Original Research Article

~~DETECTION OF B1 GENE IN~~ TOXOPLASMOSIS ~~USING PCR AMONG~~INFECTION OF PREGNANT WOMEN ATTENDING ANTENATAL CLINIC IN KADUNA STATE NORTHWEST NIGERIA, AS DETECTED BY SEROLOGY AND PCR.

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~~ recent *Toxoplasma gondii* (IgM) and detect the B1 gene of the organism using Polymerase Chain Reaction (PCR) across the three Senatorial zones of Kaduna state.

RESULT: The investigation ~~however shows~~ revealed a prevalence of 2.8% (IgM). Ages 16-20 and 26-30 years ~~have~~ had the highest prevalence of 3(0.8%) positive each. While ages 21-25 and 31-35 years have prevalence of 2(0.6%) positive each. However ages 36-40 years are 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 Toxoplasmosis by detecting the B1 gene in the pheripheral blood of 9 out of 10 positive samples.

CONCLUSION: ~~Therefore t~~The chance of acquiring acute infection with *T. gondii* ~~is~~ seems to be high during pregnancy, ~~ and the infection would have~~which mat cause potential ~~tragic outcomes~~complications to ~~for~~ the mother~~,~~ and new-born ~~despite the fact that it can be prevented~~. This suggests the need for ~~aggressive awareness~~implication of preventative measures and early detection, ~~and~~ which will require necessary facilities available for screening of *T. gondii* ~~during~~ in antenatal clinics.

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

INTRODUCTION

Toxoplasmosis is a zoonosis, caused by the obligate intracellular protozoan *Toxoplasma gondii* (1,2). This disease poses major public health challenge in congenital infections as it may cause~~causing~~ seizure, mental retardation, hearing impairment and visual loss (REF)., ~~it is however~~The parasite can be 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~~feces of infected cats (5,6).

The ~~disease~~ parasite 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 c~~Since cats are~~is~~

Comment [1]: How can the prevalence of each subgroup be lower than the total?

Comment [2]: How can awareness be aggressive?

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the definite host ~~and~~ they play a significant role in the spread of toxoplasmosis ~~because they are the only animals that~~by excreting resistant oocysts into the environment. However other animals such as pigs, cattle, sheep, goats, ~~and~~ rodents and humans may play role in its transmission as intermediate hosts. 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~~toxoplasmosis in intermediate hosts is the fact they create tissue cysts…, 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~~between regions and may reach up to 50% ~~with~~ in countries where raw meat is commonly ~~eaten~~ consumed and in tropical regions of Latin America or Sub-Saharan Africa, where cats are numerous and the climate is ~~favourable~~favorable for oocyst~~s~~ survival (17).

The chance of acquiring acute infection with *T. gondii* is high during pregnancy and ~~the~~ infection ~~would~~ may have potential tragic outcomes for the mother, the foetus ~~and~~ or the new-born ~~despite the fact that it can be prevented~~ (18). Inspite 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~~ It 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~~different 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).

Epidemiological studies ~~suggest~~ revealed that the prevalence of *T. gondii* infection in pregnant women varies ~~greatly among different~~between countries, with prevalence of ~~estimates from US studies having a range of 3~~% ~~%~~ to 42% in the US, Britain 22%, Netherland 80%, Korea 3.7%,Sudan 34.1%, Senegal 40.2%, NewZealand 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~~ antibodies ~~was found to be~~were found in 52.1% and 0.54% respectively. Therefore, infections due to *T. gondii* are considered a worldwide zoonosis of great public health importance (36, 37).

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

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Comment [8]: What were the other prevalence studies? PCR?

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 Sahara (28).

In Nigeria, the serological seroprevalence ~~rates~~ of toxoplasmosis ~~by serological investigations have~~ has been estimated from 7% to 51.3% in normal pregnant women ~~to~~ and from 17.5% to 52.3% in women with abnormal pregnancies and abortions, while in Lagos ~~16.7%~~the prevalence of ~~was reported for~~ IgM antibodies was 16.7% in women in ~~F~~the first trimester and in 46.7% ~~for IgG at~~of women in the 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, d~~Despite the ~~recognized~~ public health importance of *T. gondii* in different parts of the world, studies on the prevalence of toxoplasmosis among people and the risk of related congenital diseases are scarce?~~danger posed on neonate and children there is~~ and no measures are currently implemented ~~taken~~ for the prevention of infection in~~on~~ pregnant women ~~and even~~or children, who are ~~venerable~~ vulnerable to the disease in Nigeria.

