The Journal of Animal & Plant Sciences, 24(3): 2014, Page: 676-684
ISSN: 1018-7081

REVIEW PAPER

TOXOPLASMOSIS A GLOBAL THREAT TO ALL VERTEBRATES: TRENDS IN DIAGNOSTIC METHODS

A. A. Bajwa, S. Islam, I. Rashid*, K. Ashraf*, H. Akbar*, and W. Shehzad

Institute of Biochemistry and Biotechnology, University of Veterinary and Animal Sciences Lahore, Pakistan.
*Department of Parasitology, University of Veterinary and Animal Sciences Lahore, Pakistan.
Corresponding author e-mail: wasim.shehzad@uvas.edu.pk

ABSTRACT

Toxoplasma gondii is globally widespread obligate intracellular parasitic protozoan,It affects almost all warm-blooded animals including man. More than one third of human population is infected by this parasite. It can cause life threatening diseases both in immunocompromised as well as immunocompetent hosts. The symptoms of the disease arefever, seizures and ataxia. The parasite has heteroxenous lifecycle and reproduce both sexually and asexually. Sexual cycle is limited to the members of felidae while the asexual cycle completes in mammals and birds. The transmission of parasite to human occurs either by ingestion of poorly cooked meat of infected animals or by accidental intake of food contaminated with cat feces. The infection by toxoplasma results in acute disease, latent infection may also occur. In order to diagnose the disease different diagnostic methods are used like fecal microscopy, a primitive technique is still in use. Different molecular based approaches like serological methods for parasite diagnosis including Enzyme Linked Immunosorbent Assay, Indirect Fluorescence Antibody Test, Modified Agglutination Test and many others are used for pathogen diagnosis by targeting stage specific immunoglobulins. DNA based approaches like PCR have made a significant advancement in the diagnoses of many pathogens including*T. gondii*. Nested-PCR is considered to be more sensitive in diagnosis of toxoplasmosis. For more accurate and sensitive parasite detection, Real-Time PCR is more useful. This review is an attempt to highlight the global threat of the parasite. In addition; different diagnostic approaches are reviewed ranging from classical methods to recent advancements in molecular techniques.

Key words: ELISA, Parasite, PCR, *Toxoplasmagondii*.

INTRODUCTION

*Toxoplasma gondii*is an important unicellular pathogen (Scott *et al*., 2007), affecting almost all vertebrates. It is an obligate intracellular protozoan parasite, harboring both humans and animals worldwide.This protozoan parasite belongs to the phylum Apicomplexa, causing toxoplasmosis worldwide (Mott, 1971; Reid *et al*., 2012). As compared with other apicomplexans, *T. gondii* is considered as the best model system for studying the biology of this phylum. This ease of study is due to presence of many cell markers, high efficiency of stable transfection and quick adaptiveness to genetic manipulations (Kim and Weiss, 2004).

The parasite has heteroxenous life cycle and propagates both sexually and asexually (Cleary *et al*., 2002). The sexual cycle is limited to the members of Felidae (cats) family while the asexual cycle completes in mammals and birds. The three infective stages of *T. gondii* are tachyzoites, bradyzoites and sporozoites. Bradyzoites present in tissue cystsand sporozoites present in sporulatedoocysts are the main causes of infection in humans and animals. Tachyzoites can transmit infection through blood transfusion or through placenta. After digestion of ingested tissue cysts or sporulatedoocysts external walls, the bradyzoitesor sporozoites are released respectively and penetrate intestinal wall where the conversion to tachyzoites form takes place in the lamina propria of small intestine.Bradyzoites are less infective as compared with tachyzoite stage(Dubey, 1998). As soon as the immune system of patient is activated; the tachyzoites assume latent stage known as tissue cyst containing bradyzoites. Once in the latent phase; it is then impossible to eradicate the parasite from hosts (Henriquez *et al*., 2009).

The transmission to human occurs either by ingestion of poorly cooked meat of infected animals (Swai and Schoonman, 2012), or by accidental intake of food which is contaminated with cat feces. Thepeoplehaving cats as pet are more prone to this disease (Dubey and Jones, 2008; Jones and Dubey, 2012; Lass *et al*., 2012). It is not known which route is more important for dissemination of toxoplasmosis but past studies showed that raw or semi cooked meat of pigs and sheep are considered as major cause for transmission (Dubey, 2009; Huong and Dubey, 2007; Paul, 1998). However recent studies have indicated that prevalence of *T. gondii* has been decreased over the past twenty years in meat producing animals because of improved hygienic and management conditions at livestock farms(Tenter *et al*., 2000).

