

## Review

# Emerging diagnostic approaches for toxoplasmosis: A review

Vikrant Sudan\*, Amit K. Jaiswal and Daya Shanker

Department of Parasitology, College of Veterinary Sciences and Animal Husbandry, U P Pandit Deen Dayal Upadhyaya Pashu Chikitsa Vigyan Vishwavidyalaya Evam Go Anusandhan Sansthan (DUVASU), Mathura-281001, India.

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*Toxoplasma gondii* is one of the most widely prevalent cyst forming apicomplexan parasite present worldwide. Felines serve as definitive hosts, while all non-feline vertebrates including humans, act as intermediate hosts of the parasite. There are several serological tests available for the detection of *T. gondii* antibodies such as the dye test (DT), indirect haemagglutination test (IHA), complement fixation test (CFT), modified agglutination test (MAT), latex agglutination test (LAT), indirect immunofluorescent test (IFAT) and direct agglutination test (DAT). The dye test once considered as gold standard is seldom used nowadays, owing to potent hazardous effects because of involvement of live tachyzoites. Of late, enzyme linked immune sorbent assays (ELISA) have gained a lot of utility in diagnosing toxoplasmosis. Other than serological tests, bioassays, polymerase chain reaction (PCR) based assays and histopathologies are also used. The various tests used for diagnosis of toxoplasmosis, along with various molecules being employed, coupled with their advantages and limitations, are described and discussed in detail in the present review.

**Key words:** *Toxoplasma gondii*, diagnosis, enzyme linked immune sorbent assays (ELISA), molecular techniques, polymerase chain reaction (PCR).

## INTRODUCTION

*Toxoplasma gondii* is one of the most widely prevalent cyst forming apicomplexans parasites and have been recorded worldwide. Felines serve as definitive hosts, while all non-feline vertebrates, including humans act as intermediate hosts of the parasite with disseminated tissue infections. The parasite is distributed worldwide in the human population and is estimated to affect more than a billion individuals. The parasite has significant impact not only on animal production but also on public health throughout the world.

Nicolle and Manceaux (1908) provided first comprehensive description of *T. gondii* tachyzoites from the spleen, liver and blood of Gundi (*Ctenodactylus gundi*), a rodent species of North Africa. They named the genus *Toxoplasma* in 1909 and *T. gondii* became the type species of the genus. Electron microscopic (EM) studies conducted during 1960s provided evidence of the coccidian nature of *T. gondii* and revealed ultrastructural

similarities between extra-intestinal merozoites of *T. gondii* and intestinal merozoites of *Eimeria* species (Mehlhorn, 1972). The heterogeneous life cycle of *T. gondii* was elucidated in the late 1930s when it was found that the faeces of cats may contain an infectious stage that is, the isosporan oocysts of *T. gondii*, which can induce infection when ingested by intermediate hosts (Hutchison, 1965).

In 1970, knowledge of the coccidian life cycle of *T. gondii* was completed by the discovery of sexual stages in the small intestine of cats (Dubey and Beattie, 1989; Jackson and Hutchison, 1989; Dubey, 1998). It was further attributed that *T. gondii* is a tissue cyst, forming coccidium with a heteroxenous lifecycle in which an asexual phase of the development in various tissues of herbivorous, carnivorous or omnivorous intermediate hosts is linked to a sexual phase of development in the intestine of carnivorous definitive host, felids (Tenter et al., 2000).

## Diagnostic techniques

Various laboratory techniques are used in diagnosis of

\*Corresponding authors. E-mail: [viks.sudan@gmail.com](mailto:viks.sudan@gmail.com). Tel: 09045414200.

toxoplasmosis that is, isolation, serology, protein analysis and molecular tests.

### **Isolation of the agent**

Isolation of *T. gondii* from aborted ovine and caprine fetuses and fetal membranes is best made by inoculation of the tissues in laboratory mice. The best tissues for inoculation are fetal brain and placental cotyledons, and optimum results are obtained with fresh samples free from contamination. Bioassays in murine models can further be used for confirmation about the presence of the parasite in the suspected material.

