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REVIEW ARTICLE

Toxoplasmosis: The value of molecular methods in diagnosis compared to conventional methods

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ABSTRACT

Toxoplasmosis is a parasitic infection due to *Toxoplasma gondii* an obligate intracellular protozoan parasite. It is considerate one of the most common parasite worldwide. The contamination of the parasite is generally occurred via consumption of infected food or water or, undercooked contaminated meat. *Toxoplasma gondii* infection may lead to serious illness when the organism is contracted while pregnancy or when it is reactivated in immune-suppressed persons. Diagnosis of toxoplasmosis in humans is elaborated using various techniques such as detection of anti-Toxoplasma antibodies, mouse inoculation, histological revelation of tachyzoites in tissue sections or smears of body fluid, but the detection of *Toxoplasma gondii* DNA by molecular methods has revolutionized prenatal diagnosis of congenital toxoplasmosis and in immunocompromised patients. In this paper we will discuss the parasite and different methods of diagnosis including the usefulness of molecular methods. *J Microbiol Infect Dis 2013; 3(2): 93-99*

Key words: Toxoplamosis, *Toxoplasma gondii*, diagnosis

Toksoplazmozis: Tanıda moleküler yöntemlerin değeri geleneksel yöntemlere göre

ÖZET

Toksoplazmoz, zorunlu hücre içi bir protozoan parazit olan *Toxoplasma gondii*'nin etken olduğu bir parazit enfeksiyonudur. Dünyada en yaygın parazitlerden bir olarak kabul edilir. Parazitin bulaşması genellikle enfekte yiyecek/su tüketimi veya az pişmiş kontamine et ile elde olmaktadır. *Toxoplasma gondii* enfeksiyonu hamilelik döneminde geçirilirse veya bağışıklığı bastırılmış kişilerde alevlenirse ciddi bir hastalık tablosuna yol açabilir. Insanlarda toksoplazmozis tanısı; anti-Toxoplasma antikorlarının saptanmasıyla, fare inokülasyon deneyiyle, doku kesitlerinden histolojik değerlendirmeyle veya vücut sıvılarından hazırlanan yaymalarda takizoitlerin araştırılması ile konabilir. Öte yandan *Toxoplasma gondii* DNA'sının moleküler yöntemlerle tespiti konjenital toksoplazmozun prenatal tanısında ve immün sistemi baskılanmış hastaların tanısında devrime yola açtı. Bu yazıda paraziti ve moleküler yöntemler dahil değişik tanı yöntemlerinin kullanışlılığını tartıştık.

Anahtar kelimeler: Toksoplazma, *Toxoplasma gondii*, tanı

INTRODUCTION

Toxoplasmosis is an infection caused by *Toxoplasma gondii* (*T. gondii*). This obligate intracellular protozoan parasite was identified in 1908 by Nicolle and Manceaux, in a North African rodent.[1] Its intermediate hosts could be humans or other warm-blooded animals. The infection has a distribution worldwide. Almost one-third of the population has been confronted to this protozoan.[2] *T. gondii* infection occurs usually while ingesting tissue cysts in undercooked meat, or contaminated water and food with oocysts.[3] Because of the risk of congenital infection and its sequelae in the newborn, the diagnosis of toxoplasmosis should be done timely and correctly. There are various techniques of diagnosis but the molecular methods as PCR amplification have revolutionized prenatal diagnosis of congenital toxoplasmosis and in immunocompromised patients thanks to its excellent sensitivity and specificity to determine the presence of parasites in clinical sample in much reduced time.

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PARASITE

T. gondii belongs to the phylum, Apicomplexa, which includes also Plasmodium and Cryptosporidium species.[4] There are three infectious forms of this protozoon including oocysts, tachyzoite and bradyzoites. Oocysts are formed exclusively in felines and necessitate sexual reproduction of *T. gondii*. After sporulation, it contains two sporocysts each enclosing four sporozoites.[5]

The tachyzoite form is considerate the fast replicating one, and leads to systemic dissemination and subsequent active tissue infection in intermediate hosts. As dormant form of the parasite, the bradyzoite is characteristic of chronic infection. This form can persist in cells of various organs for the lifetime of the host within tissue cysts.[5]