The toxoplasma infection covers a wide range of hosts and it is estimated that one third of human population is affected by this disease (Hill and Dubey, 2002; Hollings *et al*., 2013). It can cause life threatening diseases like encephalitis, retinitis, myocarditis and pneumonia (McAllister, 2005). The symptoms of toxoplasmosis arefever, seizures and ataxia(Hill and Dubey, 2002). The infection by toxoplasma tachyzoitesresults in acute disease, latent infection may also occur. *T. gondii* is able to cross the biological barriers like blood retina, blood brain and blood placental barrier.These events are responsible for severity of the infection (Barragan and Sibley, 2003). As the protozoa can cross placental barrier, the infection may propagate tosubsequent generations. It can cause severe congenital infections or abortions in pregnant women. Mostly the mothers infected during third trimester are more likely to transmit the disease to their newly borne babies; those in second trimester are at intermediate risk while the risk factor is lowest during first trimester. Conversely, the risk of abortion is higher during first trimester and lower in third trimester (Menzies *et al*., 2008).Trophoblast cells are important maternal-fetal barriers with concentrated monocytes around them. As trophoblast cells are able to modulate monocyte activities, it results in the control of toxoplasmosis and thus maintaining pregnancy (Castro *et al*., 2013).

Toxoplasma infection also activates host immune system. There is elevated secretions of IL-12, IFN-gamma and TNF-alfa resulted from the activation of immune system of the host in the protective mechanism against the parasite (Nguyen *et al*., 2003; Ram *et al*., 2013). Once the immune system of the host becomes activated, the parasite goes to its latent stage, which is more hazardous and prevails permanently. *T. gondii*forms a cyst around itself for protection from the host immune system. This cyst formation takes place in body and brain tissues (Silva and Langoni, 2009). Some drugs are designed to target the tachyzoites, which cause direct tissue damage due to their rapidly dividing nature. This tissue damage results in inflammation which in turn activates the immune system (Hitziger *et al*., 2005; Nguyen *et al*., 2003). Thiocarbazones are being used as an alternative for treatment of various diseases. Due to its anti-toxoplasma activities,thiocarbazones have been investigated for the biological effect on *T. gondii* and response of the parasite in the presence of this drug has also been studied (Gomes *et al*., 2013). Bisphosphonic acids have been synthesized as principal drugs and also as potential chemotherapeutic agents for toxoplasma infection (Recher *et al*., 2013). In addition, sulfachloropyrazine is suggested as one of the new therapeutic drug for the infection to treat animal toxoplasmosis (Zeng *et al*., 2012). Yet there are no drugs available to kill the tissue cysts of *T.gondii* in hosts. However gamma radiation (0.5 kGy), freezing to -12°C or cooking of food to an internal temperature of 67°C can kill the tissue cysts in meat (Dubey, 1996).

The patients infected with toxoplasmosis remain mostly asymptomatic (Kaye, 2011). The immunocompromised individuals (especially those suffering from AIDS) are more susceptible to the infection. Early diagnosis of maternal infection is critical for an effective prevention. Congenital infections may result in abortions, still birth, prematurity etc (Gunel *et al*., 2012). Generally, the serological tests based on protein molecules detection are used as the diagnostic tool for toxoplasmosis (Liesenfeld *et al*., 2001a). Reactivity of antigens can be used as a diagnostic tool for identifying the acute phase of infection (Béla *et al*., 2008). The PCR based diagnoses have made a significant advancement in the diagnoses of many pathogens including *T. gondii*. In past few years the diagnostic process has been improved and PCR is being used for the diagnosis of congenital and acute infections. Toxoplasma DNA has been detected using different molecular techniques like nested PCR and RT-PCR. Loop mediated isothermal amplification (LAMP) has emerged as the most promising new molecular assay to detect the parasite from environmental samples (Gallas *et al*., 2013).This review will provide an insight into the biology and pathogenesis of toxoplasmosis and will mainly focus the different approaches proposed for diagnosis of toxoplasmosis according to their efficacy in comparison with other methods as well as their limitations.