### **Serological tests**

There are several serological tests available for the detection of *T. gondii* antibodies such as DT (Sabin and Feldman, 1948), IFAT, IHA (Jacobs and Lunde, 1957), CFT, MAT (Desmonts and Remington, 1980), LAT and DAT (Dubey and Desmonts, 1987; Devada et al., 1998).

**Dye test:** It is the 'gold standard' serological test for *T. gondii* antibody detection in humans. Live *Toxoplasma* tachyzoites are incubated with a complement-like accessory factor and the test serum at 37°C for 1 h before methylene blue is added. Specific antibody induces membrane permeability in the parasite so that the cytoplasm is able to leak out and the tachyzoite does not incorporate the dye and so appears colorless. Tachyzoites not exposed to specific antibody (that is, a negative serum sample) take up the dye and appear blue. It is highly specific and sensitive in humans, but may be unreliable in other species and is potentially hazardous owing to the use of live parasites. It is expensive and requires a high degree of technical expertise.

**Indirect immune fluorescent test:** IFAT (Munday and Corbould, 1971) is a simple test and has been used extensively in detection of *T. gondii* antibodies in animals and man. Whole, killed *Toxoplasma* tachyzoites are incubated with diluted test serum, the appropriate fluorescent anti-species serum is added, and the result is then viewed with a fluorescence microscope.

Fluorescent-labeled antibodies are available commercially for a variety of animal species, the method is relatively inexpensive and kits are commercially available. However, the method requires a fluorescence microscope and the results are read by eye, so individual variation may occur, besides the use of expensive reagents.

Adding fluorescent-labeled anti-species immunoglobulin

G (IgG) or whole immunoglobulin and viewing under a fluorescent microscope enhances antibody detection. A modification of IFA (IgM-IFA) was developed by Remington (1969) to detect immunoglobulin M (IgM) antibodies in congenitally infected children and could detect 75% of congenital infections. Pappas et al. (1986) used 1.5% formalin fixed tachyzoites of *T. gondii* (RH strain) as antigen in IFAT and found four out of 56 humans sera positive for IgM and 51 out of 56 (91%) for IgG antibodies at a reciprocal titre of  $\geq 16$ .

In United States, a manual of the Department of Health, Education and Welfare (USDHEW) provided a procedural guide for the performance of IFAT in detecting *T. gondii* antibodies. Kumar (2004) screened 150 field goat sera collected from a local abattoir by IFAT and found that *T. gondii* antibodies could be detected in 42.66% of animals, and found that the acetone fixed whole tachyzoite antigen in IFAT could detect the earliest antibody on 8th day post infection (DPI) with mean titres ranging from 1:18 to 1: 2594.

**Modified agglutination test:** MAT (Desmonts and Remington, 1980) is both sensitive and specific. Formol-fixed *Toxoplasma* tachyzoites are added to U-shaped well microtiter plates and dilutions of test sera are then applied. Positive samples will produce agglutination that can be graded, while negative samples will produce a 'button' of precipitated tachyzoites at the bottom of the well. The test is simple and easy to perform although relatively large amounts of antigen are required. Commercial kits are commonly available in the market.

**Enzyme-linked immunosorbent assay:** ELISA uses crude soluble antigens adsorbed onto the walls of microtiter plate wells and the antigen-antibody reaction is enhanced by the addition of a secondary enzyme-linked antibody, and the reaction can be assessed objectively by quantization of the colour that developed by an ELISA reader. The procedure is simple to perform, economical and easily adoptable for field use (Anthony et al., 1980; Spencer et al., 1980). The original ELISA (Voller et al., 1976) uses a soluble antigen preparation made from *Toxoplasma* RH strain tachyzoites and layered into wells in a microtitre plate. Test sera (for example, ovine in origin) are added followed by an anti-species enzyme-labeled conjugate such as horseradish peroxidase labeled anti-ovine-IgG. Any attached conjugate causes a color change in the substrate that is directly related to the amount of bound antibody, and which can be read with a spectrophotometer at the absorbance specific to the substrate used. The assay is simple, can readily test a large number of samples and is easy to perform with the chosen anti-species conjugate. Defined anti-species conjugates, substrates and whole kits are commercially available. However, the assay does require a spectrophoto-

meter. The ELISA is well suited to laboratories required to analyze large numbers of samples.

