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Detection of acute toxoplasmosis in rural women in Sudan using different diagnostic tests

Khalil Mohamed[1,2]*, Petr Kodym[3], Marek Maly[4] and Intisar EL Rayah[5]

[1]Umm AL Qura University, College of Public Health & Health Informatics, Department of Epidemiology, Makkah, Saudi Arabia.
[2]Commission for Biotechnology & Genetic Engineering, Department of Medical Biotechnology, Khartoum, P.O. Box 2404, Sudan.
[3]National Institute of Public Health, National Reference Laboratory for Toxoplasmosis, Prague, Czech Republic.
[4]National Institute of Public Health, Department of Biostatistics and Informatics, Prague, Czech Republic.
[5]Tropical Medicine Research Institute TMRI, Khartoum, Sudan.

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Blood samples from 254 childbearing age women from two villages (EL Nuba and EL Massoudia) were collected after seeking their consent; their written consent form was obtained. Detection of IgM for acute toxoplasmosis was done using two different enzyme-linked immunosorbent assay (ELISA) techniques. Prevalence of IgM was 18.9 and 20.3%; this was obtained by using ELISA IgM in Khartoum for all samples and ELISA IgM in Prague for selected samples, respectively. Diagnostic test was used to confirm acute toxoplasmosis. This consists of detecting IgA and IgE using ELISA technique. The result was the same in both techniques (6.8%). Confirmation tests were used for different purposes. IgG avidity was used to determine the exact time the selected samples had the infection. Six cases had recent infection; five cases had old infection and two cases were in between. Western blot (WB) was used to confirm the antibodies detected by screening and diagnostic tests. Western blot confirmed that 61.5% of samples had antibodies against P30 gene.

Key words: Acute toxoplasmosis, ELISA- IgG avidity, Western blot, rural women, Sudan.

INTRODUCTION

Toxoplasmosis is one of the more common parasitic zoonoses world-wide and one of the most common human infections. Infection is acquired by ingestion of viable tissue cysts in meat or oocysts excreted by cats that contaminate food or water (Jones et al., 2009). Diagnosis of toxoplasmosis in humans is performed using different techniques. A few examples of these techniques are mouse inoculation, detection of anti-*Toxoplasma* antibodies, histological demonstration of tachyzoites in tissue sections or smears of body fluid, and detection of *Toxoplasma gondii* DNA by molecular methods (Montoya, 2002; Lappalainen and Hedman, 2004; Remington et al., 2004). Many serological methods for the diagnosis of toxoplasmosis have been established over the years. Most have been developed for detection of *Toxoplasma* infection in human and are commercially available in kit form (Buxton and Brebner,1998). The diagnosis of infection caused by *T. oxoplasmagondii* is carried out by the detection of specific anti-*Toxoplasma* immunoglobulin (IgM and IgG); and to discriminate chronic from reactivated infection IgG avidity is also determined with VIDAS instrument (bioMerieux, France) (Calderaro et al., 2009). Detection of anti-*Toxoplasma* antibodies indicates that a person

*Corresponding author. E-mail: kmismail@uqu.edu.sa or khali72@gmail.com.

has been infected with *T. gondii* some time in the past. There are many different serological techniques available; for example, the dye test, complement fixation test, indirect immunofluorescent test, latex agglutination test, enzyme linked immunosorbent assay, and immunosorbent agglutination test (Hill and Dubey, 2002; Montoya, 2002).

In asymptomatic infections, the only indication of a primary infection is seroconversion indicated by anti-*Toxoplasma* IgM or IgG antibodies. IgM antibodies usually become detectable within days after infection, while IgG antibodies become detectable after 1-2 weeks and may have lifelong persistence. The presence of IgM antibodies indicates a recent infection, but the tendency of IgM to remain detectable for a long time has been demonstrated (Lappalainen and Hedman, 2004; Petersen et al., 2005). Detection of anti-*Toxoplasma* IgM and IgG is essential for the diagnosis of *Toxoplasma* infection in pregnant women (Panah et al., 2013). Measure of IgA or IgE antibodies is also helpful in the diagnosis of acute infections (Ronday et al., 1995).

