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RESEARCH PAPER

PRENATAL DIAGNOSIS AND GENETIC CHARACTERIZATION OF TOXOPLASMOSIS IN IMMUNOCOMPETENT AND IMMUNOCOMPROMISED PREGNANT WOMEN AND THE RISK OF CONGENITAL TOXOPLASMOSIS: A PROSPECTIVE STUDY IN GHANA

Bhavana Singh[1,2*], Linda Batsa Debrah[1,3], Georgina Isabella Djameh[4], Alexander Yaw Debrah[3,5]

[1]Department of Clinical Microbiology, School of Medicine and Dentistry, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana.
[2]University Hospital, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana.
[3]Kumasi Centre for Collaborative Research into Tropical Medicine, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana.
[4]Parasitology Department, Noguchi Memorial Institute for Medical Research, College of Health Sciences, University of Ghana, Legon, Accra, Ghana
[5]Faculty of Allied Health Sciences, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana.

***Corresponding author:** littlebhavanasingh@gmail.com

ABSTRACT:

Background: Toxoplasmosis, a protozoan parasitic disease caused by Toxoplasma gondii is of public health concern. Studies on trans-placental transmission of T. gondii is limited in Africa. This study aimed at assessing the seroprevalence and genetically characterize T. gondii infection among pregnant women.

Methods: HIV-negative pregnant women in their first trimester and HIV-positive pregnant women attending Antenatal Clinic at a University Hospital were screened for anti-Toxoplasma antibodies (IgG and IgM) using ELISA. Multi-locus gene detection was done using nested Polymerase Chain Reaction to target the Surface Antigen Gene 3 and the Dense Granule Antigen protein 6 of T. gondii parasite, followed by sequencing to identify the prevalent T. gondii strain in Ghana.

Results: The prevalence of acute T. gondii infection (ATI) among the HIV-negative pregnant women was 1.5% (6/400), transmission rate was 50% (3/6) and prevalence of congenital toxoplasmosis (CT) was 0.75% (3/400). From HIV-positive women, the prevalence of ATI was 56% (14/25), transmission rate 57% (8/14) and prevalence of CT 32% (8/25). Older aged and unemployed HIV positive women were significantly associated with T. gondii seropositivity (p=0.021) and (p=0.016) respectively. T. gondii DNA was detected in 16.7% (12/72) samples and were clonal type GRA6 type II sequences.

Conclusion: Seroprevalence of toxoplasmosis were higher in HIV-positive pregnant women. The seropositivity to T. gondii specific antibodies in the babies did not fully translate into clinical Toxoplasmosis. The strain identified was the clonal type GRA6 type II sequence. It is important to follow newborn with CT for 10 years for clinical toxoplasmosis.

Keywords: Clonal types, Ghana, Immune compromisedImmune competent, *Toxoplasma gondii*

BACKGROUND

Toxoplasmosis, a protozoan parasitic disease caused by *Toxoplasma gondii*, is of public health concern. The parasite may be acquired through a number of ways including consumption of raw or undercooked meat contaminated with oocyte or tissue cyst as well as ingestion of cat-shed oocysts via contaminated soil, food or water (Pomares and Montoya, 2016). Vertical transmission is another route of acquisition of the disease in pregnancy and the consequence of this is congenital *toxoplasmosis* (CT) (Ayi *et al*., 2016, Smit *et al*., 2017). CT is asymptomatic in most cases but can result in fetal or neonatal death or various congenital defects, such as hydrocephalus, central nervous system abnormalities and chorioretinitis (Hill and Dubey, 2002). There is an estimated global incidence of 1.5 cases per 1000 live births recorded for CT globally, resulting in a burden of 9.6 Disability-Adjusted Life Years (DALYs) per 1000 live births (Torgerson and Mastroiacovo, 2013). In immunocompromised patients such as HIV positive patients, *toxoplasmosis* can cause serious disease especially by reactivation of a latent infection leading to life threatening encephalitis (Lago *et al*., 2009, Montoya and Liesenfeld, 2004).

There are three clonal lineages of *T. gondii*, types I, II, and III previously identified using bi-allelic polymorphism characteristics, which are known to be associated with human *toxoplasmosis* (Howe *et al*., 1997) and lead to different clinical outcomes from asymptomatic, benign, or severe infection of the newborn or even death. The various *T. gondii* clonal types I, II, and III are known to be well associated in primarily diseased humans, domestic animals, and livestock. Type I clonal type, which is known to be very virulent in mice and type II are predominantly associated with human infection. The clonal types circulating in populations in some countries have been determined and hence the severity of the disease and its outcome (Ayi *et al*., 2016, Barragan and Sibley, 2002). Detection of anti-Toxoplasma antibodies is the most widely used approach in diagnosing toxoplasma infection. Classification of detected antibodies is important in determining if the condition is recent or past infection and to estimate the potential risk of CT. The presence of anti-Toxoplasma IgG antibodies represents past infection while the detection of anti-Toxoplasma IgM antibodies indicates recent infection. However, IgM antibodies remain for several months or years after initial infection, hence, poses a limitation in diagnosis as well as misdirection in treatment (Andiappan *et al*., 2014). Women who acquire primary *T. gondii* infection during pregnancy pose a risk for CT.

It is thus important to assess the serological status of pregnant women at the beginning of pregnancy. This procedure is usually not done in most health facilities in developing countries. Seropositivity is regarded as having past/previous infection and therefore considered to have immunity to the parasite; hence reduced risk for CT. Conversely, patients who are seronegative are at greater risk of infection and CT; thus, early identification and prompt preventive measures during pregnancy for seronegative mothers can markedly reduce the risk of infection and CT (Smit *et al*., 2017, Furtado *et al*., 2011).