EPIDEMIOLOGY

T. gondii is distributed worldwide because a lot of animals can accommodate the parasite and follow its dissemination. Numerous factors might influence its broad geographic location such as food habits and variations in climate, and ingestion of mature oocysts after contact with infected cat feces.[6] The climate variation has a marked influence on the habitat of *T. gondii*; for example, an elevation in ambient temperature and precipitation can modify the soil humidity, so that the sporulated oocysts persist for a long time viable in the moist environment.[7]

Transmission

The *T. gondii* infections in humans are conducted from different ways: by consuming raw or undercooked meat that contains *T. gondii* tissue cysts or eating food that has been cross contaminated with raw/undercooked meat; by ingesting oocysts via soil (for example, by way of gardening, handling/ eating unwashed vegetables, or changing a cat litter box); Of the estimated 750 deaths caused by toxoplasmosis in the United States each year, 375 are thought to occur from eating raw or undercooked meat; this makes toxoplasmosis the third-leading source of US food-borne mortality.[8] Another way of transmission of Toxoplasma is represented by transplantation of organs from a seropositive donor into a seronegative recipient. Transmission of Toxoplasma in seronegative recipients is more than 80% but the clinical presentation, is very rare.[9,10] Infection with *T. gondii* could be transmitted vertically from mother to child, even if a minority of infected individuals worldwide acquires the parasite congenitally.[11]

Congenital toxoplasmosis concerned seven children per 1000 births in France in the 80s. Nowadays, it is considered that this rate would be less than one child per 10 000 births. Transplacental passage of Toxoplasma and therefore the child infection occur on average in 30% of cases. The risk is higher during the last trimester of pregnancy, near 70%, while it is only 5% first trimester. That risk reaches 90% during the last week of pregnancy.[12] *Toxoplasma gondii* infection acquired in the course of pregnancy may lead to fetal loss or lesions which often involve the brain and the eyes.[13]

Seroprevalence

Not many current reports focus on the seroprevalence of toxoplasmosis in Africa. One study from Northeast Nigeria reported a seroprevalence of 23.9% of 180 individuals aged 10 and older, who were screened by enzyme-linked immunosorbent assay (ELISA) for *T. gondii*-specific immunoglobulin IgG.[14] In Iraq, the toxoplasmosis seroprevalence in wives was only 30.7%, while 13.1% in husband's only.[15] In a study achieved in 2005-06, a seroprevalence of 15.33% has been revealed in pregnant women in India.[16] In a new study from France, 47% of 273 people tested positively for IgG-*T. gondii* antibodies by ELISA.[17]

In Turkey, the seroprevalence of *Toxoplasma gondii* varies significantly between different regions which range from 30.7% to 69.5% [18-21]. In United States only 16% of people aged 12 to 49 have a positive serology for toxoplasmosis.[22] However, the seroprevalence in developed countries is generally lower than before thanks to the improvement of hygiene condition and widespread freezing (which leads to the destruction of cysts in contaminated meat).[12]

CLINICAL PRESENTATIONS

Immunocompetent adults are usually asymptomatic or have symptoms such as fever, malaise, and lymphadenopathy that resolve spontaneously.[8]

In pregnant women: Generally, acute acquired infection does not lead to expression of obvious symptoms or signs.[23] In a minority of them may appear malaise, low-grade fever, and lymphadenopathy. Rarely, as a result of recently acquired infection or reactivation of a chronic infection while pregnancy, women will present visual changes due to toxoplasmic chorioretinitis.[24] In immunocompromised, chronically infected pregnant women, congenital transmission of *T. gondii* to the fetus result of reactivation of latent parasite.

