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Detection of B1 Gene in Toxoplasmosis using PCR among Pregnant Women Attending Antenatal Clinic in Kaduna State Northwest Nigeria

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Authors' contributions

This work was carried out in collaboration among all authors. Author IUE designed the study. Author IBS performed the statistical analysis. Author DSE wrote the protocol and author GI wrote the first draft of the manuscript. Authors MK and EII managed the analyses of the study. Author IUE managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Background: Acute infection of *Toxoplasma gondii* can be transmitted during pregnancy to the foetus vertically which may cause congenital complications like abortion, stillbirth, visual impairment, seizure, hearing impairment and other neurological disorders.
Methodology: A total of 357 pregnant women were screened using ELISA method for acute *Toxoplasma gondii* (IgM) and the detection of the B1 gene, using Polymerase Chain Reaction (PCR) across the three Senatorial zones of the Kaduna State.

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Results: The investigation shows an overall seropositive prevalence of 2.8% (IgM). Ages 16-20 and 26-30 years have the highest prevalence of 0.8% positive each, while ages 21-25 and 31-35 years have a prevalence of 0.6% positive each. However ages 36-40 years were all negative, p> 0.05.This did not show any statistical significant with the age groups. PCR analysis confirmed toxoplasmosis by detecting the B1 gene in the peripheral blood from 9 out of 10 IgM positive samples.

Conclusion: There is a chance of acquiring acute infection of *T. gondii* during pregnancy and the infection may have potential tragic outcomes for the mother, and new-born despite the fact that it can be prevented. This suggests a need for aggressive awareness and necessary facilities available for antenatal screening of *T. gondii* at the maternity clinic.

Keywords: Toxoplasmosis; IgM; PCR; B1 gene.

1. INTRODUCTION

Toxoplasmosis is a zoonosis, caused by the obligate intracellular protozoan [1,2]. This disease poses major public health challenge in congenital infections causing seizure, mental retardation, hearing impairment and visual loss, it is however transmitted to humans by ingestion of oocysts, or through accidental ingestion of sporulated oocysts from the environment [3,4].Alternatively, it can result from consumption of water or food contaminated by oocysts excreted in the faeces of infected cats [5,6].

The disease is an important food-borne pathogen and may also be transmitted by blood and blood products, organ transplants or by the ingestion of tachyzoites in unpasteurized milk [7,8]. In fact, toxoplasmosis was once a leading infectious cause of food-borne death after Salmonellosis and listeriosis in the USA [9]. Among several domestic animals cats are the definite host and play significant role in the spread of toxoplasmosis because they are the only animals that excrete resistant oocysts into the environment. However pigs, cattle, sheep, goats and rodents may play roles in its transmission. Rats and mice are thought to be persistent wildlife host reservoirs of *T. gondii* [10,11]. One of the major challenges of the parasite in human is once they are infected with the parasite, they continually harbour the organism throughout life since human defence mechanisms cannot eliminate the cyst of *Toxoplasma* [12].

Globally approximately 10% of congenital *Toxoplasma* infections result in abortion or neonatal death. In 10-23% of congenital infections, signs are present at birth; these may include hydrocephalus, chorioretinitis, hepatosplenomegally, and microcephally. Clinical signs of congenital *Toxoplasma* infection are not apparent at first in 67-80% of cases [13]. A significant proportion of encephalitic patients can also present with neuropsychiatric disorders including psychosis, dementia, anxiety, and personality disorder [14]. Ocular toxoplasmosis may occur in up to one third of children that survive congenital infection and is the most common cause of intraocular inflammation in the world [15]. Hearing loss has also been reported in 10%-30% and developmental delay in 20%-75% of this group of patients [16]. Seroprevalence varies considerably high up to 50% with countries where raw meat is commonly eaten and in tropical regions of Latin America or Sub-Saharan Africa where cats are numerous and the climate is favourable for oocysts survival [17].

The chance of acquiring acute infection with *T. gondii* is high during pregnancy and the infection would have potential tragic outcomes for the mother, the foetus and new-born despite the fact that it can be prevented [18]. The wide practice of keeping cats as domestic animals in a suitable climatic conditions favoring survival of the parasite in an area, increases the prevalence of *T. gondii* infection among pregnant women [19]. Research has been shown that over 90% of women who contract *T. gondii* infection remain asymptomatic and spontaneously recover, while only a small proportion will develop clinical signs of the disease [20,21].

