

Recent and past toxoplasmosis infections, associated factors, and awareness among pregnant women in Nigeria

SAGE Open Medicine

Volume 11: 1–8

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DOI: 10.1177/20503121231202230

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Abstract

Objectives: The study determined the rate of recent and past *Toxoplasma gondii* infections, associated factors, and awareness among expectant mothers assessing antenatal care in a tertiary hospital in Nigeria.

Methods: This prospective cohort study was conducted among pregnant women. A questionnaire was used to gather information on socio-demographics, infection risk factors, and awareness of the infection. Using an ELISA kit, the IgG and IgM antibodies against *Toxoplasma gondii* were assessed in blood samples from these women. For samples that tested positive for IgM, a real-time polymerase chain reaction was used to amplify the DNA. SPSS version 23 was used for data entry and analysis. The p -value < 0.05 was adjudged to be significant.

Results: A total of 250 pregnant women participated in the study. The rate of recent infection (IgM antibody positivity) was 3.6% (9/250), while past infection (IgG antibody positivity) was 68.4% (171/250). Polymerase chain reaction confirmed 5/9 recent infections as positive. Factors significantly associated with toxoplasmosis were gardening ($p = 0.037$) and undercooked meat ($p = 0.023$). Only 27 out of 250 pregnant women in this research had heard of toxoplasmosis, which translates to a low awareness rate of 10.8%.

Conclusions: The recent infection among pregnant women in this study indicates the possibility of mother-to-child transmission with attendant sequelae. There was a significant association between past *Toxoplasmosis gondii* infections and stillbirth. Routine screening for toxoplasmosis should be incorporated into the antenatal program since none of the symptoms could significantly predict illness. In addition, regular antenatal care instruction should cover toxoplasmosis education.

Keywords

congenital toxoplasmosis, recent infections, associated factors, awareness, molecular detection

Date received: 20 March 2023; accepted: 31 August 2023

Introduction

Maternal vertical transmission of infections to their newborns contributes significantly to childhood disease burden and associated sequelae of *Toxoplasma gondii* infection, one of the diseases that are transferable from a mother to her unborn baby in utero is estimated to infect one-third of the global population.^{1,2} Toxoplasmosis is a zoonotic and opportunistic infection that affects people worldwide.^{3,4}

Most humans contract *T. gondii* infection through eating raw or undercooked meat that contains tissue cysts, drinking water or food contaminated with oocysts from the soil, transplacental transmission from the mother to the fetus (congenital toxoplasmosis), or, less frequently, through organ transplantation from a seropositive donor, infected blood transfusions, or injuries from needle sticks.⁴

Although the infections are mainly asymptomatic with little complication, maternal transmission of infection to offspring is associated with complications that can lead to

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intrauterine death and other long-term complications in infected babies. Congenital toxoplasmosis is even more severe when contracted in the first or, in rare circumstances, the second trimester.⁵ Congenital *T. gondii* infection causes significant health issues such as mental retardation, blindness, epilepsy, death, and lethal harm to a fetus.⁶ Toxoplasmosis lowers the affected person's quality of life. A transplacental transmission rate of 55% for untreated mothers and 25% for treated mothers has been documented, despite the organism only causing minor or asymptomatic infection in pregnant women.⁵

Congenital toxoplasmosis is estimated to occur in 1.5/1000 live births, and it is estimated that approximately 200,000 neonates are affected by congenital toxoplasmosis annually.³ Strategic approaches to preventing congenital toxoplasmosis include serological screening, which is the primary method of diagnosis for this illness.⁷ To diagnose all recent *T. gondii* infections, polymerase chain reaction (PCR) has been employed to identify *T. gondii* DNA in bodily fluids and tissues. For the diagnosis of this infection, real-time (quantitative) PCR has taken the role of traditional PCR because it makes it easier to quantify the infection burden, gauge the severity of the disease and the effectiveness of the therapy, and act as a prognostic indicator.⁸ *T. gondii* DNA amplification from blood, bodily fluids, placenta, or fetal tissues is diagnostic of acute infection.⁴ Several studies have demonstrated the value of utilizing an ELISA or IgG avidity test in conjunction with a real-time PCR to diagnose congenital toxoplasmosis.⁸⁻¹¹