Several studies on *toxoplasmosis* in humans have been conducted elsewhere (Andiappan *et al*., 2014, Cong *et al*., 2015, El Deeb *et al*., 2012, Mozzatto and Procianoy, 2003, Neto *et al*., 2000); however, in Ghana, there is limited published data (Ayi *et al*., 2016, Ayi *et al*., 2009, Ayi *et al*., 2010, Kwofie *et al*., 2016, Abu *et al*., 2015). Additionally, studies on trans-placental transmission of *T. gondii* is limited in Africa (Simpore *et al*., 2006) and only one has been conducted in Ghana, in the Greater Accra region (Kwofie *et al*., 2016). The limited number of studies conducted in Ghana makes it difficult to formulate and adopt a national policy for *toxoplasmosis* management. Evidence suggests that in addition to host immunity, the genotype of the parasite play key roles in the disease progression, yet studies characterizing *T. gondii* is limited in Ghana. Previous studies in Ghana (and other countries) predominantly utilize serology-based diagnosis (Ayi *et al*., 2016, Ayi *et al*., 2009, Abu *et al*., 2015). Indeed, an advantage of serology-based diagnosis such as the use of ELISA is that it has the ability to detect specific anti-toxoplasma IgG and IgM antibodies with a good accuracy, although skilled personnel is required (Robert-Gangneux and Dardé, 2012). However, complementation with molecular-based detection tools is warranted considering the fact that there is no screening program in place and a higher sensitivity in PCR assays has been observed (Remington *et al*., 2011, Liesenfeld *et al*., 2001). We therefore prospectively assessed the seroprevalence of *T. gondii* infection from first to third trimesters of pregnancy and evaluated the rate of CT at a tertiary care center in the Ashanti region of Ghana using serology, PCR and sequencing. Given that *T. gondii* infection in immuno-compromised patients such as those with HIV/AIDS manifests largely as a life-threatening condition and trans-placental transmission is reportedly increased in pregnant women with chronic or immunosuppressive conditions, we also evaluated *T. gondii* infection and the rate of CT among HIV positive patients. This study therefore, assessed the seroprevalence and genetically characterized *T. gondii* infection among pregnant women and the risk of congenital *toxoplasmosis* in their babies.

MATERIALS AND METHODS

Study Design

This was a prospective study conducted in Kwame Nkrumah University of Science and Technology (KNUST) Hospital, Kumasi from August 2017 to January 2019.

Study setting

The KNUST Hospital is located on the Kumasi Accra high way. It renders services to the university staff, students and its neighboring communities. It also serves as a referral center for other health facilities in the region. It is endowed with healthcare professionals with both in general and specialist care services. It serves an average population of about 180,000 every year.

Study Population

The study included two different cohorts of pregnant women:

The first cohort consisted of 400 healthy pregnant women attending ANC in their first

trimester who were HIV negative. They were selected using simple systematic sampling technique. The sample size was calculated using Fischer's sampling formula ($N = Z^2PQ/d2$), where Z is the critical value of the normal distribution (1.96 at 95% CI); P is the estimated prevalence of *T. gondii* among pregnant women in Ghana (51.2%) (Ayi *et al*., 2016); $Q = 100-P$; and d is the absolute precision or sampling error tolerated = 5%. From the above equation, minimum sample size for this study was 384. However, in an effort to increase the statistical power of the study and also to take care of the drop outs due to the follow ups, 400 consecutive consenting pregnant women attending antenatal care at the KNUST hospital were recruited.

Given the higher rate of *T. gondii* infection among immunocompromised relative to the general population, the second cohort of twenty-five HIV positive pregnant women (HIV positive) in their third trimester were also included. Purposive sampling method was employed for the 25 HIV positive mothers and it was based on getting participants wherever they could be found during their clinic days at the Infectious disease unit at the KNUST hospital. Since they were recruited during their third trimester, purposive sampling method was adopted with no exclusion criteria identified prior to the selection of subjects. All subjects who were HIV positive, and in their third trimester were invited to participate.

Inclusion criteria

Women attending ANC at KNUST hospital and were residing at the study area, women who were expecting to be resident for the duration of the study and women who were eligible for enrollment to sign an informed consent.

Exclusion criteria

Women who did not continue their ANC at the KNUST hospital, and women who tested positive for IgG in the first trimester were excluded from the study.

Serological classification of women for *T. gondii* antibodies

All enrolled women were tested serologically for *T. gondii* IgG and IgM and based on their serological status they were classified accordingly:

1. Women who tested IgG positive and IgM negative in the first trimester were classified as chronic carriers and considered those who cannot transmit the infection to their new born. They were therefore not followed up in the study.
2. Women who tested IgG positive and IgM positive in their first trimester were retested for IgG and IgM in their third trimester to determine their status for acute toxoplasma infection (ATI). Avidity testing was also done on this group at first trimester and those who tested positive for both IgG and IgM at third trimester as well to determine recency of the infection.
3. Women who tested IgG negative and IgM positive at first trimester were retested for IgG and IgM in their third trimester to determine for ATI
4. Women who tested negative for both IgG and IgM in their first trimester were retested in their third trimester to determine seroconversion to ATI

Given the higher rate of *T. gondii* infection among immunocompromised relative to the general population, the second cohort of twenty-five HIV positive pregnant women (HIV positive) in their third trimester were also included. Moreover, latent toxoplasma infection is more prevalent in women with HIV in pregnancy; hence these women with IgG and/or IgM positive were recruited in their third trimester [40]. The women were tested

for *T. gondii* IgG and IgM. Women who tested positive for IgG and/or IgM were classified as women with ATI.

Babies delivered to women having ATI were followed-up at birth and two monthly till 12 months in the case of HIV negative mothers. However, from HIV-positive mothers' babies were followed at birth and every two months till 9 months after birth to evaluate the presence of CT, both serologically and clinically.

Serological classification for CT in the babies was based on presence of *T. gondii* specific IgM and or IgG at birth and persistence of IgG at 9 months (HIV-positive women babies) and 12 months (HIV-negative women babies). Babies with clinical triad of *toxoplasmosis* with or without *T. gondii* specific IgG were also classified with CT.

Clinically, all babies were examined for classical symptoms of CT including retinochoroiditis, intracranial calcifications, microcephaly and hydrocephaly by physical examination, cranial ultrasonography, ophthalmoscopy and psychoneurological assessment by a pediatrician neurologist.

Serological detection of *T. gondii*

Anti-*Toxoplasma* IgG and IgM antibody screening, Vidas testing and *T. gondii* infection classification:

Five milliliters (5ml) of venous blood were collected from all mothers (both HIV positive and negative) into a serum vacutainer. It was then centrifuged at 1500g for 5 mins to obtain serum for specific anti-*Toxoplasma* IgG and anti-*Toxoplasma* IgM testing using commercial ELISA Kit (INVBIO IgG and IgM ELISA, Innovation Biotech Beijing Co. Ltd, China) following the manufacturer's instructions. The absorbance of each well was measured spectrophotometrically at 450 nm using Thermo Electron Multiskan EX plate reader (Shanghai, China).

Women who tested positive for both IgG and IgM and those who seroconverted (HIV negative women) were classified as women with Acute *Toxoplasma* Infection (ATI) (both first and third trimester results for HIV negative women, and third trimester results for HIV positive mothers).

Women who tested positive for IgG only in the first trimester were not considered to have ATI but classified as chronic carriers. Women who were IgG and IgM positive were retested again in their 3rd trimester. An IgG negative and IgM negative test result in the first trimester in a woman who became toxoplasma specific IgG positive and IgM positive test results in their 3rd trimester is indicative for seroconversion. Avidity testing using VIDAS test kit (VIDAS TOXO IgG Avidity (Bio-Meriux France) was also done to measure the strength with which the antibodies bind to the antigens in women who tested positive for both IgG and IgM in their third trimester to determine recency of infection.