The clinical presentation in pregnant women is not more severe than in non-pregnant women and most often occurs as an influenza-like illness with an incubation period of 5-18 days following exposure [22].Seroprevalence varies greatly in geographical regions within a country and within different ethnic groups according to different environments, social customs, and habits of different populations [23-26].

Epidemiological studies suggest that the prevalence of *T. gondii* infection in pregnant

women varies greatly among different countries with the prevalence estimated in US studies having a ranging from 3%–42%, Britain 22%, Netherland 26%-80%, Korea 3.7%,Sudan 34.1%, Senegal 40.2%, NewZealand 33%, Iran 38.1%, Ethiopia 93.3% and 41.6-66.9% in other Asian countries such as India and Jordan [27-31,17,32-35]. In Southern Turkey anti-Toxoplasma IgG and IgM antibody was found to be 52.1% and 0.54% respectively [35]. Therefore, infections from *T. gondii* are considered to be worldwide zoonosis and of great public health importance [36,37].

The worldwide prevalence rate of latent *Toxoplasma* infections in HIV-infected patients varies greatly from 3% to 97% [38,19]. In sub-Saharan Africa, toxoplasmosis often remains undetected and untreated due to insufficient diagnostic procedures [39]. Several studies have shown a consistently high *T. gondii*-seroprevalence for this region, ranging from 35% - 84% in different African countries south the Sahara [28].

In Nigeria the seroprevalence rates of toxoplasmosis by serological investigations have been estimated from 7%-51.3% in normal pregnant women to 17.5% -52.3% in women with abnormal pregnancies and miscarriage, while in Lagos 16.7% there is a prevalence reported for IgM antibodies in the first trimester and 46.7% for IgG at third trimester [40,41]. A study conducted in Zaria also reported prevalence of 29.1% for chronic and 0.8% for acute infections respectively [40,41,38,42].

However, despite the recognized public health importance of *T. gondii* in different parts of the world, studies on the prevalence of toxoplasmosis among people and congenital disease danger posed on neonate and children there is no policy for prevention on pregnant women and even children who are venerable to the disease in Nigeria.

2. MATERIALS AND METHODS

2.1 Study Area

The cross sectional study has been carried out in selected hospital in Kaduna State that were, spread across the three Senatorial political Zones. Kaduna State the old capital of Northern Nigeria, where it is located in the north-western geopolitical zone of Nigeria and lies between longitude 605 and 838 east of the Greenwich meridian and has a latitude of, 903 and 1132 north of equator. It has an estimated population of six million people with a total land mass estimated at 46,020sqKm in 23 local government areas. It shares borders with Zamfara, Katsina, Kano, Bauchi, Plateau, Nasarawa Niger States and Abuja [43].

2.2 Study Population

The study population consisted of pregnant women attending antenatal clinics in selected State General Hospitals in the three Senatorial district of Kaduna State.

2.2.1 Inclusion criteria

Pregnant women of all ages and at all trimesters.
Those attending antenatal clinic in Government Hospitals selected in Kaduna State.
Those who gave consent for the investigation.

2.2.2 Exclusion criteria

Non pregnant women.
Those not attending antenatal in Government Hospitals selected within Kaduna State.
Those that decline consent for the investigation.

2.3 Study Population

Multistage sampling was used in the selection of the study hospitals from one of the three Senatorial district of Kaduna State using a random sampling method. Participants were selected from Gambo Sawaba General Hospital in the northern Senatorial District, Yusuf Dan Tsoho General Hospital in the central Senatorial District and Kafanchan General Hospital in the southern Senatorial District.

2.4 Sample Size

The sample size was calculated using the descriptive study formula as described by Ishaku, et al.,2009

$$n = \frac{z^2 pq}{d^2}$$

Where the P = Value of proportion of interest (If no information is known about p then p= 0.5).

A prevalence of 29.1% was used for the calculation [38].

d= Tolerance eg: within 0.05

Hence:

$$n = \frac{1.96^2 \times 29.1/100\ (1-29.1)}{0.05^2} = 317 \text{ samples.}$$

Therefore a total of 357 samples were collected across the three geopolitical zones of Kaduna State due to additional 10% anticipated non response rate and to minimize sampling error in the study.