Primary prevention and anti-parasitic therapy of affected mothers during pregnancy may lower the risk of transmission to the fetus if detected early.^{10,11} Close contact with cats and dogs has been linked to the gestational *T. gondii* infection.¹² Other risk factors include living in areas with flea, cockroach, and rat infestations; poor food storage; low educational attainment; unhygienic living circumstances; and consuming undercooked meat.⁴

One of the first steps in disease prevention should include raising awareness and methods of disease transmission, treatment, and prevention.^{13,14} There is a need for periodic review to generate up-to-date data on the rate of new infections, prevalence, treatment, and preventive strategies, which will provide information for policy formulation geared toward reducing the public health disease burden of congenital toxoplasmosis. This study determined the rate of recent and past *T. gondii* infections, associated factors, and awareness among expectant mothers assessing antenatal care in a tertiary hospital in Nigeria.

Methods

Study design

This prospective cohort study was conducted among women attending antenatal care (ANC) at Nnamdi Azikiwe University Teaching Hospital Nnewi (NAUTH), Anambra, Nigeria.

Pregnant women who attended ANC at NAUTH, Nnewi, between February 2018 and May 2018 were recruited.

However, enrollment was open to all expectant mothers. We used a systematic random sampling strategy to enroll participants who presented for ANC throughout the study until the desired sample size was attained.

Ethical approval was obtained from the institution's research ethics committee (reference number NAUTH/CS/66/VOL.9/99), and the research was conducted in compliance with the declaration of Helsinki.

Approximately 50 participants were recruited each week. Hence, the sample fraction (k) was the number of patients recruited each week/total number of patients who appeared each week, or $50/150 = 1/3$. Thus, $k = 3$. The first subject was chosen randomly from the range of 1 to 3. After that, the researchers recruited every third person into the sample.

The sample size of 217 was calculated using a prevalence of 83% in a previous study,¹⁵ providing a 95% confidence interval and a significance level of 5%. Assuming an attrition rate of 10%, this increased to 241 and rounded to 250.

Pregnant women who consented after counseling were recruited for the study. Women who had underlying diseases or were below the age of 18 years were excluded from the study.

A semi-structured questionnaire was used to collect relevant information from the participants. The information collected includes socio-demographics and associated factors. The questionnaire was pre-tested for clarity, validity, and reliability. In addition, four physicians received training to help with data collection and questionnaire administration.

Serum collection and screening for anti-*T. gondii* IgM and IgG antibodies:

Blood samples were obtained from study participants using a plain tube to draw 5 ml of venous blood. Centrifugation at 2800 gravitational force for 10 min was used to separate the serum from the other components of the blood after it had clotted at room temperature. Clean Pasteur pipettes were used to separate the sera before transferring them into serum containers and frozen at -20°C until processing.

Pregnant women were tested for IgM and IgG antibodies using ELISA test kits (BIOGENIX INC, Lucknow India) per the protocols provided by the manufacturers. Before usage, the developing plates, cards, reagents, and samples were warmed to room temperature between 22°C and 26°C .

After adding diluted serum (1:100) to the wells, they were incubated for 15 min for IgG and 30 min for IgM. Antibodies bind to the antigens immobilized in the wells to form stable complexes, while unbound antibodies were removed by washing them with distillate water. Next, the wells were filled with horseradish peroxidase-labeled anti-human IgG or IgM conjugate, which was then incubated for 15 min for IgG and 30 min for IgM. The conjugate that was not bound was removed using distilled water. After adding the 3,3',5,5'-Tetramethylbenzidine (TMB) enzyme substrate, the plates were left in the dark for 5 min for IgG and 10 min for IgM antibodies. Then using sulfuric acid, the wells'

Table 1. Cross tabulation between IgG and IgM.

IgG (%) <i>n</i>	Variable	IgM (%) <i>n</i> =250		χ^2 -value	<i>p</i> -value
		POS	NEG		
IgG (%) <i>n</i> = 250	POS	9 (100%)	162 (67.2%)	4.313	0.038*
	NEG	0 (0.0%)	79 (32.8%)		
	Total	9 (100%)	241 (100%)		

*Significant *p*-value (<0.05).

POS: Positive/reactive; NEG: Negative/non reactive.

reaction was halted, and the optical densities of controls, standards, and samples were determined with a microplate reader.

