

Seroprevalence of low avidity anti-*Toxoplasma* IgG in pregnant women and its relationship with their age and contact with cats

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Abstract. *Toxoplasma gondii* is a protozoan parasite that can infect all mammals, serving as intermediate hosts. The cause of congenital toxoplasmosis is transplacental transmission of the parasite to the foetus, resulting in wide range of manifestations from mild chorioretinitis to miscarriage. Its frequency can be reduced by early screening of pregnant women which is based mainly on tests for anti-*Toxoplasma* antibodies. We collected serum samples of 594 pregnant women (subjects) after taking their consent over a period of two years (2016-2018) and analyzed them for anti-*Toxoplasma* IgG by ELISA. The positive samples were then analyzed for IgG avidity test which could differentiate between recent and past infections. The seroprevalence was also correlated with the age of the subjects and their contact with cats. 162 subjects were found positive out of which only three showed a recent infection. After following up until delivery, one of them delivered a baby who had jaundice and was diagnosed with anti-*Toxoplasma* IgM at birth. The foetus of the second subject died *in-utero*, while the third woman delivered a normal baby after being given spiramycin when diagnosed with toxoplasmosis in the first trimester. It was found that most of the positive subjects had frequent contact with cats. Invasion of the parasite during third trimester resulted in death *in-utero* and jaundice. Most common cause of pregnancy wastage during our study was spontaneous abortions while pregnancy loss due to congenital anomalies was rare.

INTRODUCTION

It is known that *Toxoplasma gondii* which is an obligate intracellular protozoan parasite is able to infect all warm-blooded animals who serve as intermediate hosts and almost one-third of world's human population (Wam *et al.*, 2016; Iddawaela *et al.*, 2017; Khan and Khan 2018). The disease caused by this parasite is called 'toxoplasmosis' which is associated with congenital anomalies (Hoffman *et al.*, 2012). The definitive hosts of the parasite are felids in which oocysts are produced and defecated out. Oocysts are resistant against extreme environmental conditions and become highly infectious after getting matured in soil under favourable

conditions (Puvanesuaran *et al.*, 2012; Yolken *et al.*, 2017). Once the oocysts are ingested by mammals, they undergo development and finally give rise to tachyzoites which get propagated through the blood and consequently reach all tissues mainly the muscles, eyes, CNS and placenta thereby infecting them (Many and Koren 2006; Khan and Khan 2018). Humans can get the infection of *T. gondii* through two most common routes: either through ingestion of oocysts in water or food contaminated with infected cat's feces or through the ingestion of undercooked or raw meat infected with tissue cysts. If the infected individual is a pregnant woman, this infection can be transmitted through the placenta to the unborn developing foetus

thereby obstructing its growth. When *T. gondii* crosses the placenta and reaches the foetus, it may cause congenital toxoplasmosis in the foetus. Women who acquire toxoplasmosis during pregnancy can transmit infection to their foetus through the placenta as the tachyzoites are capable of bypassing the placental blood barrier and attack the foetal organs. Tachyzoites proliferate and interfere with the developmental process in the foetus which results in foetal loss, repeated abortions and stillbirths in pregnant women. Thus, it becomes necessary to assess the immunological status of pregnant women against toxoplasmosis as consequences of congenital infection are devastating (Malarvizhi *et al.*, 2012; Sarkar *et al.*, 2012; Malik *et al.*, 2014; Sarkari *et al.*, 2014). Congenital toxoplasmosis may be present without signs or symptoms, or present with clinical symptoms like intrauterine growth restriction, hepatosplenomegaly, jaundice (Mohanty *et al.*, 2012) or neurological and ocular manifestations like hydrocephalus, microcephaly, seizures, epilepsy, intracranial calcification, mental retardation, chorioretinitis and blindness (Rodrigues *et al.*, 2014). Other modes of infection include organ transplantation (Lopes *et al.*, 2019) and blood transfusion (Amoo *et al.*, 2019).

In the United States, where toxoplasmosis is considered as the second most important cause of foodborne deaths, 22.5% of the 12 years and older population is infected with *Toxoplasma gondii* (Hökelek *et al.*, 2017). This parasite is widespread throughout the world with incidence as high as 95% in some populations. Areas that are situated at low altitudes and have hot and humid climates witness highest infection rates (CDC 2015). The precise seroprevalence of *Toxoplasma gondii* infection in India is unknown. Even so, with the advent of various diagnostic tests, it is documented to be as low as 5% and as high as 80% in adults (Singh 2003). As per the recent reports of World Health Organisation, the incidence of congenital infection depends on the infection pressure of that particular area i.e., when the infection pressure is high, maximum women are exposed to the infection and become seropositive and

when they reach the child-bearing age, they become less susceptible to the infection as they have already been exposed. When the infection pressure is low, the opposite is the case. Hence, we can say that although seronegative women are at a greater risk, the actual population that is at risk is low overall (WHO 2013; Khan and Khan 2018).