Different Diagnostic Methods for Toxoplasmosis

A-Fecal Microscopy: Primarily, the light microscopy is extensively used for detection of oocysts in case of highly contaminated samples (i.e. cat faeces). This technique is based upon morphological characteristics of *T. gondii*oocysts observed in a smear prepared directly from feces (Foreyt, 2001). Though, primitive but this method is still a most common practice, being cost effective and requires less equipment. Alternatively, the simple fecal smear (also called direct method) was further refined by floatation techniques, based on separation of oocysts from fecal debris on the basis of specific gravity (Dabritz *et al*., 2007). Different floatation solutions (e.g. sugar or zinc sulphate) are used to overcome the fecal debris and the oocystsof *T. gondii* are swum at the top layer of these solutions. This approach makes the fecal smears more clear and helps further in diagnosis (Amany and Merwad, 2012). Another modification based upon examination of fecaloocystsunder an ultraviolet beam can facilitate the examination of both sporulated and unsporulatedoocysts(Berlin *et al*., 1998). However, the typical blue autofluorescence observed in case of *T. gondii*oocystscan be similar for other coccidian oocysts like Neospora, Hammondia, and Cyclospora species, resulting in confusion of diagnosis. Besides this, all oocysts in the same suspension do not exhibit

autofluorescence under ultraviolet excitation which leads towards false negatives in case of low numbers of oocysts(Dumetre and Darde, 2003). As fecal microscopy is basedupon the structural characteristics, there are false positive results in cases where the oocysts of other parasites have very close or similar morphological characteristics. The technique is time-consuming as considerable numbers of slides are required to confirm a parasite (Parajuli *et al*., 2009; Schares *et al*., 2005).

B-Serological Assays: These are considered as the first line method for diagnosis of toxoplasma infection by determining the presence of specific antibodies (Candolfi *et al*., 2007; Sensini, 2006). Generally, the levels of the circulating IgG and IgM are being considered as important element to diagnose toxoplasmosis (Press *et al*., 2005; Tekkesin, 2012). Their level riseswithin two weeks of infection. The presence of IgM antibody in sera is becoming an inadequate criterion for diagnosis of acute infection. The avidity of IgG in serum antibodies has become a very important diagnostic tool (Liesenfeld *et al*., 2001b). However, their increased level in blood cannot differentiate between acute and chronic infections. So, the antibody testing is always questioned for its accuracy and sensitivity(Kaye, 2011).

i-ELISA Based Approaches: Enzyme-linked immunosorbent assay (ELISA) is a fundamental tool of clinical immunology, used as an initial screen for detection of an infection. Reactivity of IgG and IgG1 antibodies has been evaluated using ELISA and immunoblot assays in patients with chronic and acute toxoplasma infection against two recombinant antigens(Gras *et al*., 2005). It has been found that patients with acute toxoplasmosis showed much strong reaction of IgG and IgG1 with both SAG2A and STAg antigens than as compared with chronically infected patients.Indirect ELISA of IgG1 using recombinant SAG2A antigen has been developed as a diagnostic tool for characterization of the acute infection. Recombinant SAG2A has been reported as a molecular marker for diagnosis of acute toxoplasmosis especially for IgG1 antibodies. The indirect ELISA of IgG using recombinant SAG2A antigen has shown more sensitivity than as compared with ELISA of immunoglobulin with STAg (Béla *et al*., 2008).

Modified agglutination test (MAT) involves the detection of *T. gondii* specific IgG in the serum. The comparison of ELISA with Modified Agglutination Test (MAT) has been done to evaluate the ability of these two techniques for detection of toxoplasma antibodies (Glor *et al*., 2013; Zhu *et al*., 2012). The results have suggested that the performance of ELISA is slightly better than MAT. A good correlation has been found between titre of MAT and optical density of ELISA. The sensitivity of *T. gondii* ELISA is considerably low for tissue fluids than as compared with serum. ELISA has appeared to be more useful for routinely screening tests while there is difficulty in interpretation of results using MAT (Gamble *et al*., 2005).

ii-Direct Agglutination Test (DAT): The process involves the use of whole organisms as a means of looking forserumantibodies. It has been found that the results of direct agglutination test are less reproducible than those obtained from Dye tests. Mercaptoethanol is used in Direct Agglutination Test (DAT) but the findings suggest that this test is not a replacement of Dye test as it is highly specific and sensitive in toxoplasma diagnosis. It has been observed that during chronic infection the antibodies titers are often higher than compared with Dye test but lower in case of acute infection of toxoplasma. It has been reported that the variation of titers determined by DAT and Latex agglutination test (LAT) during the course of infection is not comparable. The DAT is regarded as an alternative to LAT for diagnosis of toxoplasmosis. Although sensitivity of DAT is lower than LAT but it is more specific than LAT(Johnson *et al*., 1989). The results produced by DAT are more false negative than LAT. More false positive results of LAT are associated with immunoglobulin IgM, but specificity of these immunoglobulins has always remained uncertain (Oshima *et al*., 1982).