Handling of IgM- and IgG-positive results

Each of the women who tested positive for *T. gondii* IgM and IgG antibodies was further counseled individually. Samples were then collected from IgM-positive participants for real-time PCR analysis. They were subsequently commenced on pyrimethamine and sulfamethoxazole as a treatment for the infection and prevention of mother-to-child transmission of the toxoplasmosis infection.

Molecular analysis

All samples that tested positive for IgM antibodies underwent additional molecular analysis using real-time PCR to test for *T. gondii* DNA. For the real-time PCR, the AccuPower Greenstar qPCR Master mix (IONEER Daejeon 34302 Korea) was used.

Using a high-purity extraction kit, DNA was isolated from each of the nine samples.

20 μ l of Proteinase K was added to 1.5 μ l clean tubes, sealed, and vortexed for approximately 10 s, before adding 200 μ l each of serum and buffer to each of the nine tubes, which were then incubated at 60°C for 10 min and briefly centrifuged thereafter, to get rid of any remaining droplets on the lid. Each tube had 100 μ l of isopropanol added to it before being vortexed and centrifuged for a minute. The lysate was then gently transferred to the binding column tubes' top reservoir and centrifuged at 8000 rpm for 1 min to receive all the filtrates in the collection tubes, which were then discarded.

The DNA in the binding column tubes had 500 μ l of washing buffer 1 added to it and centrifuged for 1 min at 8000 rpm. To eliminate all traces of ethanol, 500 μ l of washing buffer 2 was added to the binding column tubes, centrifuged at 8000 rpm for 1 min and then again at 12,000 rpm for 1 min. New collecting tubes were used to elute the tubes from the binding column. After adding 200 μ l of elution buffer, the mixture was centrifuged at 8000 rpm for 1 min after being kept at room temperature for 1 min. Analysis was performed on the genomic DNA that was eluted.

The primer sets TOX-9 5'AGG AGA GAT ATC AGG ACT GTA G nucleotide; positions 143–164 and TOX-11 5'GCG TCG TCT CGT CTA GAT CG nucleotide; positions 304–285 were used for PCR amplification.

In a reaction mixture including 25 μ l of 2X Greenstar master mix and 2 μ l of assay mix of the forward primer (10 pmole), the reverse primer (10 pmole), and the Accupower dye-labeled 50X ROX dye (1 μ l), the clinical samples were added. DNA polymerase was first pre-desaturated (activated/optimized) for 10 min at 95°C. The cycle threshold value (Ct) was then derived after 40 PCR cycles at 95°C for 15 s and 60°C for 45 s.

Handling and follow-up of confirmed who tested positive for IgM antibodies

The results were given to each of the women after individualized counseling. The confirmed positive participants were followed up in the antenatal clinic and through phone calls. They received treatment for toxoplasmosis. The neonates were examined for features of congenital toxoplasmosis.

Statistical analysis

All data were recorded, edited, and entered using Statistical Package for the Social Sciences (SPSS) version 23 (IBM Chicago USA) which was used to analyze the data. Chi-square tests were used to examine the association between categorical variables, and a significance level of *p* < 0.05 was used to indicate statistical significance. Logistic regression was used to determine the independent risk factors.

Results

A total of 250 pregnant women participated in the study. The seroprevalence of IgM antibody positivity was 3.6% (9/250), while 68.4% (171/250) tested positive for IgG antibody. All who tested positive for IgM antibodies also tested positive for IgG antibodies (Table 1).

The result of real-time PCR revealed that amplification of *T. gondii* DNA was positive in 5/9 (55.6%) of the total nine samples positive for IgM antibodies (Table 2). From the real-time PCR result, the prevalence of confirmed acute toxoplasmosis was 2% (5/250).

Table 2. Association between IgG antibodies, IgM antibodies, and socio-demographics.