Our present study aims at finding out the seroprevalence of low avidity anti-*Toxoplasma* IgG in the blood samples of pregnant women attending the antenatal clinic of Jawaharlal Nehru Medical College, Aligarh and correlating its relationship with their age and contact with cats and to understand the antagonistic role of progesterone towards chronicity and tissue damage during pregnancy.

MATERIALS AND METHODS

The protocol was ethically approved by the Institutional Ethics Committee, Faculty of Medicine, Jawaharlal Nehru Medical College. All experiments were carried out in compliance with the relevant laws and guidelines, in accordance with the ethical standards of the Declaration of Helsinki.

Five hundred and ninety four blood samples of pregnant women were collected in sterile vials over a period of two years i.e., 2016 to 2018. An informed consent was obtained from each subject after explaining them the purpose of the study. Data regarding their medical history and socio-economic background were also obtained. The subjects were placed under different age groups viz., 21-25 years, 26-30 years, and 31-35 years. The blood samples were centrifuged at 2000 rpm for 5 minutes to extract the serum which was stored in numbered aliquots at -20°C till assayed.

ELISA was performed to find out the seroprevalence of anti-*Toxoplasma* IgG using human anti-*Toxoplasma gondii* IgG ELISA kit (ab108776, Abcam, Massachusetts U.S.A.). The assay was performed according to the manufacturer's instructions. A standard curve was plotted and the concentration of the immunoglobulin of unknown samples was determined by extrapolation.

All serum samples found reactive were subjected to the avidity assay using *Toxoplasma gondii* IgG avidity test kit (TOXGA460 NovaTec, Novalisa, Ukraine) and the assay was performed according to the manufacturer's instructions.

Calculations

Equation for straight line i.e., $y = mx + c$ which was obtained after plotting the standard curve gave the IgG concentration of unknown samples. Serum samples with concentration values exceeding 35 IU/mL were considered reactive.

The avidity results were calculated by the given formula:

$$\text{Avidity} = \frac{\text{[Absorbance (sample or control) Avidity reagent]}}{\text{[Absorbance (sample or control) washing buffer (diluted 1:19)]}} \times 100$$

The results were interpreted as:

Avidity% >40 → High avidity → Past infection.

Avidity % ≤40 → Low avidity → Acute or recent infection.

Those subjects who were found positive for low avidity anti-*Toxoplasma* antibodies were followed up until delivery and information about their contact with cats was also obtained.

RESULTS

All the subjects were placed under three age groups i.e. 21-25, 26-30 and 31-35 years. The overall age distribution of the subjects has

been depicted in Figure 1. One hundred and sixty two serum samples out of 594 were found reactive for anti-*Toxoplasma gondii* IgG indicating 27.27% positivity (Figure 2).

The correlation between the subjects' age and the seroprevalence of *T. gondii* infection is summarized in Figure 3. The seroprevalence of the immunoglobulin was also correlated with the contact of positive women with cats and it was found that the maximum number of subjects who were positive for IgG were among those who had frequent contact with stray or a pet cat at home and the data was compared with that of seronegative pregnant women (Figure 4).

STATISTICS

The data regarding the seroprevalence among different age groups were analyzed by chi-square test where the χ^2 value was found to be 15.99 while the P -value was 0.0003 (<0.05). Therefore, we conclude that the difference among the three age groups has a very high statistical significance. Moreover, the data regarding the contact of seropositive subjects with cats also turned out to be significantly different among the three categories [$\chi^2=204.89$, P -value <0.00001].

The above data gives us an idea that out of the 162 subjects found positive for anti-*Toxoplasma gondii* IgG, only 3 were found to have acquired a recent infection. It was found that out of these 3, one woman whose blood sample was collected in the third trimester delivered a baby who had jaundice at birth and was diagnosed positive for anti-*Toxoplasma* IgM even after treatment with

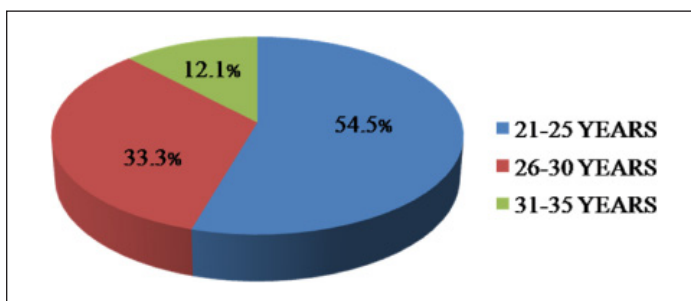


Figure 1. Proportions of the subjects in different age groups.

