Original Research Article

DETECTION OF B1 GENE IN TOXOPLASMOSIS USING PCR AMONG PREGNANT WOMEN ATTENDING ANTENATAL CLINIC IN KADUNA STATE NORTHWEST NIGERIA.

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 ~~of the organism~~ using Polymerase Chain Reaction (PCR) across the three Senatorial zones of the Kaduna Sstate.

RESULT: The investigation ~~however~~ shows an overall seropositive prevalence of 2.8% (IgM). Ages 16-20 and 26-30 years have the highest prevalence of ~~3(~~0.8%~~)~~ positive each,. ~~While~~ while ages 21-25 and 31-35 years have a prevalence of ~~2(~~0.6%~~)~~ positive each. However ages 36-40 years ~~are~~ were all negative. The Mean age of 3.76, Standard Deviation = 1.157; p> 0.05. This did not show any statistical significant with the age groups. ~~The~~ PCR analysis confirmed ~~the t~~Toxoplasmosis by detecting the B1 gene in the ~~pheripheral~~peripheral blood ~~of~~ from 9 out of 10 IgM positive samples.

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

KEY WORDS: Toxoplasmosis, IgM, PCR, B1 gene.

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Comment [CS2]: This statement must be clarified. The author did not compare the toxo infection in non pregnant women.

INTRODUCTION

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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~~is~~ the definite hosts and play significant roles 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 role 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~~defense 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~~)~~]. In spite of the wide practice of keeping cats as domestic animals and presence of stray cats around, and suitable climatic conditions favoring survival of the parasite in the study area, to our knowledge, there is no regular serological screening of pregnant women for *T. gondii* infection [~~(~~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~~,24,25,26~~, 24, 25, 26]~~)~~.

Epidemiological studies suggest that the prevalence of *T. gondii* infection in pregnant women varies greatly among different countries with the prevalence estimate~~d~~s ~~from~~ in US studies ~~having a~~ ranginge ~~of~~ from 3%–42%, Britain 22%, Netherland 80%, Korea 3.7%, Sudan 34.1%, Senegal 40.2%, New Zealand 33%, Iran 38.1%, Ethiopia 93.3%, Dutch 26%, and 41.6-66.9% in other Asian countries such as India and Jordan [~~(~~27, 28, 29, 30, 31, 17, 32, 33, 34, 35~~)~~]. In Southern Turkey, anti-Toxoplasma IgG and IgM antibody was found to be 52.1% and 0.54% respectively. Therefore, infections ~~due~~ from ~~to~~ *T. gondii* are considered to be a worldwide zoonosis ~~of~~ and a great importance of ~~great~~ public health ~~importance~~ [~~(~~36, 37~~)~~].

The ~~W~~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% ~~to~~ 84% in different African countries south of the Sahara [~~(~~28~~)~~].

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Comment [CS4]: We would say The Netherlands instead of Dutch.

Comment [CS5]: The auther should cite this data as well.

In Nigeria, the seroprevalence rates of toxoplasmosis by serological investigations have been estimated from 7% to 51.3% in normal pregnant women to 17.5% to 52.3% in women with abnormal pregnancies and abortions, while in Lagos there is a 16.7% prevalence was reported for IgM antibodies in the First first trimester and 46.7% for IgG at third trimester. 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 measures taken for prevention on pregnant women and even children who are venerable to the disease in Nigeria.

Comment [CS6]: Perhaps the author is refering to miscarriage instead of abortions?

Comment [CS7]: Author should apply these citations to the data stated in the previous sentence.

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MATERIALS AND METHOD

STUDY AREA

The cross sectional study has been was a cross sectional study carried out in some selected Hhospitals in Kaduna State that were , spread across the three Senatorial political Zones. Kaduna State is thea old Ccapital of Northern Nigeria, where it is located in the north-western geopolitical zone of Nigeria and lies between lLongitude 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 46,020 sqKm in 23 local gGovernment aAreas. It shares borders with Zamfara, Katsina, Kano, Bauchi, Plateau, Nasarawa Niger States and Abuja [(43])].