In congenital infection: The severity of congenital infections is different depending of the age of pregnancy. Infection acquired during the second and third trimesters are less severe than those acquired in the first trimester witch may result in the death of the fetus in utero and spontaneous abortion. In opposition to that late maternal infection usually lead to a normal appearing newborn.[2] The lesions most frequently associated with congenital toxoplasmosis are intracranial calcification or hydrocephalus that are generally detected during infancy, and retinochoroiditis which can appear at any age.[25] Intracranial and/or ocular lesions were diagnosed before school age in 33% (28/85) of congenitally infected children in three population-based cohort studies. Of the [28] with lesions, 3 (4%) had severe neurological complication. Retinochoroidal lesions have been found in 16% (14/85) children at 1-6 years old.[26,27] In another analysis of clinical presentation of 43 children having congenital toxoplasmosis, chorioretinitis was noticed in 95% of the cases and neurological sequelae in 54%28. Manifestations can also include prematurity, peripheral retinal scars, persistent jaundice, mild thrombocytopenia, cerebro-spinal fluid (CSF) pleocytosis.[29] As well as intrauterine growth restriction, hepatosplenomegaly, myocarditis, pneumonitis and skin rash.[30]

Immunosuppressed persons, such as those with acquired immunodeficiency syndrome, those with immunosuppressive therapy of cancer, and immunosuppressive drugs in transplant recipients, acute infection or reactivation of latent *T. gondii* may also produce severe encephalitis. Toxoplasmosis is the most common critical neurologic infection among persons with acquired immunodeficiency syndrome.8

LABORATORY DIAGNOSIS

The detection of *T. gondii* infection can be elaborated by serologic tests, amplification of specific nucleic acid sequences (i.e., polymerase chain reaction [PCR]), histologic demonstration of the parasite and/or its antigens, or animal inoculation by isolation of the organism.[31] Other technics not frequently used such as demonstration of antigenemia and antigen in serum and body fluids, a toxoplasmin skin test, and antigen-specific lymphocyte transformation.[31]

Several serological tests have been used as screening tests, like the indirect immunofluorescent antibody test (IFAT), latex agglutination (LA), indirect hemagglutination (IHA) and ELISA with high sensitivity and specificity.[32] It is generally preferred to establish a combination of serologic tests to determinate if a person has been probably infected in the distant past or has been recently infected.

IgM and IgG detection

Systematic serological screening for *T. gondii* IgG and IgM antibodies should be performed in all pregnant women as early as possible in gestation (ideally in the first trimester) and in seronegative women each month.[33]

The detection of anti-toxoplasma antibodies by ELISA is frequently performed in numerous medical centers. The results of these tests are usually well approved by clinicians thanks to their excellent sensitivities and specificities, the rapid accessibility to results, and the considerably low costs of the tests.[34]

The specific IgM can be revealed by the immunosorbent agglutination assay (ISAGA). This technique employs a monoclonal antibody to the CH2 domain of the human p chain, allowing IgM capture, while eliminating cross reactivity with IgG, IgA, or IgE. Then the specific IgM is revealed by the agglutination of whole Toxoplasma trophozoits.[35]

The existence of increase levels of Toxoplasma specific IgG antibodies means that infection was acquired at some point, but does not discriminate between a recent infection or ancient infection. While acute infection, IgM antibodies usually elevate within 1 to 2 weeks of infection.36 The timing of infection occurrence in a pregnant woman is significant because risk for transmission to the fetus is moderate if infection acquired before conception; but, the risk rise if infection is acquired after conception Toxoplasma-specific IgM antibodies constitute a help in defining the time of infection, however, it is demonstrated that IgM antibodies remain for up to 18 months postinfection. A negative IgM with a positive IgG result signifies infection at least 1 year before. A positive IgM result can indicate more recent infection or may be a false positive reaction. A negative IgM results are reassuring, whereas positive results must be interpreted attentively, validated in a toxoplasmosis reference laboratory, and complemented by serial titers at least 3 weeks after.[5]

In newborns, diagnosis of congenital toxoplasmosis is complicated because of the presence of maternal specific IgG anti-*T. gondii* antibodies in the newborn blood acquired via transplacental passage. However, IgM and IgA antibodies do not cross the placental barrier and once they do so at birth, they have a lifespan no more than five days, which provides them to be used as serological markers of

vertical transmission. The existence of specific IgG antibodies can only represent transmission of maternal antibodies, which disappear in the first year of life.[37]

Sabin-Feldman Dye test

Previously, IgG antibodies are detected by the Sabin-Feldman Dye Test (DT). The DT and its modification were defined before by Sabin and Feldman, 1948; Feldman and Lamb, 1996; Reiter-Owona et al, 1999; Udonson et al, 2008. The test consists on complement mediated cytolysis of antibody coated live *T. gondii* tachyzoites.[32] The raising of IgG antibodies generally begin within 1 to 2 weeks of the infection, peak within 1 to 2 months, decreases at variable rates, and usually remain for life. The severity of illness is not correlated to the titer of IgG. A positive indicate that the patient has contact with the parasite. A negative DT essentially excludes prior exposure to *T. gondii*.[38]