Variable	IgG		χ^2 -value	p-value	IgM		χ^2 -value	p-value
	n (%) +ve	n (%) -ve			n (%) +ve	n (%) -ve		
18–30 years	89 (67.9)	42 (32.1)	4.054	0.400	5 (3.8)	126 (96.2)	0.39	0.822
31–40 years	77 (70.6)	32 (29.4)			4 (3.7)	105 (96.3)		
>40 years	5 (55.6)	4 (44.4)			0 (0.0)	10 (100.0)		
Occupation			3.51	0.173			0.51	0.773
Unemployed	35 (68.6)	16 (31.4)			1 (2.0)	50 (98.0)		
Semi-skilled	60 (73.7)	19 (24.1)			3 (3.8)	76 (96.2)		
Skilled	76 (63.3)	44 (38.7)			5 (4.2)	115 (95.8)		
Highest level of education			5.68	0.058			0.56	0.754
No formal education	1 (100.0)	0 (0.0)			0 (0.0)	1 (100.0)		
None/primary	6 (85.7)	1 (14.3)			0 (0.0)	7 (100.0)		
Secondary	77 (75.5)	25 (24.5)			3 (2.9)	99 (97.1)		
Tertiary	88 (62.4)	53 (37.6)			6 (4.3)	135 (95.7)		
Trimester			2.179	0.997			1.965	0.374
First	26 (68.4)	12 (31.6)			0 (0.0)	38 (100.0)		
Second	60 (69.0)	27 (31.0)			3 (3.4)	84 (99.6)		
Third	85 (68.5)	39 (31.5)			6 (4.8)	118 (95.2)		
Place of residence			2.399	0.121			2.984	0.084
Rural	56 (75.7)	18 (24.3)			5 (6.8)	69 (93.2)		
Urban	115 (65.7)	60 (34.3)			4 (2.3)	171 (97.7)		

n: number; %: percentage; +ve: positive; -ve: negative.

*Significant p-value (<0.05).

The IgM seropositivity was more prevalent in the 18–30 years age group (5/9), while IgG seroprevalence was highest in the 31–40 years age group (77/171). However, there was no significant association between age and *T. gondii* IgG and IgM antibodies ($p=0.400$ and 0.822), respectively. None of the socio-demographics showed any significant association with the *T. gondii* antibodies, as shown in Table 3. There was no remarkable difference in IgG seropositivity among the three trimesters. An increase in trimester was associated with a corresponding rise in IgM antibody seropositivity, increasing from zero in the 1st trimester to 4.8% in the 3rd trimester. In addition, considering each IgG and IgM seroprevalence, infection rates are higher in women from rural communities than women from urban residences, but this was not statistically significant (Table 3).

The anti-*T. gondii* IgG seropositivity showed a statistically significant association with the risk variables “gardening and consumption of undercooked meat” ($p=0.037$ and $p=0.023$, respectively) (Table 3).

Of the pregnancy-related symptoms of toxoplasmosis analyzed, the experience of stillbirth alone showed a statistically significant association with *T. gondii* IgG antibodies with a p-value of 0.009 (Table 4).

The pregnancy outcome of the nine IgM-positive patients included seven deliveries at term, one premature neonate at 35 weeks, and one intrauterine fetal death (IUFD) at 21 weeks + 5 days. The outcome for the five confirmed PCR-positive patients included: three had term deliveries, one pre-term delivery, and one miscarriage, respectively.

Only 27/250 (10.8%) of these pregnant women had read, heard, or seen any information regarding toxoplasmosis. This is shown as shown in Figure 1. Among the pregnant women who were aware of the infection, a small percentage of them knew that toxoplasmosis can be acquired from contact with infected cats (5.2%), infected cat litter (5.6%), and contaminated water (5.2%) (Figure 2).

Discussion

The seroprevalence of IgM and IgG antibody positivity was 3.6% (9/250) and 68.4% (171/250), respectively, in this study. All who tested positive for IgM antibody also tested positive for IgG. The rate of confirmed acute toxoplasmosis with real-time PCR was 2% (5/250).

Possible acute infection was suggested by a seroprevalence of *T. gondii* IgM antibodies of 3.6% in this study. This finding is consistent with the prevalence rates of 3.9% and 4.2% seen in prior studies conducted using the same methods in India⁷ and Ethiopia,¹² respectively. The seroprevalence of IgM antibody in this study is comparable to results from Sub-Saharan Africa in a meta-analysis by Bigna et al.¹⁶ Lower values were reported from Europe while higher values were reported from the Eastern Mediterranean region.¹⁷ This highlights the heterogeneity of infection and may have been due to a varied presence of risk factors in different regions and countries of the world. It is greater than the 0.8% IgM seroprevalence reported in a previous study from Zaria, Northern Nigeria

Table 3. Risk factors and *T. gondii* IgG seropositivity.