STUDY POPULATION

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

Inclusion Criteria

Pregnant women of all ages and at all trimesters

Those attending antenatal clinic in Government Hospitals selected in Kaduna State

Those that who gave consent for the investigation

Exclusion Criteria

Non pregnant women

Those not attending antenatal in Government Hospitals selected within Kaduna State

Those that decline consent for the investigation

Study Population

Multistage sampling was used in the selection of the study hospitals from one of each in the three SenetorialSenatorial district of Kaduna sState using a random sampling method. In view of the aboveParticipants were selected from Gambo Sawaba General Hospital in the northern Senatorial District, Yusuf Dan Tsoho General Hospital in the cCentral Senatorial District and Kafanchan General Hospital in the sSouthern Senatorial District were selected.

SAMPLE SIZE

The sample size was calculated using the descriptive studyies 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: $n = \frac{1.96^2 \times 29.1/100\ (1-29.1)}{0.05^2} = 317$ samples.

Therefore, a total of 349 samples wereas collected across the three geopolital geopolitical zones of Kaduna State due to an additional 10% additional anticipated non response rate and to minimize sampling error in the study.

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MATERIALS

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

ETHICAL CONSIDERATION

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

SAMPLE COLLECTION

Five millilitres of blood was collected from participants by a qualified mMedical laboratory sScientist/tTechnician via the ante cubical vein by applying torniquet on either of the arm for visibility of the vein then swapping the area with cotton wool soaked in alcohol after which using sterile vacutainer/syringe and a needle is pierce into the vein for blood collection and then transfertransferred each the blood into a plain, sterile plain tube and 2.5 mL EDTA tubes 2.5ml each and labelled appropriately. The blood in the plain container was centrifuged at 3,000 rpm for 5 minutes and the sera was harvested into clean cryovials and stored at –20°C, while the 2.5

ml blood sample in the EDTA was also stored at -20° C ~~same temperature~~ until it was~~is~~ required for use.

QUESTIONAIRE ADMINISTRATION

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

SAMPLE ANALYSIS

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 ~~, t~~the wells ~~are~~ were coated with purified antigen. The IgM specific antibodies~~,~~ bound to ~~if present binds to~~ the antigen in the positive samples. All unbound material~~s~~ ~~are~~ was washed away and the enzyme conjugate ~~is~~ was added to bind ~~to~~ the antibody-antigen complex in positive samples~~, if present~~. Excess enzyme conjugate was~~is~~ washed off and the substrate was~~is~~ added. The plate was~~is~~ incubated ~~to~~ and allowed to ~~the hydroly~~hydrolyse~~sis~~ ~~of~~ the substrate by the enzymatic reaction~~e~~. The intensity of the colour generated at the end of the reaction ~~is~~ was proportional to ~~the among~~the amount of IgM specific antibody present in the sample.

MOLECULAR DIAGNOSIS

~~-~~IgM positive samples were ~~selected~~ identified and transported using an ice pack to maintain ~~cold chain of~~ -20° C~~e~~ until they arrived at the ~~to~~ University of Maiduguri, Biotechnology Centre Molecular Laboratory. The DNA was extracted from the samples using Phenol Extraction Method. Samples were amplified using g~~C~~el e~~E~~lectrophoresis m~~M~~achine 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*. ~~,~~(1989).

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

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

PCR was run ~~for~~ 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. ~~A~~ The primer which targets a 122 bp sequence of the Hb beta sub-unit ~~was used; primer w~~was obtained commercially from Inqaba Biotec, West Africa with the sequence as shown in the table. PCR ~~reaction~~ was carried out in 50µL reaction volumes using FIREPol® master mix (Solis BioDyne, Estonia), where each reaction contained~~ing~~ 2.5 mM MgCl and ~~,~~ 200 µM dNTPs in equimolar concentration of~~in~~ standard buffer. The following t~~T~~hermocycler (Eppendorf mastercycler nexus, Hamburg, Germany) program was used~~:~~; Initial Denaturation at 94^{0} C for 5

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min followed by 35 cycles of denaturation at 95^{0}C for ~~,~~30 ~~S~~sec; annealing at 56^{0}C for 30 sec; and extension at 72^{0}C for 30 sec.