All mothers were educated on (congenital) *toxoplasmosis* in newborn. Women who tested negative for *T. gondii* IgG and IgM in their first trimester (for HIV negative group) were advised on measures to be taken to avoid contracting *toxoplasmosis* infection during pregnancy.

Molecular detection and sequencing of *T. gondii*

DNA extraction and purification

Genomic DNA was isolated from the serum samples using the DNeasy® Blood and Tissue Kit (Qiagen). Molecular diagnostics was carried out on 72 patients. These included all 25 HIV positive mothers and their babies, although not all HIV positive mothers were serologically classified as ATI and HIV negative mothers with ATI (that is, 6 mothers with recent infection and their babies), and 5 pregnant women without ATI who tested positive for IgG/IgM

at both first and third trimester but had a high IgG avidity test in their third trimesters and their babies). This procedure was performed on the samples with high avidity to validate the efficacy of the avidity test even though they were serologically classified as women without active *T. gondii* infection.

Genomic DNA was isolated from the serum samples by using the DNeasy® Blood and Tissue Kit (Qiagen) based on the manufacturer's instructions and stored in -20*°C* until used in the PCR procedure

Multi-locus gene detection of *T. gondii* infection by nested Polymerase Chain Reaction

A nested PCR was performed to target the Surface Antigen Gene 3 (SAG 3) and the Dense Granule Antigen protein 6 (GRA 6) of *T. gondii* parasite (I. Ayi *et al*., 2016, Irene Ayi *et al*., 2016). The first step of nested PCR was performed using respective sets of external primers **(Supporting information 1: Table S1)** in a 25 µL reaction volume and the amplification reaction of mixture consisted of 12.5 µL 1X Green Gotaq mastermix®Promega, 0.2 µM each of the forward and reverse primers and 5 µL of DNA sample. Amplification was conducted at 95°C for 4 minutes followed by 25 cycles of 94°C for 30 seconds, 55°C for 1 minute, and 72°C for 2 minutes. The last extension step was at 72°C for 5 minutes. The 2nd step of nested PCR was performed using respective sets of internal primers **(Supporting information 1: Table S1)** in a 25 µL reaction volume and the amplification reaction mixture consisted of 12.5 µL 1X Green Gotaq mastermix®Promega, 0.2 µM each of the forward and reverse primers and 2 µL of PCR product. The amplification protocol was 95°C for 4 minutes followed by 27 cycles of 94°C for 30 seconds,

58°C for 1 minute, and 72°C for 2 minutes. The over-extension step was at 72°C for 5 minutes and PCR products were examined by electrophoresis on 2% agarose gel, stained with ethidium bromide and visualized under ultraviolet light.

Sequencing

The final PCR products from the 12 positive samples were purified and sequenced by macrogeneurope (https://dna.macrogen-europe.com/eng/index.jsp), using the Sanger method. Both forward and reverse orientation cycle sequencing was performed using the amplification primers. The sequences obtained were then analyzed and compared with GRA 6 partial sequences of *T. gondii* available in GenBank and deposited in the GenBank by macrogen-europe (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Data analysis

Categorical data were presented as frequencies and percentages. Flowcharts were used to trace and outline the number of pregnant women tested and their test results at all stages of the study. Continuous data were presented as mean± standard deviation and independent t-tests were used to assess significance of differences between variables. Fisher's exact tests and logistic regression analyses were used to assess the association between sociodemographic and clinical characteristics, medication adherence and *T. gondii* sero-positivity. Statistical analyses and graphical presentations were done using GraphPad Prism 8.0.2 (GraphPad Software, Inc., La Jolla, USA). For molecular and phylogenetic analysis, homologous sequences were downloaded from the GenBank database at the NCBI via BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic tree was constructed using Geneious Prime version 2020.2.4 (www.geneious.com). Multiple sequence alignment was done using Clustal Omega and the tree was built using the neighbor-joining method with maximum

composite likelihood distance correction, with bootstrap consensus tree inferred from 1,000 replicates.

Ethical considerations

Ethical clearance for this study was granted by Committee on Human Research, Publications and Ethics, School of Medical Sciences, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana (Reference number: CHRPE/AP/541/17). This was done after obtaining permission from the management of the study site. The objective of the study was explained to all the women in Twi (local dialet). All the women were screened and those that met the inclusion criteria and accepted to take part in the study were recruited into the study. All the mothers who verbally agreed to take part in the study were made to either thumb print or sign an informed consent form.

RESULTS

Baseline characteristics of the study population

A total of 400 HIV negative and 25 HIV positive pregnant women were included in this study. A higher proportion of the participants were between 30-39 years old (non-HIV:50.7% and HIV: 60.0%), married (non-HIV: 86.8% and HIV: 64.0%), and employed (non-HIV: 86.2% and HIV: 72.0%). Most of the HIV negative women were nulliparous and had tertiary education whereas that for HIV positive women was multiparous and had secondary education (Table 1).

Table 1. Baseline characteristics of the study population

Variables	Non-HIV (n=400); N (%)	HIV (n=25); N (%)
Age (years)		
20-29	182 (45.5)	7 (28.0)
30-39	203 (50.7)	15 (60.0)
≥40	15 (3.8)	3 (12.0)
Parity		
0	158 (39.5)	0 (0.0)
1	90 (22.5)	4 (16.0)
2	94 (23.5)	14 (56.0)
3	42 (10.5)	3 (12.0)
>3	16 (4.0)	4 (16.0)
Marital Status		
Single	46 (11.5)	6 (24.0)
Married	347 (86.8)	16 (64.0)

Separated	7 (1.8)	3 (12.0)
Educational Level		
Primary	26 (6.5)	5 (20.0)
Junior High	98 (24.5)	14 (56.0)
Senior High	96 (24.0)	3 (12.0)
Tertiary	159 (39.8)	2 (8.0)
No formal education	21 (5.3)	1 (4.0)
Employment status		
Employed	345 (86.2)	18 (72.0)
Unemployed	55 (13.8)	7 (28.0)

‡; 39 of the non-HIV group did not report their family size. HIV negative data collection was at first trimester whereas HIV positive data was collected at third trimester.

Figure 1 represents the distribution of 400 HIV-negative healthy pregnant women who were enrolled into the study from the first trimester of their pregnancy. Among them, 229 pregnant women were seropositive for *T. gondii* IgG at first trimester, 11 out of these mothers were also IgM positive. Avidity testing among these 11 women showed high IgG avidity, however, they were still followed up to the third trimester to ensure they were not acutely infected.