Dubey (1995) used *T. gondii* tachyzoite lysate (9.2 µg of protein/ml equivalent to  $5 \times 10^8$  tachyzoites/ml) in ELISA and found 24% out of 1,000 sows, positive for *T. gondii* antibodies with a sensitivity and specificity of 72.9 and 85.9%, respectively. Vitor et al. (1999) detected specific antibodies in goats subcutaneously infected with *T. gondii* C4 strain, tachyzoite ( $10^7$ ) by 12 DPI that reached a peak between 19 and 62 DPI.

In a study conducted by Tenter et al. (1992), the ELISAs based on the recombinant antigen H4/GST or a mixture of the two recombinant antigens H4/GST and H11/GST gave consistent results with those obtained with the ELISA based on traditional ELISA antigen (TEA). Both the recombinant antigens or their combinations showed much higher sensitivity, specificity, positivity and negative predictive values. Using an improved ELISA format, it is possible to target *T. gondii* specific IgM, IgG and IgA antibodies (Takahashi and Ross, 1994).

### **Protein analysis**

**Native protein:** Various sero-epidemiological studies were conducted in different animals and human beings by using native tachyzoite protein as a diagnostic antigen in ELISA, LAT, IHA and MAT. A comparative study of the IHA, IFAT and ELISA tests was carried out to determine the prevalence of *T. gondii* antibodies in goats. A high and significant positive correlation was found between the titers obtained by the IHA versus IFAT, IHA versus ELISA, and ELISA versus IFAT. Therefore, it can be concluded that the three analyzed tests have shown to be highly concordant and appropriate for epidemiological surveys of *Toxoplasma* infection in goats (Figueiredo et al., 2001). Meireles et al. (2004) tested sera samples from stray cats and dogs for antibodies to *T. gondii* by indirect IHA and ELISA. Antibodies to *T. gondii* were found in 40% of the cats, less than 50.5% in dogs by ELISA. Haemagglutination showed low resolution and concordance, precluding their use for diagnosis of *T. gondii* infection compared with ELISA. The validation of a *T. gondii* IgG avidity ELISA was reported by Sagar et al. (2003) based on the use of an affinity-purified, native *T. gondii* SAG1 antigen. The assay was used to examine sera from sheep experimentally infected with *T. gondii*. High IgG avidity was found in 80% of the seropositive lambs, about 90% of the clinically healthy ewes and 97% of the ewes with abortion problems.

**Recombinant proteins:** Since a number of the serological targets for the detection of *T. gondii* antibodies are sequestered within internal organelles, the method of preparation of the tachyzoite antigen has a significant effect on assay performance, as shown by the

differential agglutination assay (Dannemann et al., 1990). The most important recombinant proteins which are presumed to be the candidates for diagnosis, as well as subunit vaccine development, are surface antigen 1 (SAG1), surface antigen 2 (SAG2), microneme protein (MIC3), rhoptry proteins (ROP2), GRA1 and GRA7. SAG1 is highly abundant and the most immunogenic surface protein of *T. gondii* tachyzoites present during the acute phase of the disease, but it is not expressed in bradyzoites during the chronic stage of infection. Therefore, the SAG1 antigen has been the focus of intensive research with the aim of developing a diagnostic tool against *T. gondii* infection (Marti et al., 2001).