The detection of *T. gondii* specific IgM antibodies is the most common method used to determine the infection (Tekkesin, 2012). Positive IgM results are not sufficient as evidence of recent infection, as these antibodies are often present for many months (Kaul et al., 2004; Ali-Heydari et al., 2013). Repeat testing of IgM is not sufficient to confirm acute infection (Bobic et al., 1991; Gorgievski-Hrisoho et al., 1996; Meek et al., 2001). There is need to detect specific IgG, IgM, and IgA antibodies in order to increase diagnostic sensitivity (Partanen et al., 1984; Wilson et al., 1987; Wilson and Auley 1991; Roos et al., 1993). As is true of IgM antibodies to the parasite, IgA antibodies may persist for many months or more than a year. For this reason, they are of little additional assistance for diagnosis of acute infection in the adult. In contrast, the increased sensitivity of IgA assays over IgM assays for diagnosis of congenital toxoplasmosis is advancement in the diagnosis of the infection in foetus and newborns with congenital toxoplasmosis and negative IgM antibodies. Serologic diagnosis has been established by the presence of IgA and IgG antibodies (Stepick-Biek et al., 1990).

The persistence of specific IgE several months after seroconversion is suggestive of active toxoplasmosis and should be taken into account. Specific IgE is almost present in symptomatic acquired toxoplasmosis and can thus contribute to the etiologic diagnosis of lymphadenopathy (Foudrinier et al., 2003). Detection of IgA has been introduced as supplementary method to detect the acute phase of the disease (Jenum and Stray-Pedersen, 1998).

Measurement of *T. gondii* IgG avidity is a powerful tool for distinguishing recent from past infection. *T. gondii* IgG avidity measurement is particularly valuable for approximating the time of infection in pregnant women (Prince and Wilson, 2001; Sadraie et al., 2013). IgG avidity measurement is based on the differential elution of antigen bound IgG using urea-containing wash buffer; low avidity IgG dissociates from antigen in the presence of urea, whereas high avidity IgG remains bound to antigen (Hedman et al., 1989). Results for avidity assays are generally expressed as an avidity index (AI).

Since the original description of immunoblotting for diagnosis of congenital toxoplasmosis was done (Remington et al., 1985), almost 10 years elapsed before additional reports for its use appeared in the literature (Remington et al., 2004). Western blot is a sensitive and specific assay for diagnosis of congenital toxoplasmosis (Gallego-Marin et al., 2005). It is utilized as confirmation test, particularly in mothers and newborns. Immunoblotting technique is easy to perform and might be useful as additional serological assay for routine diagnosis of *T. gondii* infection. However, it can not replace other serological test system but can serve as additional method to improve diagnosis (Gross et al., 1992).

The specific aim of this study was to differentiate between acute toxoplasmosis and chronic toxoplasmosis in Sudanese women using immuno techniques.

MATERIALS AND METHODS

Study area and population

The study was performed in two villages: EL Massoudia and EL Nuba in the North of EL Geizera State (middle Sudan) located near Blue Nile. These villages belong to EL Kamleen Province about 50 km South of the Capital, Khartoum. Most of the population in these villages belongs to the same ethnic group. People in these areas have low socio-economic status. They are farmers, animal breeders or workers in a big industry, newly established near this area. Women live simple life; although most of them do not work, they lend a hand to improve the economic situation by bringing water from river, wood for cooking or taking care of animals; they also help animals to give birth or participate in farms and agricultural process.

Study design

The study was a cross-sectional study. The sample size was 254; a prevalence of 20% was obtained from first 10 samples collected (d = 0.05 at a confidence level of 95%). A total of 5% of the sample population was added to the sample size. One sample was missed due to lack of plasma. From the total samples size, 254 samples were detected for IgM in Khartoum and 59 samples were examined by using ELISA IgM, IgA, and IgE in Prague. About 13 samples which were highly positive by CFT, ELISA IgG, and ELISA IgM, IgA, IgE were chosen to confirm the acute and recent infection.

Data collection

Consent form and questionnaire were filled for each individual. Consent form was signed and fingerprinted by each one after agreeing to participate in the study. Data were collected after convenient interview.

Table 1. Detection of Acute Toxoplasmosis in EL Nuba and EL Massoudia Villages Using Different Investigation Tests.