Comment [9]: Can be removed

Comment [10]: In pregnant women?

Comment [11]: What is the aim of this study?

MATERIALS AND METHOD

STUDY AREA

The study was a cross sectional study carried out in some selected Hospitals in Kaduna State, spread across the three Senatorial political Zones. Kaduna State is an old Capital of Northern Nigeria, is located in the north-western geopolitical zone of Nigeria and lies between Longitude 605 and 838 east of Greenwich meridian and latitude, 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).

STUDY POPULATION

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

Comment [12]: Which hospitals? Please specify

Inclusion Criteria

Pregnant women of all ages at all trimesters

Those attending antenatal clinic in Government Hospitals selected in Kaduna State

Those that 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

Comment [13]: This repeats the previous paragraph. Just add that they had to consent

Study Population

Multistage sampling was used in the selection of the study hospitals one each in the three Senetorial district of Kaduna state using random sampling method. In view of the above 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 were selected.

Comment [14]: There is no need for the subtitle, just add this to the previous paragraph

SAMPLE SIZE

The sample size was calculated using the descriptive studies formula (Ishaku, *et al.*,2009)

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

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).

Comment [15]: Please add justification for this number

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 was collected across the three geopolital zones of Kaduna State due to 10% additional anticipated non response rate and to minimize sampling error.

MATERIALS

The materials used are 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 questionnaire.

Comment [16]: Unnecessary

ETHICAL CONSIDERATION

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

SAMPLE COLLECTION

Five millilitres of blood was collected by ~~a~~ qualified Medical laboratory Scientists/Technicians 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 transfer the blood into a sterile plain tube and EDTA tubes 2.5ml each and labelled appropriately. The blood

in the plain container was centrifuged at 3000rpm for 5 minutes and the sera was harvested into clean cryovials and stored at -20^{o}C, while the 2.5ml in the EDTA was also stored at same temperature until it is required for use.

QUESTIONAIRE ADMINISTRATION

The patient's' information were collected using a designed structured questionnaire. Age, literacy level, source of drinking water, type of meat consumed, pets contact, obstetric history and milk consumed. The study was however explained to the patients and informed consent obtained before administering the questionnaire. In order to ensure confidentiality, names of patients were not recorded. The questionnaire was interpreted in 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 detection kits were used according to manufacturer's manual (purchased from CALBIOTECH Inc., USA). The reagent contain serum diluent to remove Rheumatoid factor and human IgG interference, the wells are coated with purified antigen. IgM specific antibodies, if present binds to the antigen. All unbound materials are washed away and the enzyme conjugate is added to bind to the antibody-antigen complex, if present. Excess enzyme conjugate is washed off and substrate is added. The plate is incubated to allow the hydrolysis of the substrate by the enzyme. The intensity of the colour generated is proportional to the among of IgM specific antibody in the sample.

Comment [17]: Unnecessary. Please state, however, the method used. ELISA, I presume?

MOLECULAR DIAGNOSIS

IgM positive samples were selected and transported using ice pack to maintain cold chain of -20^{o}c to 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 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)

Comment [18]: This belongs to the nPCR section

DNA was quantified using NanoDrop 2000C spectrophotometer (Thermos Scientific, USA). Concentration was determined based on absorbance at 260nm. 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 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 pPrimers which targets a 122 bp sequence of the Hb beta sub-unit was were used; primers was were obtained commercially from Inqaba Biotec West Africa with the sequence as shown in the table. PCR reactions was were carried out in 50µL reaction using FIREPol® master mix (Solis BioDyne, Estonia), each reaction containing 2.5 mM MgCl, 200 µM dNTPs in equimolar concentration in standard buffer. The following Thermocycler (Eppendorf mastercycler nexus, Hamburg, Germany) program was used; Initial Denaturation at 94^{0}C for

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

First PCR for B1 gene

The first PCR to detect ~~t~~*Toxoplasma* DNA was carried out using a primer set as shown in the table that ~~targets~~ targeted a 197 bp section of the B1 gene. PCR reactions ~~was~~ were 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.

Second Nested PCR

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

Gel Electrophoresis

To confirm amplification of the 122 bp *Hb beta* sub unit, 197 bp B1 gene and 97 bp nested PCR, agarose gel electrophoresis was carried out on 2.5% agarose in TAE buffer according to method suggested by Green and Sambrook (2012)(45). Electrophoresis was carried out at 90V for 60min and viewed under UV trans-illuminator. A 100 kb size ladder (*NEB*) was used as the standard size DNA marker for the beta hemoglobin and B1 gene while a 50kb ladder was used for the nested B1 gene. Staining was done with Ethidium Bromide.