In an attempt to develop a recombinant protein based ELISA, the gene coding SAG1 of *T. gondii* was cloned and expressed in *E. coli* as glutathion-S-transferase (GST) fusion protein by Kimbita et al. (2001). The recombinant SAG1 (rSAG1) was used for ELISA. Results of ELISA were comparable with those of a commercially available latex agglutination test (LAT) kit. Velmurugan et al. (2008) assessed the diagnostic efficiency of the recombinant proteins SAG1 and GRA7, either individually or as a cocktail of the recombinant proteins with 56 reference goat sera by ELISA. The reactivity of the recombinant proteins as a cocktail preparation was more than that of individual proteins in ELISA and could detect accurately the infection in goats. This was the first report of serological detection of caprine toxoplasmosis by ELISA using a cocktail of recombinant *Toxoplasma* proteins.

SAG2 (P22) is major surface protein known as attachment ligand (Grimwood and Smith, 1996) that also has good antigenicity and immunogenicity (Aubert et al., 2000; Zhou et al., 2012). To evaluate the potential use of rSAG2 for diagnosis of feline toxoplasmosis by ELISA, it is shown that out of 192 samples screened, 21.9% were positive and there was significant co-relation between the titers of ELISA and LAT that led to the conclusion that ELISA with rSAG2 can be a useful method for detection of *T. gondii* in cats, as there was no cross reactivity with *Neospora caninum* (Huang et al., 2002). Sudan (2010) used rSAG1 and rSAG2 for diagnosis of toxoplasmosis in 252 field cattle sera samples and found that with rSAG1, a better diagnostic molecule was obtained as compared to rSAG2.

GRA9 (B10) exhibit strong immunoreactivity with human sera by ELISA and could be valuable in diagnostics or immunoprophylaxis (Nockemann et al., 1998). GRA7 could detect IgM-positive antibodies with high sensitivity especially high-titer IgM-positive sera (IgM titers by IF, 1/400 and 1/800). The high optical density (OD) values obtained using GRA7 seem to contradict the findings of Fisher et al. (1998) who found that GRA7 is released from bradyzoite-infected host cells (chronic phase) and not from tachyzoite-infected host cells (acute phase). If GRA7 is secreted from bradyzoites, a strong antibody

response to this antigen would be expected in sera from chronic patients. Unexpectedly, Jacobs et al. (1999) observed a low antibody response against this antigen in these patients. On the other hand, in sera from patients with acute infections, a strong antibody response was reported.

It is speculated that bradyzoites studied *in vitro* behaved differently from those found in cysts upon encapsidation of the parasite in the organs of infected patients. The fact that GRA7 was found in the parasitophorous vacuole (PV), the PV membrane, and the cytoplasm of the host cell infected with the tachyzoite stage is in agreement with the ELISA results, where high reactivity with sera from acute-stage infections is found. GRA7 was evaluated in an ELISA for detection of IgG response using two sets of IgG-positive human serum samples, an overall sensitivity of 81% was reported (Jacobs et al., 1999). For chronic-phase sera, the sensitivity of detection was 79%. When GRA7 was combined with Tg34AR (rhostry protein 2, C-terminal fragment), the sensitivity rose to 96%.

Aubert et al. (2000) evaluated the diagnostic utility of 11 *T. gondii* recombinant antigens which include P22 [SAG2], P24 [GRA1], P25, P28 [GRA2], P29 [GRA7], P30 [SAG1], P35, P41 [GRA4], P54 [ROP2], P66 [ROP1], and P68 in IgG and IgM recombinant enzyme-linked immunosorbent assays (Rec-ELISAs). Following an initial evaluation, 6 recombinant antigens (P29, P30, P35, P54, P66, and P68) were tested in IgG and IgM Rec-ELISAs with four groups of samples that span the toxoplasmosis disease spectrum (negative, chronic infection, acute infection, and recent seroconversion). Their results suggested that combination of P29, P30, and P35 in an IgG Rec-ELISA and the combination of P29, P35, and P66 in an IgM Rec-ELISA, can replace the tachyzoite antigen in IgG and IgM serologic tests, respectively. The relative sensitivity, specificity, and agreement for the IgG P29-P30-P35 Rec-ELISA were 98.4, 95.7, and 97.2%, respectively. The resolved sensitivity, specificity, and agreement for the IgM P29-P35-P66 Rec-ELISA were 93.1, 95.0, and 94.5%, respectively.

