is no need to isolate parasites, this technique has the potential to become the method of choice for typing *T. gondii* in humans and animals. However, there are some limitations to the serologic assay. The selected peptides may be low sensitive, or cross-reactive in detecting recombinant strains [186]. The immunosuppressed patients may not produce sufficient specific antibodies to reach the detection threshold (DT titer of 1:64). Importantly, infection with the rare genotypes may induce entirely different humoral responses that may not be detectable using polymorphic polypeptides [184].

Conclusion

This review has attempted to provide a survey of available and developing biotechnologies for the detection of *T. gondii* infection, the diagnosis of toxoplasmosis and typing of *T. gondii* isolates. A key to effective management of toxoplasmosis is prompt and accurate diagnosis of disease. Though diagnosis of toxoplasmosis by detection of the parasite using microscopy and bioassays is regarded as the gold standard, its clinical diagnosis is more likely made by serological methods, and various serological tests have been established for the detection of *T. gondii*-specific antibodies, or circulating antigens. The molecular technologies based on nucleic acid amplification can be used in addition to conventional serological methods for the diagnosis of toxoplasmosis, and have been the focus of continued development in recent years. The recent development of the Mn-PCR-RFLP method makes it possible to genetically characterize or classify *T. gondii* from biological samples with high resolution. Serotyping methods based on polymorphic polypeptides have the potential to become the choice for typing *T. gondii* in humans and animals. With the increased usage of genomic, transcriptomic, and proteomic technologies and development of multilocus genotyping methods, the integrated use of molecular and bioinformatic technologies will be crucial to investigate genetic characterization of *T. gondii*, and could provide prospects for the design of entirely new diagnostic methods for toxoplamosis.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

QL and XQZ conceived and designed the review, and critically revised the manuscript. QL drafted the manuscript. ZDW and SYH contributed to drafting the manuscript. All authors read and approved the final manuscript.

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Author details

[1]State Key Laboratory of Veterinary Etiological Biology, Key Laboratory of Veterinary Parasitology of Gansu Province, Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Lanzhou, Gansu Province 730046, People's Republic of China. [2]Key Laboratory of Jilin Province for Zoonosis Prevention and Control, Military Veterinary Institute, Academy of Military Medical Sciences, Changchun, Jilin Province 130122, People's Republic of China. [3]Jiangsu Co-innovation Center for the Prevention and Control of Important Animal Infectious Diseases and Zoonoses, Yangzhou University College of Veterinary Medicine, Yangzhou, Jiangsu Province 225009, People's Republic of China.

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