2.5 Materials

The materials used were Vacutainers, serum microtubes, cotton wool, methylated spirit, specific Toxo- IgM EIA Kits, micropippetes of different sizes, distilled water, absorbent paper, micro-titer plate, strip well washer and micro-plate reader with 450nm wavelength and structured participant questionnaire.

2.6 Sample Collection

Five millilitres of blood was collected from participants by a qualified medical laboratory scientist/technician via the ante cubical vein and then transferred each into a plain sterile tube and 2.5ml EDTA tubes and labelled appropriately. The blood in the plain container was centrifuged at 3000rpm for 5minutes and the sera was harvested into clean cryovials and stored while the 2.5ml blood sample in the EDTA was also stored at -20°C until it was required for use.

2.7 Questionaire Administration

The patient information was collected using a designed structured questionnaire that accessed age, literacy level, source of drinking water, type of meat consumed, type of pets contact, obstetric history and milk consumption. The aim of the study was explained to the patients and informed consent was obtained before administering the questionnaire. In order to ensure confidentiality, patients names were not recorded. The questionnaire was interpreted in the local language for those who could not understand English.

3. RESULTS

3.1 Sample Analysis

3.1.1 Serological method

Commercial sample reagent for specific detection of anti-*Toxoplasma gondii* IgM antibodies were used according to manufacturer's manual that was purchased from CALBIOTECH Inc., USA. The reagent contained a serum diluent to remove Rheumatoid factor and human IgG interference, and the wells were coated with purified antigen. The IgM specific antibodies, bounds to the antigen in the positive samples. All unbound materials was washed away and the enzyme conjugate was added to bind the antibody-antigen complex in positive samples. Excess enzyme conjugate was washed off and substrate was added. The plate was incubated and allowed to hydrolyse the substrate by the enzymatic reaction. The intensity of the colour generated at the end of the reaction was proportional to the amount of IgM specific antibody present in the sample.

3.2 Molecular Diagnosis

IgM positive samples were identified and transported using an ice pack to maintain -20^{o}c until they arrived at the University of Maiduguri, Biotechnology Centre Molecular Laboratory. The DNA was extracted from the samples using Phenol Extraction Method. Samples were amplified using gel electrophoresis machine (Cleaver scientific UK, CS 300) to detect a fragment from the T. *gondii* B1 gene, which is present in 35 copies and is conserved in the T. *gondii* genome, as described by Burg. et al., [44].

DNA was quantified using NanoDrop 2000C spectrophotometer (Thermos Scientific, USA) and concentration was determined based on absorbance at 260nm. Purity was estimated as ratio of absorbance at 260nm to Absorbance at 280nm (A260:A280).

3.2.1 Nested PCR amplification of *T. gondii* B1, (PCR for beta Haemoglobin)

PCR was run on the human Hb-beta subunit to ascertain the quality of the extracted DNA, the viability of the tissue for PCR detection of Toxoplasma DNA and as a control gene for human tissues. The primer which targets a 122 bp sequence of the Hb beta sub-unit was obtained commercially from Inqaba Biotec West Africa with the sequence as shown in the table. PCR was carried out in 50µL reaction using FIREPol® master mix (Solis BioDyne, Estonia), where each reaction volumes contained 2.5 mM MgCl and 200 µM dNTPs in equimolar concentration of standard buffer. The following thermocycler (Eppendorf mastercycler nexus, Hamburg, Germany) program was used; Initial

Denaturation at 94^0C for 5min followed by 35 cycles of denaturation at 95^0C, for 30 sec; annealing at 56^0C for 30sec and extension at 72^0C for 30 sec.

3.2.2 First PCR for B1 gene

The first PCR detected *toxoplasma* DNA that was carried out using a primer set as shown in the table that targets a 197 bp section of the B1 gene. PCR reaction was carried out as explained above using the following thermocycler conditions;Initial Denaturation at 94^0C for 5min followed by 35 cycles of denaturation at 95^0C, 30 sec; annealing at 46^0C for 30 sec and extension at 72^0C for 30 sec.

3.2.3 Second nested PCR

The nested PCR amplified a 97 bp region within the B1 gene. The PCR product obtained from the first PCR was used as template and react with the primers for this region of the B1 gene table. PCR conditions were carried out using the reaction conditions as explained above and thermocycler conditions;Denaturation at 94^0C for 5min followed by 35 cycles of denaturation at 95^0C, for 30 sec; annealing at 53.5^0C for 30 sec and extension at 72^0C for 30 sec.