At third trimester, five out of the 11 women remained IgG and IgM positive. Avidity testing among these 5 patients showed that all of them had high avidity which indicated lack of ATI and hence low neonatal transmissibility (Fig 1). On the other hand, among the 171 women who were IgG negative at first trimester, 168 were IgM-negative and three were IgM-positive. At third trimester, four of the IgG-ve/IgM-ve became IgG+ve/IgM+ve and two of the IgG-ve/IgM+ve at first trimester became IgG positive at 3rd trimester. Thus, among the 400 pregnant women, 6 pregnant women had ATI by the third trimester, suggesting the potential of high neonatal transmissibility (Fig 1).

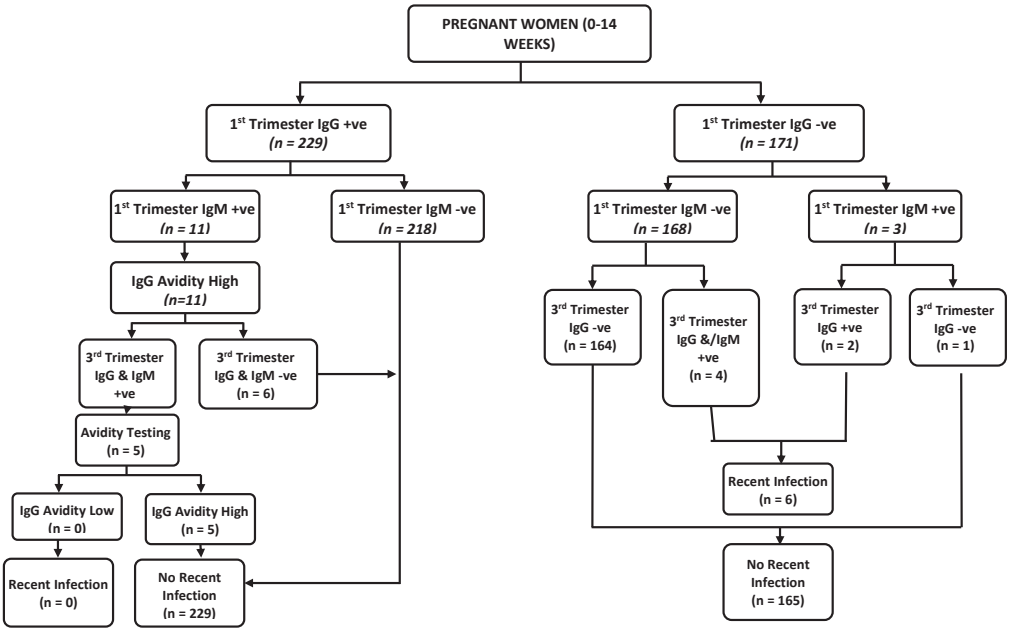

Figure 1. Flowchart showing Acute Toxoplasma Infection (Recent infection) prevalence among the HIV negative women

The prevalence of *T. gondii* IgG+/IgM+ antibodies was 1% (4/400), whereas, IgG+ antibodies was 0.5% (2/400) amongst the HIV-negative mothers at third trimester with an overall ATI prevalence of 1.5% (6/400) in this group (Figure 2). These mothers were advised regarding the available treatments covered by the national health insurance scheme. They however did not opt for the treatment.

Among the HIV positive mothers, from the 25 HIV positive pregnant women in their third trimester examined, the overall prevalence of *T. gondii* IgG antibodies were 56.0% (14/25). Among the IgG positive participants, 24.0% (6/25) were IgM positive. Hence, the prevalence of both IgG+/IgM+ antibodies was 24%, whereas, IgG+ antibodies only was 32% with an overall ATI prevalence of both IgG and/IgM was 56% (14/25) in the group (Fig 3).

Out of the 400 HIV negative pregnant women, three pregnant women (0.75%) bore babies that were serologically positive for *T. gondii* IgM antibodies. All three pregnant women who transmitted the *T. gondii* infection to their babies were among the 6 women who had acute *T. gondii* infection in the third trimester indicating a transmission rate of 50.0% (90% CI, 10.3%146.1%). However, follow-up on the babies at 2-10 days till 12 months post-delivery revealed that none of them had clinical signs of *T. gondii* infection including psychoneurological and ophthalmological signs.

However, the prevalence of *T. gondii* IgG antibodies among babies of HIV positive

women after 9 months of follow-up was 32.0% (8/25). This comprised 4 babies from the HIV positive pregnant women that were *T. gondii* IgG+ve/IgM+ve and another 4 babies

from those that were IgG+ve/IgM-ve with a transmission rate of 57%. However, none of the babies in this group also showed clinical signs of *T. gondii* infection after 2-10 days till 9 months follow-up post-delivery. All women with ATI, who delivered newborn diagnosed with CT were advised regarding the available treatments covered by the national health insurance scheme for their new born diagnosed with CT. They however did not opt for the treatment.

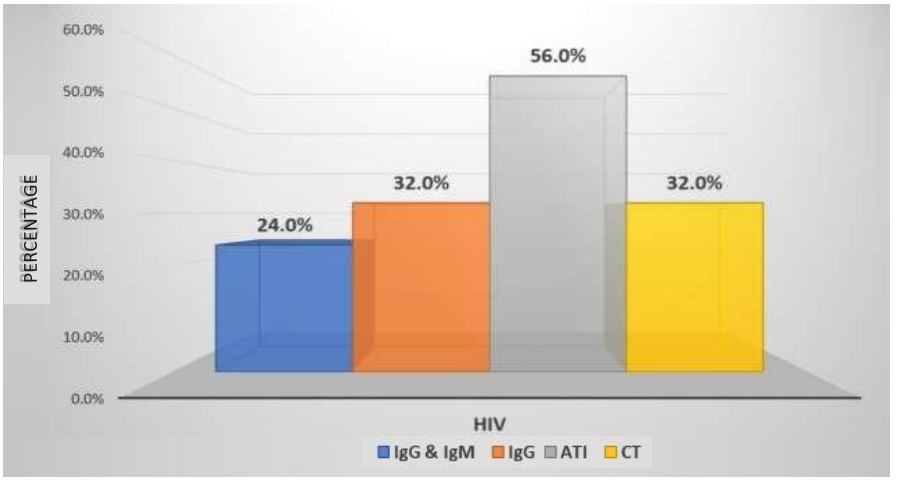

Figure 2a Prevalence of *T. gondii* IgG and IgM antibodies in pregnant women, ATI and Congenital Transmission among the newborn babies of HIV negative at 12 months