First PCR for B1 gene

The first PCR ~~to~~ detected *T~~t~~oxoplasma* 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^{0}C for 5 min followed by 35 cycles of denaturation at 95^{0}C, 30 ~~S~~sec; annealing at 46^{0}C for 30 s~~S~~ec and extension at 72^{0}C for 30 s~~S~~ec.

Second Nested PCR

The nested PCR amplifie~~d~~s a 97 bp region within the B1 gene. The PCR product obtained from the first PCR was used as template and reacted with the primers ~~used are as stated on the~~for this region of the B1 gene (~~-~~table). PCR conditions were carried out using the reaction conditions as explained above and thermocycler conditions ~~are; :~~ Denaturation at 94^{0}C for 5min followed by 35 cycles of denaturation at 95^{0}C for~~,~~ 30 s~~S~~ec; annealing at 53.5^{0}C for 30 ~~S~~sec; and extension at 72^{0}C for 30 s~~S~~ec.

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 (2012) ~~([~~45~~])~~. Electrophoresis was carried out at 90 V for 60 min and viewed under UV trans-illuminator. A 100 kb size ladder (*NEB*) 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 e~~E~~thidium b~~B~~romide.

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RESULT

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

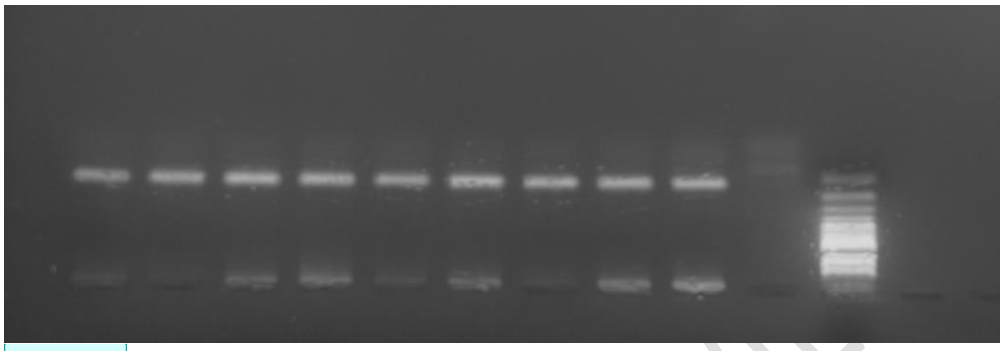

Figure 1. Gel image for amplification of human hemoglobin beta subunit (hbb).

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

Comment [CS16]: Author needs to label this gel and indicated the ladder for credibility.

Comment [CS17]: Normally we show a ladder band below the target band from the sample to give certainity of the results. The author cut the gel to illustrate this concept.