3.2.4 Gel electrophoresis

To confirm amplification of the 122 bp *Hb beta* sub unit, 197 bp B1 gene and 97 bp nested PCR, an agarose gel electrophoresis was carried out on 2.5% agarose gel in TAE buffer according to method suggested by Green and Sambrook, [45]. Electrophoresis was carried out at 90V for 60min and viewed under UV trans-illuminator. A 100 kb size ladder (New England Biolab USA) was used as the standard size DNA marker for the beta hemoglobin subunit and B1 gene while a 50kb ladder was used for the nested B1 gene. Staining was done with ethidium bromide.

This PCR shows the amplified 122 bp section of the human Hb beta subunit to ascertain that samples are of human source and that the DNA is amplifiable.

Table 1. Prevalence of *Toxoplasma gondii* (IgM) in pregnant women based on age group in Kaduna State

Age Group(year)	No. Examined	IgM pos (%)	IgM neg (%)	*P-Value*
16-20	47	3(0.8)	44(12.3)	0.630[a]
21-25	113	2(0.6)	111(31.1)	
26-30	106	3(0.8)	103(28.9)	
31-35	63	2(0.6)	61(17.1)	
36-40	22	0(0.0)	22(6.2)	
41-45	6	0(0.0)	6(1.7)	
TOTAL	**357**	**10(2.8%)**	**347(97.2%)**	

KEY: a = Pearson Chi-square test, Pos = Positive, Neg= Negative, % = Percentage

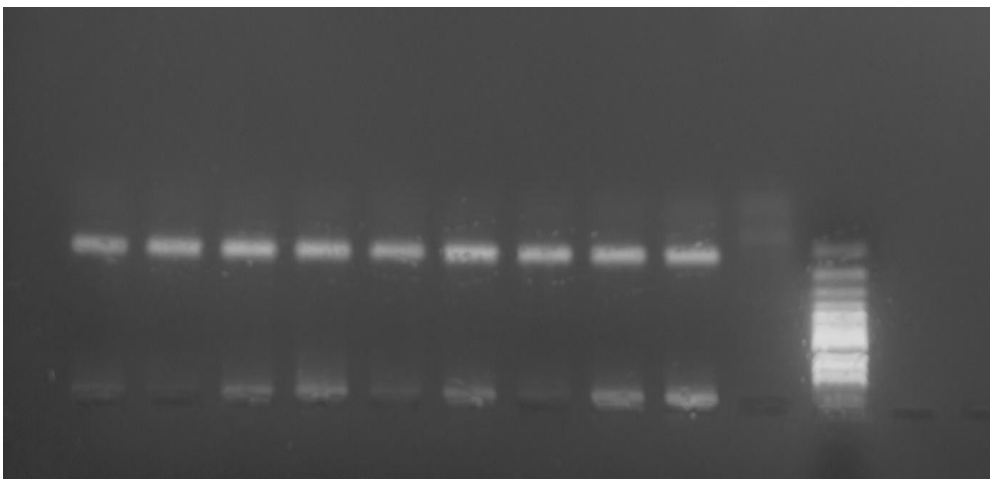

Fig. 1. Gel image for amplification of human hemoglobin beta subunit (hbb)