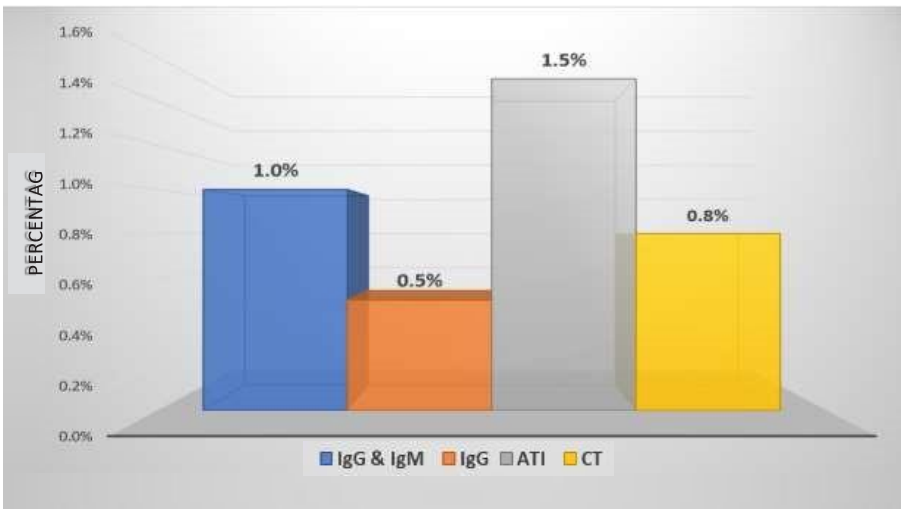

Figure 2b Prevalence of *T. gondii* IgG and IgM antibodies in pregnant women, ATI and Congenital Transmission among the newborn babies of HIV positive women at nine months

Age-specific seroprevalence of ATI among HIV-negative and positive pregnant women

The prevalence of *T. gondii* infection was statistically significant to age of pregnant women for HIV positive group (p=0.021) (Table 2).

Table 2. Age-specific seroprevalence of *T. gondii* infection among HIV-negative and positive patients

Variables	Proportion
Age-stratified Sero-prevalence HIV positive group (14/25)	
20-29	57.1% (4/7)
30-39	53.3% (8/15)
40 and above	66.6% (2/3)
HIV negative group (6/400)	
20-29	1.0% (2/182)
30-39	2.0% (4/203)
40 and above	0.0% (0/15)

Risk factors associated with *T. gondii* sero-positivity among HIV positive pregnant women

Older participants and those who were unemployed had a higher chance of being infected with *T. gondii* (p=0.021; p=0.016) (Table 3). There was little evidence to suggest that higher viral load increases the chance of a participant being infected with *T. gondii*. From the other variables there is no association between viral load and *T. gondii* infection. Nevertheless, it was identified that, two out of three women with viral loads of RNA copies more than 1000 copies/ml, gave birth to babies with CT confirmed serologically.

Table 3. Risk Factors associated *T. gondii* sero-positivity among HIV positive pregnant women

Variables	Sero-negative	Sero-positive	COR (95% CI)	p-value
Age (yrs)	28.87±4.64	34.71±5.84		**0.021***
Parity	2.18±0.88	2.50±1.07		0.432*
Family size	4.06±0.90	4.00±1.20		0.892*
Marital status				
Single	10 (58.8)	6 (75.0)	1	
Married	4 (23.5)	2 (25.0)	0.83 (0.12-6.01)	0.857 [a]
Separated	3 (17.7)	0 (0.01)	0.23 (0.01-5.23)	0.357 [a]
Educational level				
Primary	4 (23.5)	1 (12.5)	1	
Junior High	8 (47.1)	6 (75.0)	3.0 (0.26-34.19)	0.376[a]
Senior High	2 (11.8)	1 (12.5)	2.0 (0.08-51.59)	0.676 [a]
Tertiary	2 (11.8)	0 (0.0)	0.60 (0.02-20.98)	0.778 [a]
No formal education	1 (5.9)	0 (0.0)	1.00 (0.02-40.27)	1.000 [a]
Employment status				
Employed	15 (88.2)	3 (37.5)	1	
Unemployed	2 (11.8)	5 (62.5)	12.50 (1.60- 97.65)	**0.016 [a]**
Access to pipe-borne water				
Yes	10 (58.8)	5 (62.5)	1	
No	7 (41.2)	3 (37.5)	0.86 (0.15-4.89)	0.86 [a]
Access to human waste disposal facility				
Yes	10 (58.8)	4 (50.0)	1	
No	7 (41.2)	4 (50.0)	1.43 (0.26-7.73)	0.679 [a]
Pets at home				
Yes	7 (41.2)	2 (25.0)	1	
No	10 (58.8)	6 (75.0)	2.1 (0.32-13.61)	0.437 [a]
Contact with cats				
Yes	3 (17.6)	1 (12.5)	1	
No	14 (82.4)	7 (87.5)	1.5 (0.13-17.18)	0.744 [a]

Eat raw vegetables				
Yes	15 (88.2)	5(62.5)		
No	2. (11.8)	3(37.5)	4.50 (0.57-35.15)	0.152 [a]
Eat uncooked meat				
No	15 (88.2)	8(100.0)		
Yes	2 (11.8)	0(0.0)	0.36 (0.02-8.51)	0.530 [a]
Missed ART				
Yes	6 (35.3)	1 (12.5)	1	
No	11 (64.7)	7 (87.5)	3.82 (0.37-38.83)	0.258 [a]
Viral load	5887.0±0.0	1746.0±458.21		0.086*
Co-infections	0(0.0)	0(0.0)		NA
Co-morbidity	0(0.0)	0(0.0)		NA

*: Independent t-tests; NA: not applicable, a Analyzed using Fischer`s exact

Characteristics of New Born with Congenital *T. gondii* seropositivity

The birthweight of the babies born to women without HIV were all normal weight. The birthweight of 37.5% of babies born to women with HIV were low birth weight. There was no statistically significant relationship between the birth weight of the baby and the two groups (p = 0.491). The hemoglobin levels of babies born to women without HIV were all within the normal range (Hb=/more than12g/dl). The hemoglobin levels of 37.5% of babies born to women with HIV were within the moderate anemia range. There was significant difference between the hemoglobin levels of babies and the two groups (p = 0.491) (Table 4). The other parameters were all normal for both group of babies were normal. With respect to babies born to HIV positive pregnant women, there was a statistically significant relationship between the birth weight and seropositivity of the baby born to women with HIV (p = 0.024). The hemoglobin levels of 37.5% of babies born to women with HIV were below the normal range (Table 5). There was significant difference between the hemoglobin levels of babies and their seropositivity between the HIV positive group (p = 0.024) (Table 4.8). All the other parameters were normal for both groups (Table 5).