Differential Agglutination [AC/HS]

The differential agglutination test also known as the "AC/HS test" uses two antigen preparations that express antigenic determinants observed immediately after acute infection (AC) antigen or in the later phases of infection (HS). Ratios of titers using AC versus HS antigens are interpreted as acute, equivocal, non-acute patterns of reactivity or non-reactive.[39] It should be explained that the terms "acute" and "nonacute" refer exclusively to the interpretation of the agglutination pattern of the AC/HS test and not to if the patient actually had a recently acquired infection. The AC/HS tests were accomplished and read without previous information of the clinical histories of the patients.[40]

IgG avidity test

It is essential, especially during the pregnancy, to differentiate between acute and chronic stages of infection for treatment and limitation of the consequences. The parasite isolation is usually difficult, so the diagnosis is essentially focused on serological techniques, with revelation of specific Toxoplasma IgG, IgM, and IgA antibodies.[41] The IgG avidity test was created to help distinguish between past and recently acquired infection. Results are founded on the quantification of the avidity [functional affinity] of Toxoplasma-specific IgG antibodies.[42] It believed that the antibodies produced have a low average affinity following an antigenic challenge. More the immune response progress, antibody affinity increases progressively over weeks or months. Previous findings with the IgG avidity method proposed that low-avidity antibodies signified recently acquired infection. After that, studies have demonstrated evidently that low-avidity antibodies can persist for several months beyond the acute infection and because of this are not reliable for the diagnosis of recently acquired infection. According to the method used, the availability of high-avidity IgG antibodies might be used to exclude the occurrence of acute infection during the past 3-5 months. Consequently, its value is greatest when done in the first 3-4 months of gestation. A high-avidity result late in the second trimester or in the third trimester should not be interpreted to signify that the infection was not acquired in the first 3-5 months of gestation.[43]

IgA antibodies

IgA antibodies can be revealed using ELISA or ISAGA in sera of adults with acute infection and infants congenitally infected.[44] During infection it is believed that specific IgA generation parallels that of specific IgM,[45] or delays relatively behind IgM. IgA antibodies may persist for several months or more than a year. Because of this, they are of little additional assistance for acute infection diagnosis in the adult. Conversely, IgA assays have a significant interest in the diagnosis of the congenital toxoplasmosis infection in the fetus and newborn relies on the increased sensitivity over IgM assays.[44]

Animal inoculation

The isolation of *T. gondii* from tissues or body fluids is generally established with animal inoculation witch considered the most sensitive method. Intraperitoneal injections of trophozoites or bradyzoites in mice, animal most frequently used, leading to induce either an acute infection with parasite-rich ascites or a chronic infection characterized by the presence of cysts in the brain depending on the virulence of the strain. Blood, body fluids, or tissue extracts might be inoculated into mice for diagnosis.[46] However, many studies have proved that in mice, several factors such as the route of infection and the infecting dose may influence the susceptibility to Toxoplasma. As well as, the mouse virulence of the parasite may impact the capacity to isolate Toxoplasma, for the reason that some strains infect mice poorly.[47]

Polymerase Chain Reaction [PCR]

Molecular methods can allow more appropriate diagnosis of Toxoplasmosis, especially in cases in which inadequacy of conventional methods is confronted with deteriorating and potentially severe clinical outcome (congenital, ocular toxoplasmosis

and cases of immunosuppression).[48] These methods are separated into two groups. The first group contains techniques aimed at detection of *T. gondii* DNA in biological and clinical samples, such as conventional PCR, nested PCR and real-time PCR. The second group includes molecular methods such as PCR-RFLP, microsatellite analysis and multilocus sequence typing of a single copy *T. gondii* DNA and those are mainly used for strain typing.[49]