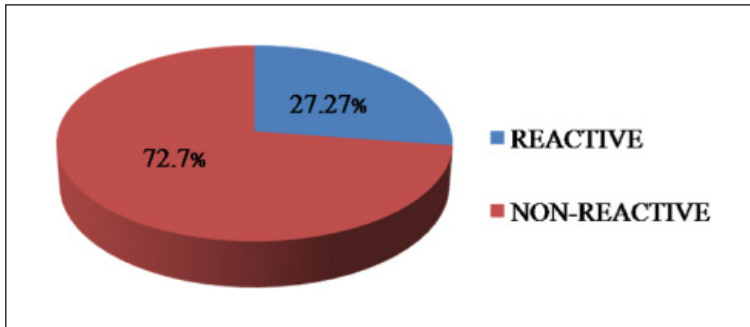


Figure 2. Seroprevalence of anti-*Toxoplasma* IgG in pregnant women.

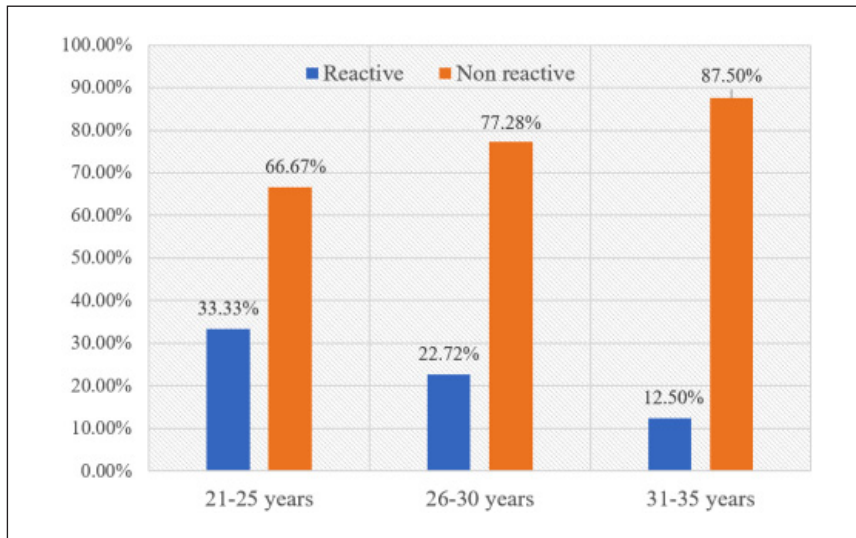


Figure 3. Seroprevalence rates among women of different age groups.

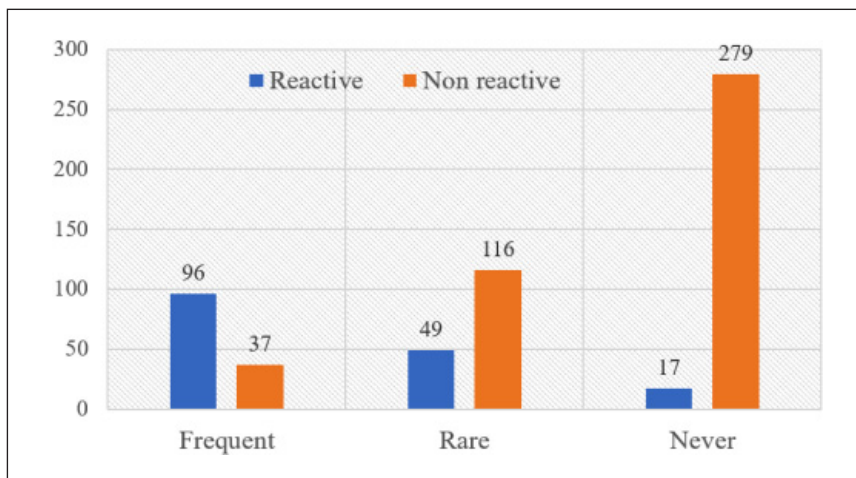


Figure 4. Seropositivity and seronegativity in pregnant women against cat contact.