Table 4: Neonatal parameters of Newborn with Congenital *T. gondii* seropositivity

Variables	Congenital *toxoplasmosis* (HIV Positive group)	Congenital *toxoplasmosis* (non-HIV group)	P – value
Hemoglobin level of baby (g/dl)			0.491
Anemic	3 (37.5)	0 (0.0)	
Non- Anemic	5 (62.5)	3 (100.0)	
Birth weight of baby (Kg)			0.491
Low birth weight	3 (37.5)	0 (0.0)	
Normal birth weight	5 (62.5)	3 (100.0)	
Jaundice at birth			
No	8 (100.0)	3 (100.0)	
Seizures at birth			
No	8 (100.0)	3 (100.0)	
Seizures in infancy			-
No	8 (100.0)	3 (100.0)	
Splenomegaly at birth			-
No	8 (100.0)	3 (100.0)	
Hepatomegaly at birth			-
No	8 (100.0)	3 (100.0)	
Petechiae skin rash at birth			-
No	8 (100.0)	3 (100.0)	
Lymphadenopathy at birth			-
No	8 (100.0)	3 (100.0)	
Ophthalmoscopy			
0-7 days, Normal	8 (100.0)	3 (100.0)	-
3 Months, Normal	8 (100.0)	3 (100.0)	-
Motor development			
1 month, Normal	8 (100.0)	3 (100.0)	-
6 months, Normal	8 (100.0)	3 (100.0)	-

9 months, Normal	8 (100.0)	3 (100.0)	-
Hearing assessment			
6 months, Normal	8 (100.0)	3 (100.0)	-
9 months, Normal	8 (100.0)	3 (100.0)	-
Hydrocephalus at birth			-
No	8 (100.0)	3 (100.0)	
USG- trans cranial at 3 and 6 months			-
Normal	8 (100.0)	3 (100.0)	

Table 5: Neonatal parameters of Newborn with Congenital *T. gondii* seropositivity in HIV positive group

Variables	**Sero-negative (n=17)**	**Sero-positive (n=8)**	**P-value**
Hemoglobin level of baby (g/dl)		**0.024**	
Anemic	0 (0.0)	3 (37.5)	
Non-Anemic	17 (100.0)	5 (62.5)	
Birth weight of baby (Kg)			**0.024**
Low birth weight	0 (0.0)	3 (37.5)	
Normal birth weight	17 (77.3)	5 (62.5)	
Other parameters			
Jaundice at birth	None.	None	
Seizures at birth	None	None	
Seizures in infancy	None	None	
Splenomegaly at birth	None	None	
Hepatomegaly at birth	None	None	
Petechiae skin rash at birth	None	None	
Lymphadenopathy at birth	None	None	
Ophthalmoscopy 0-7 days	Normal	Normal	
Ophthalmoscopy 3 Months	Normal	Normal	
Motor development 1 Month	Normal	Normal	

Motor development 6 Month	Normal	Normal
Motor development 9 Month	Normal	Normal
Speech development 6 months	Normal	Normal
Speech development 9 months	Normal	Normal
Hearing assessment 6 Months Hearing assessment 9 months	Normal Normal	Normal Normal
Hydrocephalus at birth USG- trans-Intracranial at 3 and 6 months	None Normal	None Normal

Molecular detection of *T. gondii* infection and characterization of *T. gondii* infection

From 72 participants which included 25 HIV-positive women and their babies and 11 HIV negative women and their babies (6 with recent infection and 5 who tested positive for IgG/IgM at both first and third trimesters), *T. gondii* DNA was detected in 12 (16.7%) samples using by GRA 6 gene marker. No positive sample was detected by the SAG 3 gene. Among the 12 positives, seven were from HIV positive women; two were from HIV-negative women and three babies from the HIV positive mothers (Fig. 4 and Table. 6). They were followed over 9 months period and no clinical manifestation of CT was noted in any of them.

Table 6. *T. gondii* diagnosis classification based on serological and molecular method

Diagnosis of *T. gondii* infection	HIV-Positive Women	HIV-negative Women
Serological diagnosis Mothers	14/25 (56%)	6/400 (1.5%)
Confirmation via Molecular test in mothers	7/14 (50%)	2/6 (33.3%)
Serological diagnosis of CT in newborn	8/25 (32.0%)	3/6 (50%)
Molecular diagnosis of CT in newborn	3/25 (12%)	0/6 (0%)

Table 6 displays the classification of ATI in pregnant women and CT in newborn based on the two methods used, the serological method, ELISA and molecular based PCR method. ATI was serologically diagnosed in 56% in HIV positive women whereas, only 50% percent of these women remained positive when confirmed using the molecular detection via PCR method. Similarly, only 6 (1.5%) out of 400 HIV negative women were serologically positive and out of the 1.5%, only 33.3% of the women showed positivity using the molecular method of testing.

With regards to CT, 32.0% of newborn were positive for CT using the serological method (i.e., *Toxoplasma* specific IgG and IgM antibodies positive at birth and every two monthly till 9 months of age when there was persistence of IgG without any treatment for *toxoplasmosis*) for newborn of HIV positive women, when only 12% (3/25) of them showed positivity using the molecular

method. However, 3 babies were diagnosed with CT in newborn of the HIV negative group serologically, and none (0%) showed positivity using the molecular technique.

Sequence analysis of the GRA 6 gene for the 12 PCR-positive samples confirmed the presence of *T. gondii*. The sequences ranged between 309 and 2726 bp. BLAST of all 12 sequences for homologous sequences from the GenBank resulted in a high identity hit (% identity: 93-100%) with the reference sequence (Accession number: XM_002371898.2). The phylogenetic analysis of our sequences with reference sequence revealed that sample 1-4, 6, 9-11 form one large monophyletic group. Sample 7 and 8 were more related with each other whereas sample 12 shared high homology with the reference sequence. All the isolates were evolutionarily close to the GRA6 type II sequences (XM_002371898.2) (Fig 4; Supporting information 2: Table S2).