Molecular diagnostics of toxoplasmosis is usually based on the detection of a specific DNA sequence, via different assays and protocols, principally from highly conserved regions. The first protocol for molecular detection of *T. gondii*, for conventional PCR targeting B1 gene, was introduced in 1989 and has since been improved and optimized in several laboratories. The B1 gene, although of unknown function, is mostly exploited in a variety of diagnostic and epidemiological studies thanks to its specificity and sensitivity.[48]

In addition there are certain studies in which the detection of *T. gondii* was founded on amplification of ITS-1 and 18S rDNA fragments, whose sensitivity was identical to the B1. However, the repetitive element of 529 bp in length, which was initially discovered by Homan, has showed a 10 to 100 times elevated sensitivity compared to the B1 gene.[50,51]

The detection of *T. gondii* DNA choosing the 529 bp repetitive element, and real-time PCR protocols that detect the existence of this element, is recently the most usually used molecular approach for the detection of *T. gondii*.[51,52]

Molecular detection of *T. gondii* in cases of suspected congenital toxoplasmosis could be practiced in the amniotic fluid, and fetal and neonatal blood samples. As well, it is performed in the peripheral blood of immunosuppressed patients, and in samples of humor aqueous and cerebrospinal fluid of patients suspected of ocular and cerebral toxoplasmosis, and also in bronchoalveolar lavage fluid (BAL).[48]

T. gondii DNA revealed in fetal and neonatal blood samples is one of the highest clinical importance since there is no chance of detection of DNA from earlier infections. For this reason, the most essential application of molecular methods is in the diagnosis of congenital toxoplasmosis, while the isolation of the parasite in cell culture is not satisfactorily sensitive53,54 and the isolation by bioassay requires about 6 weeks.[48]

In the last twenty years, the identification of *T. gondii* DNA in the amniotic fluid has emerge as especially important, for the reason that it allows timely diagnosis of fetal infection, and succeeding administration of adequate therapy and infection control.[55,56] In 6 European centers of reference, one study of prenatal diagnosis of congenital toxoplasmosis, it was proved that PCR from amniotic fluid has a greater sensitivity (81%) regarding both bioassay (58%) and cell culture (15%).The combination of PCR and bioassay improves the sensitivity to 91%, and corresponds to the best diagnostic approach.[54]

Cord blood is not evaluated as the best sample for prenatal diagnosis of congenital toxoplasmosis. In Ivović et al study, cord blood samples have revealed a rate of 33% positivity in real-time PCR and after inoculation into mice the rate of positivity of bioassay was 55.5%. An increased rate of isolation of viable parasites by bioassay as compared to the detection of parasitic DNA by real-time PCR can be explained by a superior sample volume employed for mouse inoculation rather than the amount used for DNA extraction, in addition to by a possibility of PCR inhibitors existence.48 It may be claimed that the diagnosis of congenital toxoplasmosis from fetal blood samples needs to be determined by the results of each bioassay and molecular detection.

Lately, molecular methods are increasingly becoming a common diagnostic approach in the diagnosis of ocular toxoplasmosis as well. Several studies has actually demonstrated that a positive PCR result is not absolutely accompanied by positive serology reflecting local synthesis of IgG antibodies [48,57,58] and therefore can be the exclusively confirmation of the diagnosis.[59]

Cerebral toxoplasmosis generally affects patients with immunosuppressive state and is mainly the result of reactivation of chronic infection that may be critical if left untreated. Certain diagnosis of toxoplasmosis can be made by the identification of tachyzoites in brain tissue samples acquired by biopsy, however this method, due to its invasiveness, is rarely applied, and absolutely not as the PCR, giving consistent and quick result, has been included in the diagnostics.[48,60] A study of cerebral toxoplasmosis in HIV-infected patients in Brazil, reported that 27.4% (14/51) of cerebrospinal fluid samples were positive for *T. gondii* DNA.[61]

It is proved that the importance of the use of molecular methods, because of their high sensitivity and specificity, in the diagnosis of toxoplasmosis. A study of PCR affirms an overall sensitivity of 64%, a specificity of 100%, a negative predictive value of 87.8%, and a positive predictive value of 100%.62 Combined with conventional parasitological diagnostic methods, PCR-based methods provide the

timely diagnosis particularly of congenital toxoplasmosis and of reactivated toxoplasmosis in immunosuppressed patients.[48]

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