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Comment [20]: Which table?

Comment [21]: I recommend joining the two paragraphs describing both stages of the nPCR

Comment [22]: Which table?

Comment [23]: Annealing temperatures of both stages are very low, which makes them less specific. Did you sequence the PCR product to confirm it is Toxoplasma?

Comment [24]: Please describe ant statistical methods used

RESULTS

Comment [25]: Please add text describing the results and refer to the figures and tables

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

Comment [26]: Why not use the age as a continues parameter and compare the mean age of positive versus negative samples using t-test?

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

Comment [27]: In the M&M section you describe collecting a lot of data in the questioners, why wasn't it used to detect risk factors? Trimester of pregnancy? Economic status?

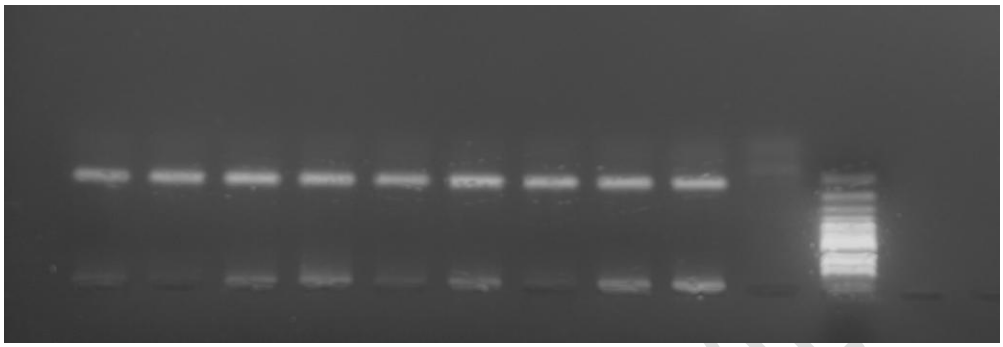

Figure 1. Gel image for amplification human hemoglobin beta (hbb).

This PCR amplifies 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 [28]: Please specify positive and negative controls

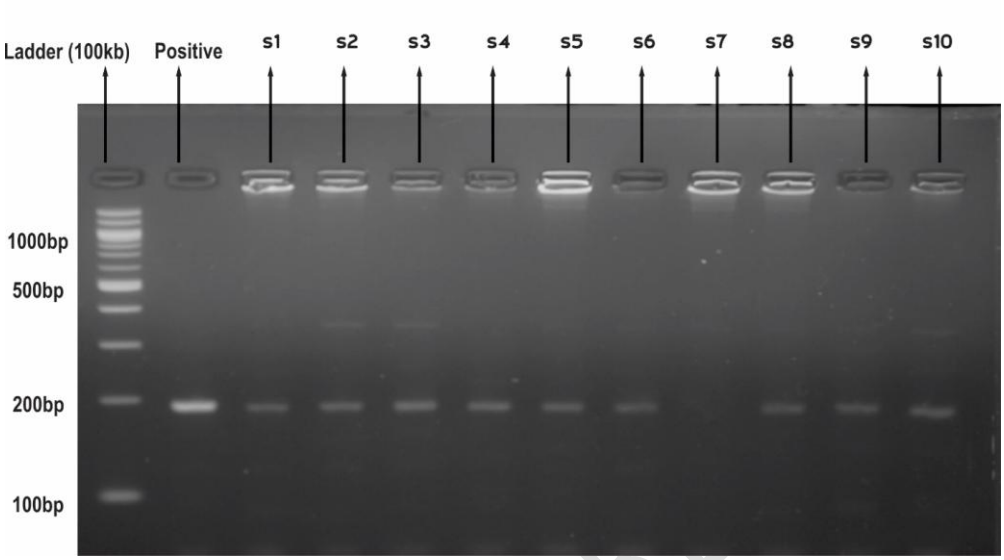

PLATE (I). Gel images of B1 gene for nested PCR showing amplification of a 197 bp fragment in samples 1, 2, 3, 4,5, 6, 8, 9 and 10.