iii-Sabin-Feldman Dye Test (DT): The Sabin-Feldman dye test has been reported in several studies and is considered as the gold-standard diagnostic test for the Toxoplasma infection. The method involves the staining of *T. gondii* cells with methylene blue, toxoplasma cells become rounded and the nucleus and cytoplasm are deeply stained(Kaye, 2011).The DAT uses whole organism as a mean of looking for serum antibodies. Comparison of Sabin-Feldmen dye test with DAThas shown that both DT and DAT are equally sensitive and specific.These tests can be employed to screen pregnant ladies, for the possible infection of toxoplasmosis. DAT test is considered as slightly more sensitive than DT, however, both tests are equally reproducible. In some sera the titres of DT were found higher than DAT, while in some other sera titres of DAT were higher than as compared with DT. The difference has suggested that antibodies are not identical in both tests. Although antibodies for both tests belong to same class (IgG) but the subclass is changed for both (Adams *et al*., 2012; Desmonts and Remington, 1980).

iv-Indirect Fluorescent Antibody Test (IFAT): The technique is used for antigen with a fluorescent antibody in which unlabeled immunoglobulin is added to tissue and combines with a specific antigen, after which the antigen-antibody complex may be labeled with a fluorescent antibody. Smear of killed tachyzoites is made on microscopic slides and can be kept at -20^{o}C for several months for further use. Although IFAT is less sensitive

but it is highly specific as compared with other serological tests. This test is considered as a confirmatory test for infection in pregnant ladies. The IFAT has some advantages over DT as former is safer and simpler to perform also it does not require living organisms (Krainara *et al*., 2004).

The evaluation of IFAT and Modified agglutination test (MAT) has been done, suggests few advantages of MAT over IFAT. The MAT results are simple to read as there is no requirement of microscope which makes it more practicable. In addition, larger number of serum samples can be analyzed at a time. The MAT can be utilized to diagnose the infection among various animal species as it does not require the presence of specific conjugates while IFAT necessarily requires the use of an anti-IgG conjugate specific for each animal species (Oksanen *et al*., 1998; Silva *et al*., 2013).

On the other hand, IFAT test also has some advantages like the results can be readily available and read after the end of performing the test. In addition, the interpretations made by IFAT are more subjective. The sensitivity and specificity of both tests have found to be same, but the antigens of IFAT are economical than MAT (Minho *et al*., 2004).

v-Flow Cytometry Based Algorithm: Flow cytometryis alaser-based biophysical technology, developed as a novel serological approach for diagnosing *T. gondii* antibodies (Pissinate *et al*., 2008). The specific IgG avidity has helped in diagnosis of acute toxoplasmosis. This technique is being used as an outstanding non-conventional alternative serological approach for diagnosis of acute toxoplasmosis in humans. Serological assays have been used widely as the main diagnostic approach against toxoplasma infection. But these assays do not necessarily differentiate between acute and chronic infection. Also the two states of infection are very different from one another having particularities in their clinical situations like congenital toxoplasmosis, ocular disease and pre transplantation.

Innovative features have been developed e.g. using wide range of serum dilutions, analysis of immunoglobulin reactivity as percentage of positive fluorescent parasites (PPEP) along with the usage of an algorithm analysis of immunoglobulin avidity. These strategieshave provided a reliable method for discrimination of acute and chronic toxoplasmosis.

Although flow cytometric based methods are costly as compared with conventional methods i.e. ELISA and immune fluorescent assay but the usage of microplate serological approach has emerged as more costeffective than routine immune fluorescent assay. Also the flow cytometry based methods are fully automated (Silva-dos-Santos *et al*., 2012).

C-PCR Based Approaches: Molecularmethods based upon the detection of toxoplasma DNA are being used as one of the most sensitive diagnostic approaches (Gunel *et al*., 2012; Hunt, 2011;Morelle *et al*., 2012). These approaches are considered as the most reliable method for diagnosis of *in utero* infections (Carlier *et al*., 2012; Montoya *et al*., 2002;Sensini *et al*., 1996). The initially reported sensitivity was as accurate as up to 100% but later studies have suggested that the accuracy is dependent on duration of the infection and on the targeted gene, which makes this approach as the most sensitive than any other available techniques(Petersen, 2007). The test can be performed in those pregnant ladies with positive serological results for confirmation of the infection. This DNA based approach can also be used with cerebrospinal fluid found in central nervous system of newborns (Kaye, 2011). Different genes are targeted for this purpose like B1, p30 and 18SrDNA.

i-Conventional PCR: Amplification of B1 gene by conventional PCR is a very useful method for detection of both congenital and acute infection of toxoplasma. Using this method B1 gene can be amplified from a crude cell lysate. B1 gene is present in all three strains of *T. gondii.* Thisgene has the potential to be amplified from purified DNA extract taken fromten parasites in the presence of thousands of human leukocytes (Burg *et al*., 1989; Chaudhary *et al*., 2006;Lee *et al*., 2012).