It has been shown that combination of two *T. gondii* antigens can increase the sensitivity of Rec-ELISAs. The combination of H4 (P25) and H11 (P41) in an IgG Rec-ELISA had combined reactivity of 81.3% versus 54 and 61%, respectively, for H4 and H11 alone (Tenter and Johnson, 1991). Likewise, the combination of GRA7 (P29) and Tg34AR (ROP2) in an IgG Rec-ELISA have a combined reactivity from 93 to 96% versus from 65 to 83% for GRA7 and from 76 to 91% for Tg34AR (Jacobs et al., 1999).

Nigro et al. (2003) reported that combination of rGRA4 with rGRA7 did not improve the difference of reactivity between serum samples produced from recently acquired and chronic cases. Whereas, the combined use of rGRA4 or rGRA7 with rROP2 or rSAG1ct decreased the signifi-

cant difference in ELISA. rGRA4 has been further reported to be useful in the diagnosis of recently acquired infections. This was in agreement with the results obtained by Li et al. (2000). GRA7 has also shown to be a good marker for recently acquired infections. The performance of the four fusion proteins GST-GRA3, GST-GRA7, GST-MIC3 and D-SAG1 was assessed using ELISA with the 60 IgG-positive samples, and for each recombinant product, the cut off value was determined as the mean plus 2 standard deviations (SD) of the absorbance readings obtained for the 30 *Toxoplasma* IgG-negative sera. D-SAG1 and GST-MIC3 fusion proteins reacted with 92 and 95% of the positive sera, respectively, and both GST-GRA3 and GST-GRA7 reacted with 85% of the positive sera (Beghetto et al., 2003)

Ferrandiz et al. (2004) developed ELISA using two recombinant antigens of *T. gondii* (GRA1 and GRA6 Nt) in order to differentiate recently acquired infection with chronic one in pregnant women, based on serological profile. For both antigens, the specificity reached 98%. In both groups of infected patients, the overall sensitivity scored was 60% for GRA1 and 83% for GRA6 Nt. Mishima et al. (2001) prepared recombinant antigens using DNA fragments corresponding to SAG1, SAG2, SAG3, SRS1 and P54 of *T. gondii* RH strain and used each of the recombinant product or their mix to vaccinate the mice. Up to 25% of the mice vaccinated with SAG2, SRS1 and P54 or mix survived, and no brain cysts were observed in them, whereas vaccination with lysate of *Toxoplasma* though almost completely protected the mice in acute phase of infection, and failed to prevent the development of cysts in the brain that resulted in gradual decrease of survivors during the experiment over period of 4 months. rMAG1 (23.8 kDa), the matrix antigen expressed in bradyzoites and tachyzoites of *T. gondii*, was applied in ELISA for detection of infection in humans and it was concluded that this antigen should be considered as specific marker for detection of IgG in persons with acute infection (Holec et al., 2007).

Of late, the seroprevalence of *T. gondii* in Dutch cattle and the correlation between detection of antibodies and parasitic DNA was analysed and it was found that DNA was detected in only two seronegative cattle (Opsteegh et al., 2011). The discordance demonstrated that seroprevalence cannot be used as an indicator of the number of animals carrying infectious parasites. Hence, the use of molecular methods is warranted.