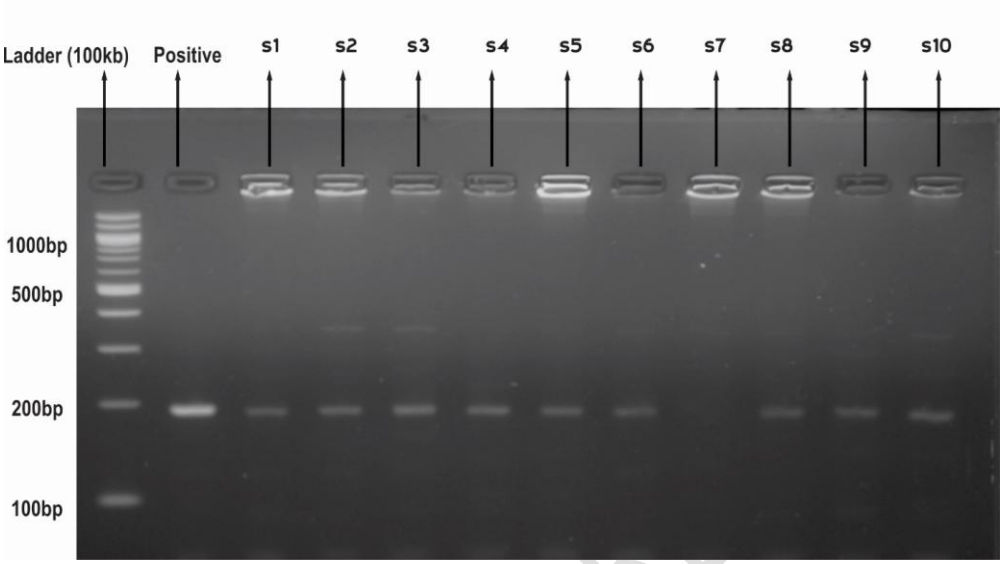

PLATE (I). Gel images of B1 gene for nested PCR showing amplification of a 197 bp fragment in samples 1, 2, 3, 4, 5, 6, 8, 9 and 10.

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 B1 gene at 197 bp

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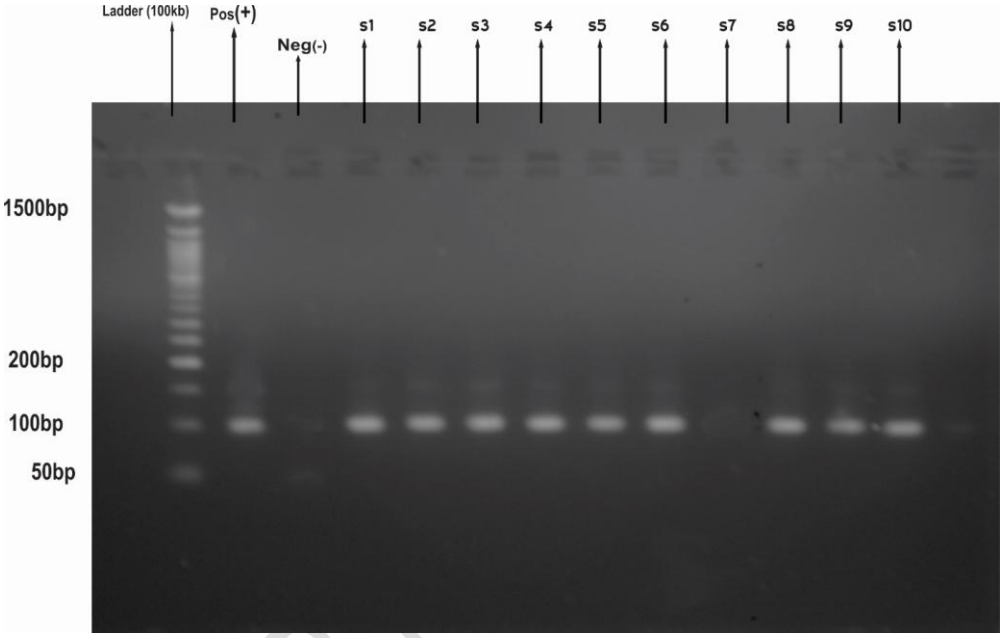

PLATE (II). Gel image of second PCR ~~which~~ with amplifieds ~~a~~ 97 bp region within the 197 bp region of the B1 gene of *Toxoplasma gondii* that was amplified during the first PCR.

KEY-: LANE 1 = -LADDER 100Kb

LANE (POS +) = POSITIVE CONTROL

LANE (NEG –) = NEGATIVE CONTROL

LANE S1-S10 = IgM POSITIVE SAMPLES 1-10

LANE S1, S2, S3, S4, S5, S6, S8, S9, and S10 show~~s~~ B1 gene at 97 bp

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

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Table 2. ~~Showing the~~ Primer S~~s~~equence and the Annealing Temperature

Primer sequence	Annealing Temp (^{0}C)	Target region	Amplicon size	Reference
5'CTTCTGACACAACTGTGTTCACTAGC3' 5'TCACCACAACTTCATCCACGTTCACC 3'	56	Human Hemoglobin beta	122 bp	[ ]
5'GGAACTGCATCCGTTCATGAG3' 5'TCTTTAAAGCGTTCGTGGTC3'	46	B1 gene	197 bp	[ ]
5'TGCATAGGTTGCAGTCACTG3' 5' GGCGACCAATGTGCGAATAGACC3'	53.5	B1 gene (nested)	97 bp	[ ]