Village	ELISA IgM Done in Khartoum		Number examined	ELISA IgM Done in Prague		Number examined	ELISA IgA		Number examined	ELISA IgE		Number examined
	Positive	Negative		Positive	Negative		Positive	Negative		Positive	Negative	
EL Nuba	24 (17.7%)	112 (82.6%)	136	6 (24.0%)	19 (76.0%)	25	1 (4.0%)	24 (96.0%)	25	1 (4.0%)	24 (96.0%)	25
EL Mass	24 (20.3%)	94 (79.7%)	118	6 (17.7%)	28 (82.4%)	34	3 (8.8%)	31 (91.2%)	34	3 (8.8%)	31 (91.2%)	34
Total	48 (18.9%)	206 (81.9%)	254	12 (20.3%)	47 (79.7%)	59	4 (6.8%)	55 (93.2%)	59	4 (6.8%)	55 (93.2%)	59

Samples collection

The blood samples were collected under direct medical supervision by medial venipuncture using 5 ml syringe in heparinized tubes. Plasma was obtained by centrifugation of the blood at 5000 rpm for 10 min. Plasma was kept in different labeled cryo tubes in -20°C until it was used. Plasma samples were sent in dry ice to National Reference Laboratory for Toxoplasmosis, Prague, Czech Republic where more investigations were done.

Detection of *T. gondii* antibodies using enzyme link immunosorbent Assay IgM (In Khartoum)

The ELISA IgM (Toxo IgM®, Human Germany) is planned for the detection of immunoglobulin M (IgM) class antibodies to *T. gondii*.

Enzyme link immunosorbent assay IgM, IgA and IgE (In Prague)

ELISA IgM, IgA, and IgE (Test-line®) demonstration of specific IgA and IgE antibodies is an indication of current or recent infection. ELISA IgM, IgA, and IgE were used as diagnostic and differential tests to see the recent and acute infection of *T. gondii* in selected patients.

Indirect immunofluorescent test

The IIFT (Test-line®) test is simple, quantitative and reproducible, and the titers can be related to clinical events. However, visual assessment of fluorescence of the whole-cell antigen is subjective (Gordon et al., 1981).

Western blot IgM

The test (Test-line®) serves for a detailed determination of specific IgM antibodies to the respective *T. gondii* antigens. The diagnosis of *T. gondii* infection using western blotting is based on the detection of specific antibodies to a number of high-specific antigens that may characterize their molecular weights (Remington et al., 1985).

Enzyme link immunosorbent assay IgG Avidity

The avidity (Test-line®) of an antibody expresses the strength of the bond between an antigen and an antibody. The methods of avidity determination are usually based on splitting the antigen-antibody bond using avidity solution (Hedman et al., 1989); and the very important and usefulness of using this test is determination of the time of infection.

Data analysis

Statistical evaluation was done by entering the data obtained into the personal computer (PC) using two different programs: (1) Statistical package for social science (SPSS) version 13.0 (SPSS Inc. Chicago, IL. USA) and (2) Statistical analysis was performed by statistical software Stata, version 9.2 (Stata Corp LP, College Station, TX).

RESULTS

ELISA IgM done in Khartoum

A total of 254 plasma samples were detected by ELISA IgM after collecting them directly. Prevalence of acute toxoplasmosis was 48 (18.9%). The numbers of cases were equal in each village; in EL Nuba village, the prevalence was 24(17.7%), while the prevalence was 24(20.3%) in EL Massoudia as shown in Table 1.

ELISA IgM done in Prague

About 59 samples were selected according to the results of screening tests done before; they were examined in the reference laboratory of toxoplasmosis in Prague including ELISA IgM of positive samples done in Khartoum. The prevalence of acute toxoplasmosis was 12 (20.3%). The prevalence was 6 (24.0%) in EL Nuba village while the prevalence was 6 (17.7%) in EL Massoudia as shown in Table 1. Significant difference was found between the result done in Khartoum and result obtained in Prague (p-value<0.05).