## Molecular methods

Parasite DNA can be extracted and purified from several tissues, including placenta, the central nervous system, heart and skeletal muscle. Several PCR based assays have been developed for detection of DNA from *T. gondii*. The main target regions are the B1 repetitive sequence,

the 18S ribosomal DNA or P30 (SAG1) gene (Pelloux et al., 1996). The B1 and P30 PCR are both widely used techniques and good diagnostic aids, but are better used in conjunction with another test, as they are insufficiently robust when used alone. Customized synthetic DNA oligonucleotides are commercially available (for example, www.sigma-geosys.co.uk) and the method described is nested form of the PCR, amplifying the B1 repetitive sequence of DNA (Wastling et al., 1993).

A PCR assay based on the detection of 18S rRNA has been investigated and may prove to be more sensitive; however, further studies are required (Ellis, 1998). PCR using the B1 gene and B22/B23 set of primers is a single, rapid and reliable method that may be valuable for discrimination between toxoplasmosis and other central nervous system (CNS) diseases (Alfonso et al., 2009). Wasting et al. (1994) attempted detection of *T. gondii* in tissues, lymph and blood of experimentally infected sheep directed to B1 and SAG1 genes, and compared with mouse inoculation test. While the PCR assays could not detect *T. gondii* in any of the tissues tested, PCR for B1 gene was found to be superior to SAG1, as it could detect parasite DNA by the 3rd day in blood and lymph. B1 gene has also been used to detect toxoplasmosis in wild birds (Darwich et al., 2012). Of late, triplex PCR using B1 primers was developed for the detection of toxoplasmosis and it provided some very good results (Rahumatullah et al., 2012).

A sensitive quantitative PCR (QC-PCR) based on amplification of a non-coding 529 bp DNA fragment that is repeated 200 to 300 times in *T. gondii* genome has been described earlier (Homan et al., 2000). Parasite DNA was detected in amniotic fluid of toxoplasmic patients, as well as in various tissues from infected mice. The assay was more sensitive than the B1 PCR and allowed a fair estimation of brain cyst numbers. The QC-PCR was successfully used to titrate tachyzoites containing samples within a range of  $10^2$  to  $10^4$  tachyzoites.

Garcia et al. (2005) used 529 bp Tox4 and Tox5 primers for detection of *T. gondii* from pig tissues and compared it with mouse bioassay and histopathology. Sudan (2010) compared the histopathological technique for diagnosing toxoplasmosis with PCR using SAG3 primers and found amplifications in tissues which were detected negative by histopathology. Nagy et al. (2006) compared the conventional PCR, fluorescent PCR with DNA fragment analysis, quantitative real-time PCR with SYBRGreen I and with fluorescence energy transfer hybridization probe detection and found that conventional PCR along with quantitative real-time PCR detection have the detection limit of 1,000 parasites, followed by fluorescent PCR with the detection limit of 10 to 100 parasites, and finally the real-time PCR using fluorescence energy transfer hybridization probes can detect one parasite.

All the methods employed have their own advantages

and lacunae. PCR is a highly sensitive, specific and rapid technique for detecting *T. gondii*. No doubt, murine bioassays are more sensitive than PCR with respect to the isolation of *T. gondii* but indeed, the ultimate significance of PCR based detection of toxoplasmosis is that PCR can detect the DNA of parasites even when the tissues available for testing are in a state of decomposition; bioassays in contrast, can only detect viable parasites. Moreover, the size of the sample analysed is very important in the detection of even bioassays and it is quite possible that the parasitic stage in the sample to be tested is either low or sparse and show focal distribution in the tissues or it may be all together dead. Bioassay will miss detection in either of these cases. But PCR will give amplification even if the parasitic stage is dead and/or very less in number. PCR can even detect 0.1 pg of DNA, even very few tachyzoites are sufficient in accurate diagnosis no matter if they are living or dead.

The Dye test though very much accurate is time consuming and often hazardous. Among all the techniques for the detection and diagnosis of toxoplasmosis, ELISA using recombinant proteins is viable under field conditions for mass screening. Accurate choice of the antigen moiety can assist in differentiation between acute and chronic infections.

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