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DISCUSSION

Acute infection ~~of~~ from *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 [~~(~~46~~)~~]. This study observed a

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2.80% prevalence of t~~T~~oxoplasmosis IgM antibodies in the pregnant women from select hospitals in the Kaduna State and was similar ~~to the work that reported~~the prevalence reported in India (~~-~~3.9%)~~ cases in India~~, Brazil (3.26%)~~ in Brazil~~, Gabon (2.6%)~~ in Gabon~~, New Zealand (2.4%)~~ in NewZealand~~, and Zaria (0.8%)~~ in Zaria,~~ ~~(~~[47, 30, 38, 48, 49, 50, 51]~~)~~. The findings in this study ~~is however not similar to studies of~~were lower that was has been reported in Kano (13.08%)~~ reported in Kano~~, Qatar (5.2%)~~ in Qatar~~, Portharcout (11.5%)~~ in Portharcout~~, ~~11.9% in~~ Trinidad Tobago (11.9%), Gabon (5.4%)~~ in Gabon~~, ~~7.6% in~~ Lagos (7.6%), ~~and~~d ~~7.2% in~~ Maiduguri (7.2%) ~~(~~[52, 42, 53, 54, 55~~)~~]. The difference in ~~the various~~ prevalence rates ~~could~~ has been suggested in the literature to be due to geographical location, climate condition, and cultural ~~behaviour~~behavior, even ~~even~~ within the same country because ~~the~~ 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*.~~,~~ (2015) ~~(~~[19]~~)~~, who reported a 20%~~,~~ prevalence in women 15-19 years and Ballah *et al*.~~,~~ (2017) ~~(~~[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 ~~the~~ family, and even the level of ~~their~~ education because most of these~~is~~ women ~~are~~ were under age~~d,~~ and had a teenage marriage, which is common in Northern Nigeria.

Several studies have shown that PCR has ~~been~~ consistently been used to detect ~~DNA of~~ *T. gondii* DNA in various biological samples ~~due to the fact that~~ since it is more sensitive~~ivity~~ for ~~in~~ diagnosis as compared to serological tests and culture [~~(~~57]~~)~~. The first PCR method for *T. gondii* detection, targeting the B1 gene, has been ~~was~~ established ~~in~~ since 1989 [~~(~~44] and is ~~).~~ ~~How ever since then this method has been w~~widely used in prenatal diagnosis of congenital toxoplasmosis and *T. gondii* infection in immunocompromised patients [~~(~~58, 59, 60, 61, 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 de~~e~~tection~~ation~~ reported ~~on~~ in toxoplasmosis investigations. In this study, however, 9 out of the 10 samples that were serologically positive ~~-serologically, only 9 that~~further tested

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Comment [CS25]: Gabon was cited to have2.6% prevalence in previous sentence. The author needs to verfy this.

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Comment [CS26]: Author needs to clarify this sentence. It is important for the paper.

Comment [CS27]: The author should further develop these statements in context of the study results, unless he/she is only aiming for a short communication.

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positive for the *Toxoplasma gondii* B1 gene ~~*Toxoplasma gondii* DNA was detected and amplified at~~*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 a recent infection that is likely to be clinically significant and confirmed the sensitivity and specificity of PCR analysis for detecting recent infection in early 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 precise than ELISA test.

Comment [CS28]: This is a run on sentence. The author should simply the sentence for clarity.

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5.2. CONCLUSION

Toxoplasmosis is important ~~in overall~~ risk ~~of its~~for congenital infection ~~from~~ from acute infection during pregnancy, which has been shown in the absence of appropriate treatment. Neonates who are infected with *Toxoplasma gondii* ~~infected neonates~~ have been shown to be at substantial risk ~~of~~ 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 ~~the~~ a need for aggressive awareness and compulsory ~~screening of~~ *T. gondii* ~~during~~ antenatal screening.

Comment [CS30]: The author could make this a more effective sentence.

Comment [CS31]: This is a fragmente, run on sentences and it needs to be clarified.

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Comment [CS32]: The references are not presented well. There appear to be many typos. Please double check them and add doi web links.

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