Using ELISA IgA

The same samples selected in ELISA IgM in Prague were detected using ELISA IgA to diagnose the acute toxoplasmosis. The samples which were positive were 4 out of 59 samples. The prevalence of IgA as indicator of acute toxoplasmosis was 6.8% from the two villages. In EL Nuba village, the prevalence was 1.7% and the prevalence was 5.1% in EL Massoudia village as shown

Table 2. Detection of Acute Toxoplasmosis in 13 samples using Diagnostic and confirmation tests in both villages.

Sample Number	Location	Age	ELISA (OD)				IIFT dilution	WB	IgG Avidity
			IgM (K)	IgM (P)	IgA	IgE			
27n	EL Nuba	30	1.632	2.063	0.841	0.799	2048	+	14
103n	EL Nuba	20	0.378	0.602	0.071	0.196	512	-	25
108n	EL Nuba	16	0.616	0.851	0.226	0.228	1024	+	28
110n	EL Nuba	35	1.439	0.472	0.129	0.095	256	+	35
124n	EL Nuba	40	0.449	0.475	0.067	0.111	512	+	24
137n	EL Nuba	35	0.518	0.692	0.071	0.080	256	-	30
1o	EL Mass.	50	0.102	0.524	0.081	0.933	256	-	35
12o	EL Mass.	40	0.472	0.140	0.288	0.171	2048	-	49
65o	EL Mass.	26	1.685	1.529	0.432	0.535	4096	+	13
66o	EL Mass.	34	0.370	0.282	0.252	0.225	1024	+	39
80o	EL Mass.	33	0.432	0.754	0.187	0.220	512	+	68
100o	EL Mass.	38	1.051	0.236	1.020	0.851	8192	-	54
118o	EL Mass.	33	0.844	0.664	0.197	0.131	512	+	42

n, code used for samples collected from EL Nuba; o, code used for samples collected from EL Massoudia; K, code for ELISA IgM done at lab. of TMRI in Khartoum; P, code for ELISA IgM done in toxoplasmosis reference lab. in Prague; + = Positive by WB; - = Negative by WB

in Table 1.

Using ELISA IgE

Samples selected were test with ELISA IgA to diagnose the acute toxoplasmosis via detection of IgE. The result is shown in Table 1. The prevalence of IgE specific antibodies for *T. gondii* was 6.8%. The prevalence was 1.7% in EL Nuba village and 5.1% in EL Massoudia village. The significant difference between ELISA IgA and ELISA IgE was high (p-value=0.001); also, there was high significant difference between ELISA IgA and ELISA IgM (p-value=0.001). There was significant difference between ELISA IgE and ELISA IgM (p-value=0.02).

Confirmation of acute toxoplasmosis

A total of 13 cases were suspected to be in acute phase of toxoplasmosis from both villages. For confirmation of these cases, several confirmation tests were done as shown in Table 2. Two cases were positive in all diagnostic and confirmation tests with low avidity.

Indirect immunofluorescent test (IIFT)

This test was done for the 13 cases with high dilutions to confirm the presence of antibodies against *T. gondii*. The results shown in Table 2 confirmed the positivity of all cases and some cases were positive in higher dilutions as shown in Table 2.

Western blot IgM test

Western blot IgM test was done on the suspected acute cases. The positive cases were 8/13; 4 cases from each village (Table 2). The prevalence of acute toxoplasmosis was 61.5%. The results show the reaction between antibodies found in the plasma and the target gene of *T. gondii* P30.

Avidity IgG Test

This test was done to determine the exact time of infection. The results confirmed six cases were in low avidity; five of them in EL Nuba village and five cases in high avidity; all of them in EL Massoudia village (Table 2).

DISCUSSION

The prevalence of acute toxoplasmosis using ELISA IgM and different kits in this study ranges between 18.9 and 20.3%. In some countries, the prevalence of IgM has been reported to be as high as 2.4% (Svobodova and Literak, 1998). In the United States, the incidence of acute *Toxoplasma* infection during pregnancy has been estimated to be between 0.2-1.0% (Wong and Remington, 1994). In Iran, prevalence of IgM in women was 11.7% (Saeedi et al., 2007) and in Mexico none of the women studied had IgM anti-*T. gondii* antibodies (Alvarado-Esquivel et al., 2006); also in pregnant women in Turkey (Ertug et al., 2005). In Saudi Arabia, the prevalence of IgM in pregnant women was 5.6% (Al-Harthi et al., 2006). Similar results were obtained in differ-