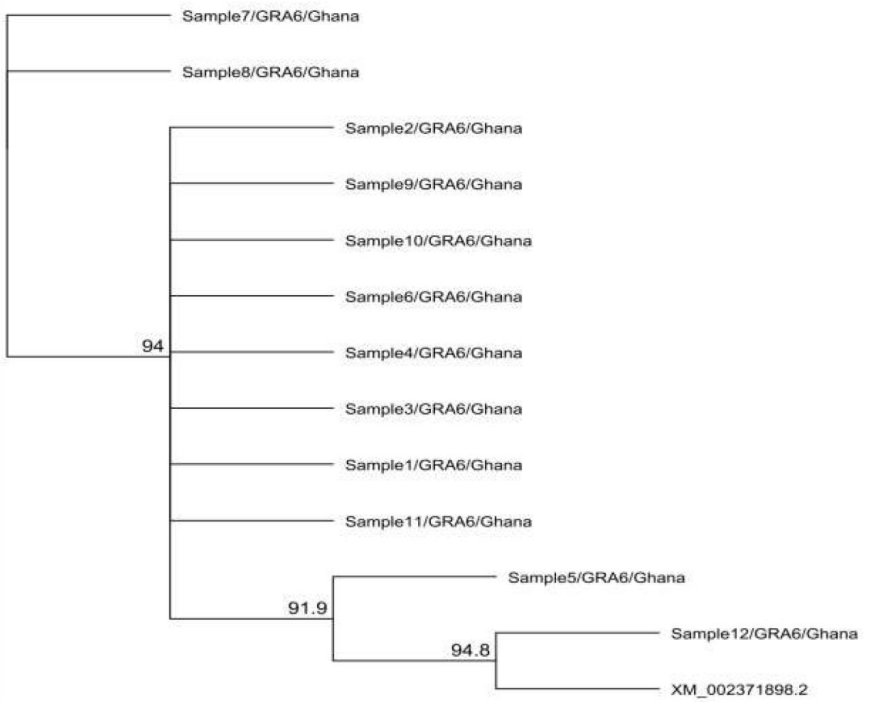

Figure 4: Sequencing and phylogenetic analysis of the GRA 6 gene

DISCUSSION

The prevalence of acute *Toxoplasma* infection (ATI) in pregnant women worldwide is 1.1% with a higher prevalence in low-income countries and the lowest in the European region (Rostami *et al*., 2019). As consistent with the global picture, the prevalence of ATI among HIV-negative pregnant women found in this study was 1.5%. On the contrary, Ayi and colleagues in Ghana did not find any woman with both IgM and IgG in their study (I. Ayi *et al*., 2016). The prospective study design employed in the current study as well as the bigger sample size could have accounted for the relatively high prevalence. Ayi and colleagues did not follow their study participants for seroconversion. Moreover, a sample size of 400 was used in the current study compared to 125 could also be a reason (I. Ayi *et al*., 2016). On the other hand, a study in coastal Ghana by Abu *et al*.(Abu *et al*., 2015) reported an IgM+/IgG+ *T. gondii* seroprevalence of 5.0%. The higher prevalence could be due to the differences in the geographical location since the predominant profession for people from coastal Ghana is associated with high exposure to soil, a risk to *T. gondii* infection (Abu *et al*., 2015, Ayi *et al*., 2016). Moreover, the general prevalence of *T. gondii* infection in the coastal region of Ghana has been consistently high (Ayi *et al*., 2009, Kwofie *et al*., 2016). In other regions of the world, varying seroprevalence rates have been reported. For example, in Eastern China, the prevalence is 2.9% (Cong *et al*., 2015, Aqeely *et al*., 2014, Chaves *et al*., 2008). We attribute these differences to several factors such as differences in study design, test methods, geographical and climatic factors which are known to influence *T. gondii* seroepidemiological studies (Afonso *et al*., 2013).

If left undiagnosed and untreated, ATI can lead to CT with significant morbidity and mortality of the fetus (Thalib *et al*., 2005, Gras *et al*., 2004). Serological detection of specific IgM and IgA anti-*T. gondii* antibodies and the persistence of specific IgG after nine months to one year in a child antibody have been considered an indicator of diagnostic method for CT (Singh, 2016, Hohlfeld *et al*., 1995, N Hezard, 1997, Torgerson and Mastroiacovo, 2013b). The 0.75% prevalence of CT in the current study is thus low but consistent with a study in Trinidad by Adesiyun *et al*.(Adesiyun and Ramsewak, 2007) who also reported as low as 0.4% prevalence of CT. This finding suggests that serology-based *T. gondii* diagnosis of mothers does not fully translate into an increased risk of maternal-fetal transmission of *toxoplasmosis*. Our finding of 50% transmission rate was comparable to the 57.5% *T. gondii* infection in infants reported in Accra-Ghana by Kwofie *et al*. (Kwofie *et al*., 2016). Though the seroprevalence indicated by Kwofie *et al*. was based on only anti-*T. gondii* IgG detection at two weeks and six weeks postnatally, the prevalence in this study was based on testing positive for both anti-*T. gondii* IgG and IgM and following their serological status till 12 months of age; thus, the conservative criteria used in this study could account for the lower seroprevalence observed. Studies in different parts of the world have reported different prevalence rates for CT. In Brazil, the prevalence of CT was reported to be 0.08-1.2% (Neto *et al*., 2000, Mozzatto and Procianoy, 2003). In another study in Poland, Paul reported the prevalence of *Toxoplasma*-specific IgA and/or IgM in filter-paper specimens at birth of 1 per 929 live-born infants (1.08/1,000) and 1 per 523 deliveries (1.9/1,000) (Paul, 2007). In India, a prevalence rate of 22.4% (8.8-37.3%) has been reported with an overall IgM positivity of 1.43% (Singh, 2016). The differences in the prevalence of CT across different countries could be due to several factors including the differences in the number of women who become pregnant without previous *T. gondii* infection the level of exposure to *T. gondii* during pregnancy and

differences in the specificity and sensitivity of the test methods used. *T. gondii* infection is reported to be more common in hot climates and at lower altitudes than in cold climates and mountainous areas (McAuley, 2014), as such, differences in a geographical location with different environmental conditions could influence the rate of infection and consequently, the possibility of CT.

In some countries, serologically-positive pregnant women are advised to abort the pregnancy for the fear of the child developing clinical symptoms such as retinochoroiditis, intracranial calcifications, microcephaly and hydrocephaly. However, this study showed that serologically determined CT may not lead to clinical CT. Upon clinical examination; none of the newborns had clinical CT as they did not exhibit the classical symptoms of CT up to a year of follow-up in HIV negative women group and till 9 months follow-ups in the HIV-positive women group. Cranial ultrasonography and ophthalmoscopy were all normal and the babies presented normal neuropsychomotor development. Nonetheless, given that the annual incidence of CT is estimated to be 190,100 cases (179,300–206,300), accounting for 1.2 million DALYs each year (Torgerson and Mastroiacovo, 2013a) and the asymptomatic presentation of ATI in most pregnant women as noted in this study, the adoption of universal prenatal screening strategies will facilitate early diagnosis and treatment in the majority of ATIs during pregnancy.