Risk factors	IgG		χ^2 -value	p-value
	n (%) +ve	n (%) -ve		
Indulge in outdoor gardening				
Yes	87 (75.0%)	29 (25.0%)	4.362	0.037*
No	84 (62.7%)	50 (37.3%)		
Eating undercooked meat				
Yes	10 (55.6%)	8 (44.4%)	5.150	0.023*
No	69 (29.7%)	163 (70.3%)		
Washing of hands before eating				
Yes	168 (69.4%)	74 (30.6%)	3.651	0.056
No	3 (37.5%)	5 (62.5%)		
Cat contact				
Yes	12 (66.7%)	6 (33.3%)	0.027	0.870
No	159 (68.5%)	73 (31.5%)		
Cat possession				
Yes	1 (33.3%)	2 (66.7%)	1.727	0.189
No	170 (68.8%)	77 (31.2%)		
Cat litters				
Yes	2 (100.0%)	0 (0.0%)	0.931	0.334
No	169 (68.1%)	79 (31.9%)		
Cat proximity				
Yes	14 (70.0%)	6 (30.0%)	0.026	0.873
No	157 (68.3%)	73 (31.7%)		
Stray cats				
Yes	28 (75.7%)	9 (24.3%)	1.064	0.302
No	143 (67.1%)	70 (32.9%)		
Farm work				
Yes	33 (76.7%)	10 (23.3%)	1.673	0.196
No	138 (66.7%)	69 (33.3%)		
Drinking untreated water				
Yes	105 (69.5%)	46 (30.5%)	0.132	0.716
No	66 (67.3%)	32 (32.7%)		
Sachet water				
Yes	98 (65.8%)	51 (34.2%)	1.072	0.301
No	72 (72.0%)	28 (28.0%)		
Boil, filter, or use a water guard				
Yes	31 (72.1%)	12 (27.9%)	0.282	0.595
No	140 (68.0%)	66 (32.0%)		
Eat unwashed vegetables and fruits				
Yes	39 (66.1%)	20 (33.9%)	0.189	0.664
No	132 (69.1%)	59 (30.9%)		
Sex partners**				
One partner	160 (68.4%)	74 (31.6%)	0.309	0.578
More than one partner	6 (60.0%)	4 (40.0%)		

+ve: positive; -ve: negative.

*Significant p-value (<0.05); **Missing values.

in 2009.¹⁸ This may point to an increasing rate of infection. Although the 2% of confirmed acute infections appears low, it may have significant public health importance considering the high fertility rate in Nigeria.¹⁹ Thus, there is a need to scale up preventive strategies among pregnant women in Nigeria.

T. gondii IgG antibody seroprevalence was found to be rather high (68.4%) among pregnant women in this study. It was difficult to determine whether these infections were in previous pregnancies. However, the significant association with previous stillbirth suggests that some of the women may have contracted the infection in their previous pregnancies. Several

Table 4. Symptoms of toxoplasmosis.

Variable	IgG		χ^2 -value	p-value	IgM		χ^2 -value	p-value
	n (%) +ve	n (%) -ve			n (%) +ve	n (%) -ve		
Fever								
Yes	49 (28.7)	22 (27.8)	0.046	0.831	3 (33.3)	68 (29.5)	0.096	0.757
No	119 (70.3)	57 (72.2)			6 (66.7)	170 (70.5)		
Headache								
Yes	81 (47.4)	36 (46.6)	0.032	0.859	3 (33.3)	114 (47.3)	0.699	0.403
No	90 (52.6)	42 (53.4)			6 (66.7)	126 (52.7)		
Cough								
Yes	53 (31.6)	299 (36.7)	0.747	0.387	3 (33.3)	79 (33.2)	0.001	0.979
No	117 (68.4)	50 (63.3)			6 (66.7)	161 (66.8)		
Miscarriage								
Yes	51 (29.8)	17 (21.5)	2.028	0.154	2 (25)	66 (27.4)	0.024	0.876
No	118 (70.2)	62 (78.5)			6 (75)	174 (72.6)		
Stillbirth								
Yes	29 (17.5)	4 (5.1)	6.751	0.009*	1 (11.1)	32 (13.3)	0.037	0.847
No	141 (82.5)	75 (94.9)			8 (88.9)	208 (86.7)		
Previous baby with an eye infection								
Yes	6 (3.5)	4 (5.1)	0.340	0.560	0 (0.0)	10 (4.1)	0.389	0.533
No	165 (96.5)	75 (94.9)			9 (100)	231 (95.9)		

+ve: positive; -ve: negative.