ent target groups in Khartoum State (Adnan, 1994; Khalil 2004). IgM test is still used by most laboratories to determine if a patient has been infected recently or long ago; and because of the hurdles posed in interpreting a positive IgM test result, confirmatory testing should always be performed (Montoya, 2002). Detection of IgM antibodies was performed only in samples with high titration by CFT and IgG positive, because the presence of IgM antibodies alone is rarely seen. Anti-*T. gondii* IgG antibodies appear very early after infection (Pfrepper et al., 2005); therefore, the period between the appearance of IgM and the appearance of IgG is extremely short and the probability to find an IgM positive/IgG negative infected subject seems to be quite low (Alvarado-Esquivel et al., 2007). In addition, seropositivity to IgM alone is not considered an acceptable diagnostic criterion for acute infection. Anti-*T. gondii* specific IgM antibodies are detectable early after infection and can persist for prolonged times after infection (Liesenfeld et al., 1997; Montoya and Liesenfeld, 2004).

IgM antibodies are the first to be produced between 7 and 15 days; IgA antibodies are produced at the end of the first month (Bessieres et al., 1992). IgM and IgA antibodies increase in parallel. Several reports have indicated the role that specific IgA plays in the acute infection process (Decoster et al., 1988; Huskinson et al., 1990; Stepick-Biek et al., 1990; Decoster et al., 1992; Gross et al., 1992; Saathoff and Seitz 1992; Gross et al., 1993). During the course of toxoplasmosis infection, the kinetics of IgA antibodies are similar to those displayed by IgM antibodies (Decoster et al., 1988; Wong and Remington, 1994; Remington et al., 1995); therefore, joint detection of IgA and IgM antibodies may be useful regardless of whether it may be discriminated at the expense of which antibody of reaction proved positive.

IgE antibodies are detectable by ELISA in sera of acutely infected adults, congenitally infected infants, and children with congenital toxoplasmic chorioretinitis (Pinon et al., 1990; Wong et al., 1993). Their demonstration does not appear to be particularly useful for diagnosis of *T. gondii* infection in foetus or newborn when compared with IgA tests. The duration of IgE seropositivity is briefer than that with IgM or IgA antibodies and hence appears useful for identifying recently acquired infection (Wong et al., 1993; Montoya and Remington, 1995).

Confirmatory testing should be done for all IgM positive cases (Liesenfeld et al., 1996; Wilson et al., 1997; Liesenfeld et al., 2001a). Accordingly, Western blot is performed because it detects antibodies against *T. gondii* P30. P30 is the major surface antigen of *T. gondii*. It is a very abundant protein (Kasper et al., 1983), conserved in most strains (Ware and Kasper, 1987), and is present both in the vesicular network of the parasitophorous vacuole and on the surface of the parasite (Sibley et al., 1986). It is very immunogenic, eliciting high titers of antibodies in infected individuals (Potasman et al., 1986). The gene coding for P30 has been cloned and completely sequenced (Burg et al., 1988). Western blot proved more sensitive than ELISA (Remington et al., 2004). Western blot was performed after PCR was done. The results obtained by PCR were negative using P30 gene primer; therefore, WB was used to confirm the antibodies against which antigen to interpret the result obtained by PCR. The WB confirmed that 61.5% of samples that were negative by PCR had specific antibodies against P30 surface gene of *T. gondii*.

Recently, a number of tests for avidity of *T. gondii* IgG antibodies have been introduced to help discriminate between recently acquired and distant infection (Hedman et al., 1989; Liesenfeld et al., 2001b). It has been observed that the functional affinity of specific IgG antibodies is initially low after primary antigenic challenge and that it increases during subsequent weeks and months by antigen-driven B cell selection. Protein-denaturing reagents including urea are used to dissociate the antibody-antigen complex. The avidity result is determined using the ratios of antibody titration curves of urea treated and untreated samples (Montoya, 2002). Therefore, IgM positive results are not sufficient as evidence of recent infection, as these antibodies are often present for many months (Kaul et al., 2004). The results we obtained show that there is high prevalence in EL Massoudia village, but the recent infection and new cases are found in EL Nuba village.

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