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 B1gene at 197bp

Comment [29]: Negative control is missing

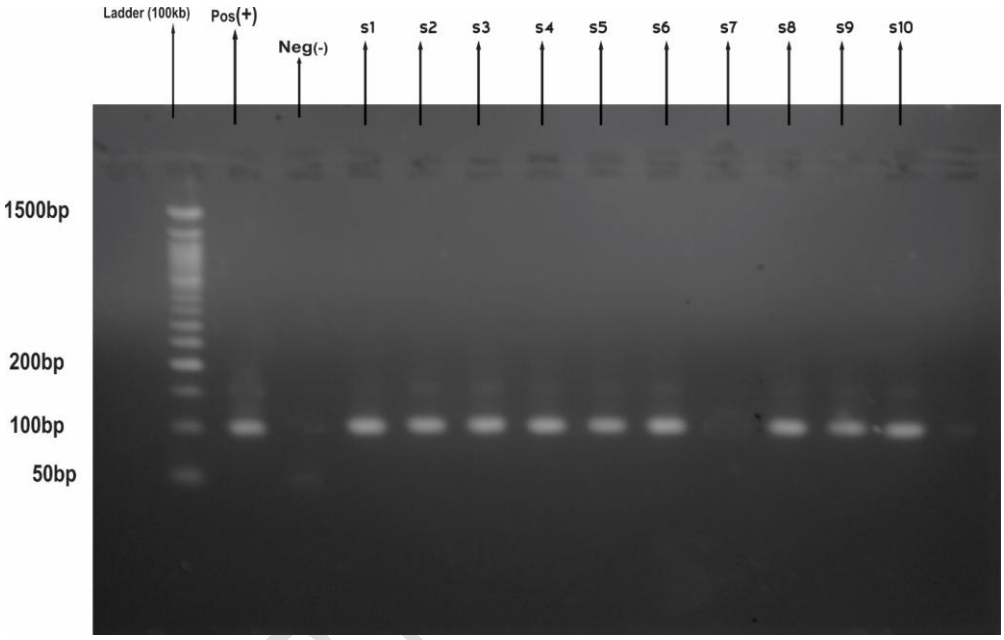

PLATE (II). Gel image of second PCR which amplifies a 97 bp region within the 197 bp region of the B1 gene of *Toxoplasma gondii* amplified during the first PCR.

KEY : LANE 1 = LADDER 100Kb

LANE (POS +) = POSITIVE CONTROL

LANE (NEG -) = NEGATIVE CONTROL

LANE S1-S10 = 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

Table 2. Showing the Primer sequence 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	

DISCUSSION

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 (46). This study ~~observed~~ revealed prevalence of 2.80% ~~prevalence~~ of anti-*Toxoplasmaosis* IgM antibodies in pregnant women, similar to the work that reported 3.9% cases in India, 3.26% in Brazil, 2.6% in Gabon, 2.4% in NewZealand, and 0.8% in Zaria, –(47,30,38,48,49,50,51). The findings in this study ~~is~~ are, however, not similar to studies of 13.08% reported in Kano, 5.2% in Qatar, 11.5% in Portharcout, 11.9% in Trinidad Tobago, 5.4% in Gabon, 7.6% in Lagos, and 7.2% in Maiduguri (52,42,53,54,55). The difference in the various prevalence rates could be due to geographical location, climate condition, and cultural behaviour even within same country because the parasite oocyst sporulation is prevalent in warm and humid condition (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 20%, prevalence in 15-19years 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 variation among the different age groups. Some of which may include the level of maturity, personal hygiene and socio-economic status of the family and even the level of their education because most of this women are under aged, and had teenage marriage which is common in Northern Nigeria.

Several studies have shown that PCR has been consistently used to detect DNA of *T. gondii* in various biological samples due to the fact that is more sensitivity in diagnosis compared to serological tests and culture (57). The first PCR method for *T. gondii* detection, targeting the B1 gene, was established in 1989(44). How ever since then this method has been 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 ~~dectation~~ data reported on toxoplasmosis investigations. In this study however out of 10 samples

Comment [30]: I suggest adding confidence interval, This also helps comparing to the data from the liturature

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that were positive serologically, only 9 that B1 gene *Toxoplasma gondii* DNA was detected and amplified at 97 bp region within the 197 bp region, 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 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.

5.2. CONCLUSION

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

Comment [32]: Same as in the abstract

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ACKNOWLEDGEMENT: This is to acknowledge WHO Measles laboratory at Yusuf Dansoho Hospital Tudun Wada Kaduna State. where the ELISA analysis was done under the supervision of MLS Peter Elisha.