*Significant p-value (<0.05); **p-value < 0.001

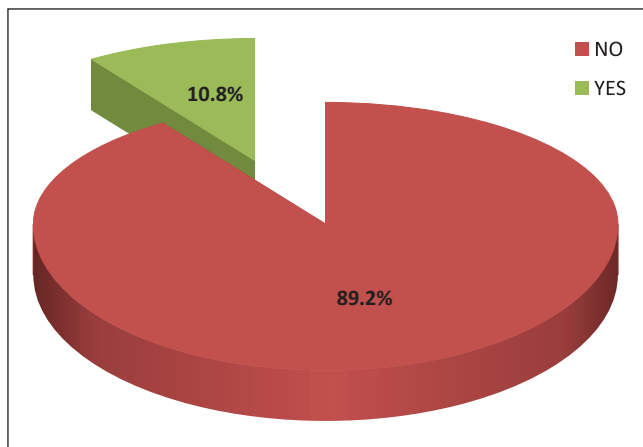


Figure 1. Percentage of pregnant women aware of toxoplasmosis.

epidemiological investigations revealed comparable findings of high prevalence rates in previous studies in Southwest (78%)⁴ and South-South (83%)²⁰ Nigeria. Results from the Southwest, South-South, and our own research in the Southeast may all have been influenced by the same climatic environment in the South, which is ideal for the sporulation of *T. gondii* oocysts. The frequency of IgG in this research, however, is greater than previous estimates from Nigerian authors (29.1%, 32.6%, and 40%),^{18,21,22} This is consistent with results from the meta-analysis that showed an increased rate of infection in recent studies. This call for more vigilance in detecting and treating toxoplasmosis in pregnancy to reduce associated adverse outcome.

This research also found that two major risk factors for *T. gondii* infection were gardening in contaminated soil and eating undercooked meat. Studies conducted in both the United States and India showed comparable results.^{6,12} Safety measures include avoiding contact with stray cats or cat excrement, using gloves when gardening, and washing hands thoroughly after handling raw meat or working in the dirt. Proper cooking of meat should be encouraged to reduce the risk of disease transmission. Although other risk variables were not significant in this investigation, their role in transmitting *T. gondii* infection should also form part of health education.

We observed a significant association between past *T. gondii* infection and history of stillbirth. This is consistent with the observation in the literature.⁴ None of the symptoms of toxoplasmosis has any significant association with either IgG or IgM antibodies. This shows that screening and treatment of the infection cannot be carried out based on clinical symptoms alone.

About 9 in 10 pregnant women (89.2%) were unaware of this parasitic infection. However, 96.3% of those who were aware had completed post-secondary education or above. This study highlights the significance of formal education and literacy in gaining awareness about *T. gondii* infection and how to avoid it.

Pregnant women should have serological screening for *T. gondii* infection as part of antenatal care testing where the prevalence of acute infection in pregnancy is high. Further care should be taken to avoid toxoplasmosis during pregnancy by counseling pregnant women on the risk factors and