Since *toxoplasmosis* in immuno-compromised patients manifests primarily as a life-threatening condition and trans-placental transmission is reportedly increased in pregnant women with chronic or immunosuppressive conditions (Shimelis *et al*., 2009), the prevalence of *T. gondii* among HIV positive pregnant women and factors determining their seropositivity and the rate of congenital transmission in this group was accessed. These mothers were included during their third trimester because latent *toxoplasmosis* is more common in HIV positive pregnant women as compared to acute *toxoplasmosis* (Nourollahpour Shiadeh *et al*., 2020) and followed their newborn from birth to nine months postnatally. The overall *T. gondii* IgG antibody positivity was 56% (14/25). This finding is similar to a study among HIV-positive patients by Bavand *et al*. (Bavand *et al*., 2019) who reported a 46.3% prevalence of *T. gondii* IgG positivity in Iran. Also consistent with our findings, previous reports indicate higher *T. gondii* IgG than IgM positivity in HIV (Bavand *et al*., 2019, Nissapatorn, 2011). Of importance, out of the total 14 women, who were positive for IgG antibodies, a total number of 8 babies who were all seropositive for *T. gondii* IgG were reported at birth and persistence of IgG at 9 months post-delivery. Of the 14 mothers, 6 mothers were also positive for IgM antibodies and 4 out of these 6 mothers gave birth to babies (4/8) who were positive for IgG antibodies at birth and at 9 months post-delivery. Out of the eight babies, none of them showed clinical signs of CT which reaffirms our previous deposition that serologically determined CT may not translate to clinical CT. The prevalence of CT in the current study, is generally low. In a study by Lago *et al*. (Lago *et al*., 2009b), only one infant among babies born to 103 HIV-positive pregnant women had CT, by a woman who had received prenatal and intrapartum ART had CT. This child was positive for *T. gondii*-specific IgG at a low concentration (40 UI/mL) and negative for *T. gondii*-specific IgM. *T. gondii*-specific IgG remained positive for over 12 months, and increased after the discontinuation of treatment for *toxoplasmosis*, reaching 941 UI/mL at 17 months. In keeping with previous reports, there is not enough data to conclude that the risk of vertical transmission of *T. gondii* infection in HIV positive pregnant women is high that translates to clinical CT in their babies (Lago *et al*., 2009b); although

there is a widespread impression that the risk is high (Helfgott A, 1999, Azevedo *et al*., 2010).

Lago *et al*. (2009) also found a significant relationship in the HIV pregnant women with a viral load greater than 2,000 viral RNA copies/ml having a two times greater probability of having *toxoplasmosis* with high *T. gondii*-specific IgG concentration greater than 300 UI/mL (prevalence ratio 2.0, 95% CI 1.0–4.2). Although, our study there was not significant evidence to suggest that HIV mothers with high viral load have a high risk of acquiring *toxoplasmosis* infection, all 3 women out of 25 with a viral load of RNA copies 1000/ml were positive for *T. gondii*-specific IgG.

Another finding of this study is that HIV-positive patients with *T. gondii* infection were unemployed and may have been involved in other house chores that might have put them at a higher risk of acquiring *toxoplasmosis*. Furthermore, the prevalence of *toxoplasmosis* was higher among older pregnant women. Although there are conflicting reports (Rostami *et al*., 2019), this finding is in harmony with a study by Anuradha and Preethi in India who found the seropositivity of *T. gondii* to be most common in older participants (Anuradha and Preethi, 2014). Also in line with our findings is a study by Meisheri *et al*. who showed that the highest prevalence of *T. gondii* occurs in the third and fourth decade of life (Meisheri, Mehta and Patel, 1997). The relationship between increasing age and *T. gondii* prevalence could be due to the increased risk of exposure with age (Anuradha and Preethi, 2014, Basavaraju, 2016).

It is known that in some cases, serology-based diagnosis of infections is not robust enough; hence there is the need to use other tools such as molecular-based detection methods which is more sensitive and specific. Increased risk of *T. gondii* infection in the immunocompromised and the likelihood of vertical transmission (Bavand *et al*., 2019, Nissapatorn *et al*., 2004, Nissapatorn, 2011, Helfgott A, 1999, Anuradha and Preethi, 2014) is evident in from the current study where 7 pregnant women out of the 14 with *T. gondii* positive outcomes using PCR-based diagnosis tool were from HIV-positive women and three babies out of the eight babies with *T. gondii* also belonged to babies from HIV-women group. This corroborates the previous reports of BLAST analysis revealing no significant differences with already published sequences. All of the isolates shared high similarity with the GRA 6 type II sequence (XM_002371898.2) with percentage identity ranging from 93.0 to 100.0%. This was not surprising since the clonal type II strain is frequently associated with disease in humans and it has been identified in Ghana as well as from human samples in Europe (Howe *et al*., 1997, Ajzenberg, 2015, Khan *et al*., 2005, Galal *et al*., 2019). Moreover, the abundance of type II strains has similarly been reported among immune compromised individuals from Europe and the USA and most often associated with human *toxoplasmosis* (Howe *et al*., 1997, Sibley and Ajioka, 2008, Howe *et al*., 1997). Given that the type II strain has been implicated in severe *Toxoplasma* encephalitis in murine models (Araujo and Slifer, 2003), there is the need to follow up with the children at specific intervals to assess the development of late sequel of the disease. It is also important for the diagnostics in the country to consider molecular-based assessments following serological diagnosis.

The strength of this study is in being the first study to prospectively evaluate the *toxoplasmosis* and the possibility of CT using both serological and molecular diagnostics in the Ghanaian population. However, some limitations need to be acknowledged. First, this study was conducted at a single center and the findings might not be generalizable to other areas of the country. Population-based multicenter studies are highly recommended. We could not report analysis for risk factors for toxoplasma seropositivity among HIV negative group due to the limited number of positive

cases. The follow-up of the newborn of the HIV positive women group was also done for 9 months after birth although the newborn from HIV negative women group was followed for 12 months after birth, due to time constraints with the study. However, several studies have diagnosed CT based on serological antibody test at both 9 months and 12 months after birth, hence, the diagnostic criteria still fall within the scientific time frame.

CONCLUSION

This study found a low sero-prevalence of *toxoplasmosis* among both HIV positive and negative pregnant women in Kumasi-Ghana. The sero-positivity to *T. gondii*-specific antibodies did not fully translate into CT. The GRA6 type II strain is the most prevalent in Kumasi. The prevalence of congenital *toxoplasmosis* in an infant born to an HIV positive mother is higher than those born to HIV negative mothers; however, the clinical manifestation of congenital *toxoplasmosis* was absent in all babies. Because, type II strains are the most prevalent cause of human *toxoplasmosis* both in congenital infection and HIV patients, and usually present asymptomatically at birth, it is important to follow newborn with CT up to 10 years of their lives for neuropsychological and ophthalmological complications later in their lives.

Given that the prevalence of serology-based CT was common among the HIV- positive cohort, future studies may need to focus more on people with immunocompromised states, such as people living with HIV.

DATA AVAILABILITY

The datasets used for this study is available upon request from the corresponding author.

CONFLICTS OF INTEREST

All authors have declared no competing interest exist for this study.

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AUTHORS' CONTRIBUTIONS

This study was conceptualized by BS, LBD, and AYD. BS, LBD, AYD, and GID were involved in data collection, formal analysis, report writing. All authors reviewed were involved in the review.

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SUPPORTING INFORMATION

Supporting information 1 Table S1. Summary of primer sequences used.

Supporting information 2 Table S2. Sequence analysis of the GRA6 gene for each sample

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