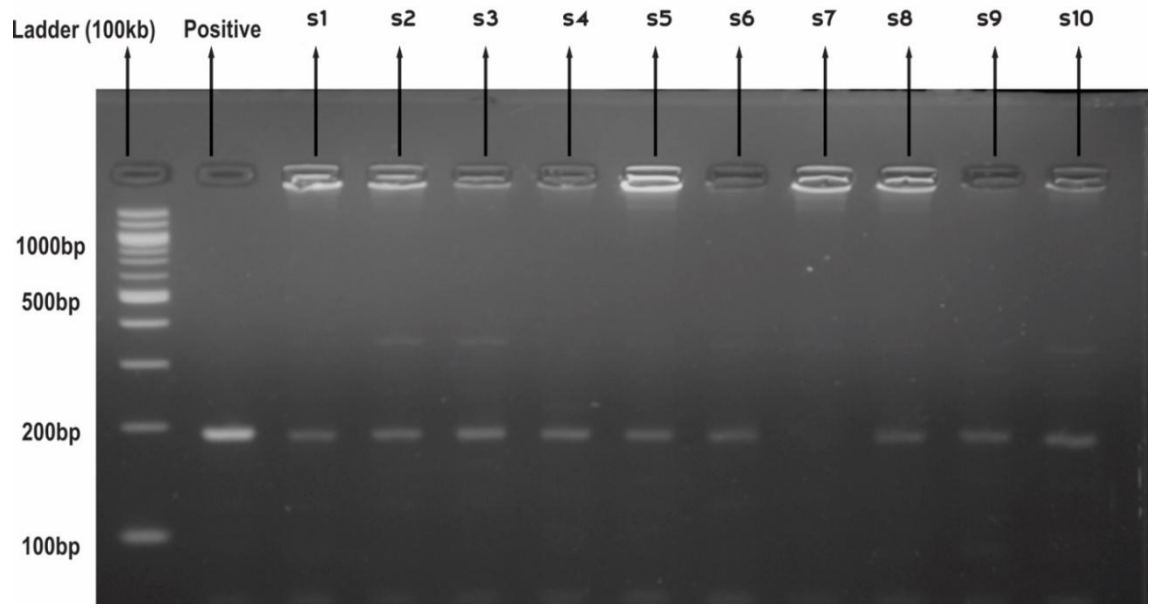

Fig. 2. Gel images of B1 gene for nested PCR showing amplification of a 197 bp fragment in samples 1, 2, 3, 4,5, 6, 8, 9 and 10

KEY : *LANE 1 = LADDER 100Kb, LANE 2 = POSITIVE SAMPLE, LANE S1-S10 = SAMPLES 1-10 LANE S1,S2,S3,S4,S5,S6,,S8, S9, and S10 shows B1gene at 197bp*

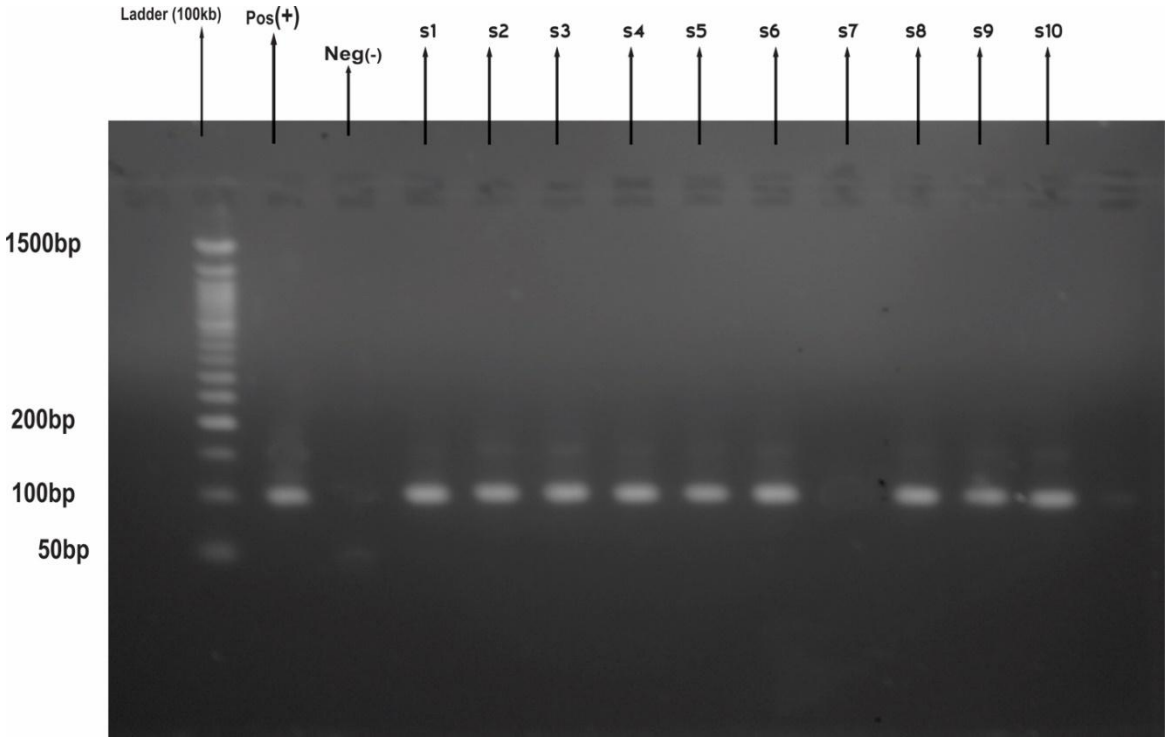

Fig. 3. Gel image of second PCR with amplified a 97 bp region within the 197 bp region of the B1 gene of *Toxoplasma gondii* that was amplified during the first PCR