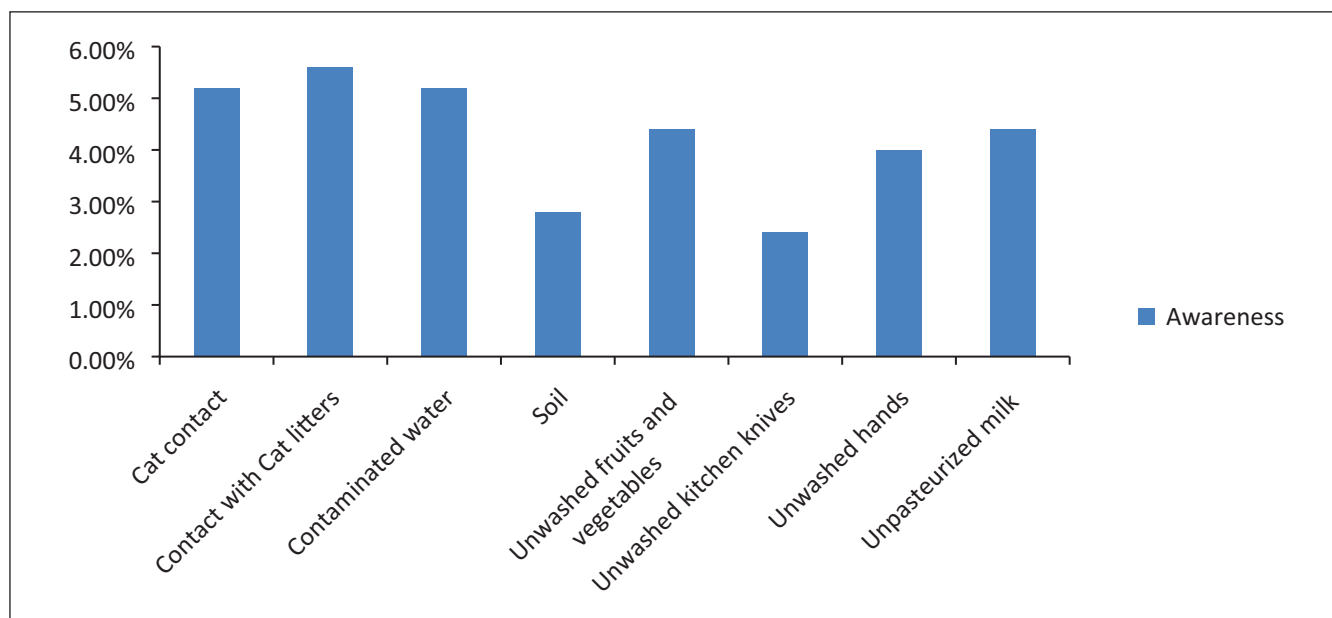


Figure 2. Percentage of women who knew different modes of *Toxoplasma gondii* transmission.

possible routes of transmission. For those who tested positive for *toxoplasma* antibodies, therapy with the necessary medications should be administered. Preventing complications from toxoplasmosis, particularly in newborns, requires raising awareness by educating the public and prompt diagnosis and treatment of infected pregnant mothers.

Because of its high sensitivity and quantifiability, real-time PCR may be useful as an adjunct to ELISA screening, especially as a quality assurance measure to make sure that sensitivity and specificity are optimal. Due to the high cost,²³ using real-time PCR for routine diagnosis of *T. gondii* infection may not be feasible, especially in developing countries. There is also an urgent need to develop IgM antibody rapid point-of-care test kits with high sensitivity and specificity for effective and efficient screening, diagnosis, and treatment, especially in developing countries with high disease burdens.

One of the strengths of this study was the detection of IgM antibodies and confirmation of diagnosis with a real PCR machine. This helped us to rule out false-positive results and avoid unnecessary treatment of the patients. The administration of therapy to the confirmed positive women helped in the reduction of mother-to-child transmission and its complications.

Despite the obvious strengths of the study, it has some limitations. It is a single hospital study and findings should be interpreted with caution.

Conclusion

Acute *T. gondii* infection, which might be passed on to the baby, was found in 2% of the pregnant women in the research. More than two-thirds of the women in the study were positive for *T. gondii* IgG antibody which signified previous infection. There is poor awareness about this illness. Because of the high

seroprevalence and low levels of awareness, preventive actions should include educating the populace in general and pregnant women on modes of prevention of the disease with an emphasis on the risk factors. Prevention of infectious diseases should be the core component of the antenatal care class. There is a need to develop an IgM rapid test kit that helps to scale up the diagnosis and treatment of *T. gondii* infection. Thus, reducing the burden of mother-to-child transmission of the infection and its attendant complications.

Acknowledgements

Nil.

Authors contributions

This work arose from original ideas/concepts by NPO and ACN. All the authors contributed to the study design. Data collection was done by NPO, NC, and OIN while analysis was done by PNO, NC, and MI. The initial draft was done by NPO assisted by NC and edited by MI and ACN. All the authors approved the final draft of the manuscript.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) received no financial support for the research, authorship, and/or publication of this article.

Ethical approval

The institution's ethics review board was obtained with reference number NAUTH/CS/66/VOL.9/99. The study was conducted

within the safe limits of objectivity, integrity, and respect for the subjects used in the study according to the ethical declaration of Helsinki.

Informed consent

Written informed consent was obtained from all subjects before the study.

Trial registration

Not applicable.

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Supplemental material

Supplemental material for this article is available online.

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