Recently, a sensitive, rapid and specific conventional PCR has been optimized for detection of *T. gondii* genome. In this study, a new set of primers have been used against B1 and ITS1 region of toxoplasma. The method of diagnosis is sensitive enough to detect the parasite in 10ng of DNA (Rahumatullah *et al*., 2012).

ii-Nested-PCR: It is the modification of conventional PCR intended to increase the specificity of amplified product. Even with high copy numbers, the region of B1 gene is highly conserved in all strains of *T. gondii*. As compared with other genes i.e. P30 and 18SrDNA, this gene shows more sensitivity and specificity. Because of these properties this gene has been targeted to find the prevalence of *T. gondii*. Nested PCR of B1 gene has been useful for early detection of the parasite infection (Hierl *et al*., 2004; Horiuchi *et al*., 2010;Lee *et al*., 2008). The comparison of B1 with P30 gene has been done through RT-PCR in order to determine the utility of a single copy gene with that of a 35 fold B1 gene. Quantitative assay of P30 is also useful for diagnostic purpose(Buchbinder *et al*., 2003).

iii-Real Time PCR: As compared with conventional PCR, the Real Time-PCR (RT-PCR) is considered as more sensitive, accurate and rapid molecular method. Congenital infections are responsible for 2-3% of all congenital anomalies,making the prenatal diagnosis important. Targeting B1 gene, the RT PCR is presently being used for detection of prenatal infection of toxoplasmosis (Delhaes *et al*., 2013). The amniotic fluid

has also been tested for detection of the infection using RT-PCR of B1 gene of the parasite (Gunel *et al*., 2012).

In another study RT-PCR of *T. gondii*has been done using Taqman probe for detection of the infection. In this approach a set of primers is used along with a fluorogenic probe, targeting B1 gene for molecular diagnosis of the protozoan infection. This process is highly sensitive and reproducible (Kompalic-Cristo *et al*., 2007; Reischl *et al*., 2003).

iv-Loop Mediated Isothermal Amplification (LAMP): The LAMP has been reported as a recent molecular approach for early diagnosis of parasite infections. Recently LAMP has been reported as a useful tool for routine diagnosis of toxoplasma infection as well as for evaluation of therapy effectiveness of human toxoplasmosis. Like previous studies B1 gene is targeted in this method (Xin *et al*., 2012). In another study a 529bp repeat element has been used for LAMP assay. The method is cost-effective with high specificity (Homan *et al*., 2000; Kong *et al*., 2012). Real Time-Loop mediated isothermal amplification (RT-LAMP) has been reported first time for detection of toxoplasma infection. In a recent study conserved region of 18s rRNA was targeted for the parasite detection. This molecular approach is more sensitive, fast, with high specificity and more reliable. Instrumentation of RT-LAMP is also very basic and results can be directly visualized (Daofeng *et al*., 2013).

Conclusion: The threats associated with *T. gondii*are worldwide in nature also all vertebrates are at risk. Different diagnostic approaches are available in order to evaluate their utility for diagnostic purpose. Traditionally microscopy is used as the first examination of parasite then serological methods are given an edge for further confirmation of the parasite infection. Different immunoglobulins are targeted for this purpose but the problem arises with the level of IgM that persist in the host for a long period even after acute infection is over, producing false positive results. DNA based approaches like PCR, nested PCR, RT-PCR and LAMP has revolutionized the field of diagnosis due to their robustness and accuracy. But unlike other parasitic infections molecular diagnosis of toxoplasmosis has not attained enough sensitivity, a lot of research is yet to require in this field to develop even sensitive and cost effective methodologies. Improvement in the available diagnostic methods is required in addition to explore certain other techniques which can better diagnose the toxoplasma infection. The acute and latent infections should be differentiated as requires different line of action to counter the disease. Similarly, accurate quantification of the disease is also pre-requisite to separate clinical and sub-clinical infections.

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