KEY : *LANE 1 = LADDER 100Kb, LANE (POS +) = POSITIVE CONTROL, LANE (NEG -) = NEGATIVE CONTROL, LANE S1-S10 IgM positive samples = SAMPLES 1-10, LANE S1,S2,S3,S4,S5,S6,S8, S9, and S10 shows B1gene at 97bp*

NOTE: *No amplification was observed in sample S7, and Negative Control (NEG-) while positive control shows distinct amplification at 97bp.*

4. DISCUSSION

Acute infection from *Toxoplasma gondii* can be transmitted during pregnancy to the foetus vertically which may cause congenital complications like miscarriage, stillbirth, visual impairment, seizure, hearing impairment and other neurological disorders [46]. This study observed 2.80% prevalence of toxoplasmosis IgM antibodies in the pregnant women select from hospitals in Kaduna State and was similar to the prevalence reported in India 3.9%, Brazil 3.26%, Gabon 2.6%, NewZealand 2.4% and in Zaria 0.8% [47,30,38,48-51]. The findings in this study were lower that has been reported in Kano 13.08%,Qatar 5.2%, Portharcout 11.5%, Trinidad Tobago 11.9%, Lagos 7.6% and in Maiduguri 7.2% [52,42,53-55]. The difference in the prevalence rates has been suggested in literature to be due to geographical location, climate condition, and cultural behavior, even within the same country because parasite oocyst sporulation is known to be prevalent in warm and humid conditions [56].

The observed prevalence of IgM antibodies in the age group 16-20 and 26-30 years is in agreement with Kefale et al. [19], who reported a 20%, prevalence in women 15-19years and Ballah et al. [55] who reported < 20 years 52.86%. This may be attributed to several factors which could have been responsible for the variation in prevalence amongst the different age groups. Some of which may have included the level of maturity, personal hygiene and socio-economic status of family and even the level of education because most of these women were under age, and had a teenage marriage which is common in Northern Nigeria.

Several studies have shown that PCR has consistently been used to detect *T. gondii* DNA in various biological samples since it is more sensitive for diagnosis as compared to serological tests and culture [57]. The first PCR method for *T. gondii* detection, targeting the B1 gene, has been established since 1989 [44], and is widely used in prenatal diagnosis of congenital toxoplasmosis and *T. gondii* infection in immunocompromised patients [58-62].

Most PCR-based techniques make use of the B1 gene, and less commonly the SAG-1 (P-30) single-copy sequence, which has been shown to be a satisfactory PCR target for the detection of *T. gondii* [63]. Unfortunately despite the several studies in Kaduna there is little or no molecular detection reported in toxoplasmosis investigations. In this study however 9 out of 10 samples that were serologically positive, further tested positive for the *Toxoplasma gondii* B1 gene that was showed the 97 bp region within the 197 bp DNA fragment region and therefore the presence of *Toxoplasma* DNA in the peripheral blood indicates the sensitivity and specificity of PCR analysis for detecting infection in pregnancy. This is in agreement with previous reports that PCR is recommended over serologic techniques for the diagnosis of toxoplasmosis [57]. However, the 1 out of 10 positive samples that B1 gene was not detected could be as result of false positive by the ELISA analysis which signifies that PCR is more sensitive and specific than ELISA test.

5. CONCLUSION

Toxoplasmosis is important risk for congenital infection from acute infection during pregnancy which has been shown in the absence of appropriate treatment. Neonates who are infected with *Toxoplasma gondii* have been shown to be at substantial risk for developing long-term sequelae when no treatment is given and the chance of acquiring acute infection with *T. gondii* is high during pregnancy which would have potential tragic outcomes for the mother, and new-born despite the fact that it can be prevented. This suggests a need for aggressive awareness and compulsory *T. gondii* antenatal screening.

CONSENT

As per international standard or university standard, patient's written consent has been collected and preserved by the author(s).

ETHICAL APPROVAL

The ethical permission letter was obtained from the Kaduna State Ministry of Health Review Ethical Committee with reference number: MOH/ADM/744/VOL. 1/527 before samples were collected from the hospitals.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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