Table 1. Avidity of anti-*Toxoplasma* IgG among the seropositive subjects and their pregnancy outcomes

Sl. No.	Age	Contact with cats	IgG Concentration (IU/ml)	Avidity (%)	Fetal outcome
1.	35	Frequent contact with stray cats	154.271	38.50	The new born baby was diagnosed positive for anti- <i>Toxoplasma</i> IgM and jaundice.
2.	24	Frequent contact with stray cats	146.054	29.36	Intra-uterine death of the foetus.
3.	25	Contact with a pet cat	156.762	34.67	Full term normal delivery (FTND)

pyrimethamine and sulfadiazine. In case of the second woman who was also diagnosed in the third trimester, the foetus died *in-utero* while the third woman delivered a normal baby whose diagnosis was done in the first trimester and treatment of spiramycin was given after diagnosis. The result of the avidity test and the pregnancy outcome is summarized in Table 1.

DISCUSSION

Toxoplasmosis is a global infection which varies widely based on social and cultural habits, climate, geographic factors and most importantly, the route of transmission. Studies have suggested that toxoplasmosis is more prevalent in warm and humid areas (Studeníèová *et al.*, 2006; Wam *et al.*, 2016). The seroprevalence of IgM and IgG found in the first nationwide survey in humans in India was 2% and 24.3%, respectively. This also varied with the geographical location with the highest prevalence rate in Southern India and the lowest in the Northern India (Dhumne *et al.*, 2007). Since highest humidity in Aligarh prevails during the months of July to September, it favours the survival and proliferation of *T. gondii* oocysts (Singh *et al.*, 2014).

Our result showed that 27.27% of the subjects with recurrent pregnancy wastage were reactive for anti-*Toxoplasma* IgG antibodies. Out of these, 66.66% were between 21-25 years of age, while 27.77% belonged to 26-30 years age group and 5.55% were under 31-35 years of age. This result was in

consensus with that obtained previously from India (Siddiqui *et al.*, 2014a) and Brazil (Spalding *et al.*, 2005) where the seroprevalence rates for the age group 21-30 years were 73.9% and 74.5%, respectively. However, a few studies have reported that the prevalence of *T. gondii* infection increases with age and the reason was thought to be increasing risk of exposure (Morris and Croxson 2004; Hung *et al.*, 2007). In Windhoek, the seroprevalence of anti-*T. gondii* IgG among urban pregnant women who attended public antenatal care in 2016 was very low (2.61%) (Van der Colf *et al.*, 2020) while in Almadinah Almunawwarah, it was found to be 21.3% (Imam *et al.*, 2016). The seropositivity of toxoplasmosis in pregnant women with bad obstetric history was studied in a tertiary care hospital in Andhra Pradesh, India by ELISA and found that there is a gradual increase in the seroprevalence with advancing age (Sarkar *et al.*, 2012). However, we have contradictory results showing that most of the pregnant women who were found positive for anti-*Toxoplasma gondii* IgG were aged between 21 and 25 years followed by women under the age group 26-30 years, while lowest seroprevalence was found in women under the age group 31-35 years. This result can be validated by the fact that most of the married women below 25 years of age came from rural background with poor hygiene facilities and had frequent contact with stray cats. Moreover, after comparing the contact of cats with seropositive and seronegative women, we found that most of them who were positive for anti-*Toxoplasma*

IgG had frequent contacts with cats (stray or pet), while most of those found negative for the same did not have contact with cats at all, gives us a convincing evidence that there is a direct established relationship between toxoplasmosis and the contact of the subjects with cats. In addition, the statistical analysis of our results showed that toxoplasmosis is dependent on the age of the subject as well as on the contact of the intermediate host (in this case, pregnant woman) with the definitive host i.e., cat. Earlier studies conducted in Aligarh (Siddiqui *et al.*, 2014b) and Tamil Nadu (Malarvizhi *et al.*, 2012) also showed similar results. Borkakoty *et al.* (2007) reported higher prevalence rate in people who had low socio-economic status in Assam which was seconded by Mohammed (2011) who reported that prevalence of *T. gondii* infection decreases with the adoption of better hygiene measures. The seroprevalence of *T. gondii* infection in Indian women of child-bearing age and its association with the social and environmental factors in the 4 different regions of the country was studied by Singh *et al.* (2014) who concluded that the prevalence was the highest in South India (37.3%) and the lowest in Western India (8.8%), while the overall prevalence rate was 22.4%. As there is a significant variation in the environmental and climatic conditions of these two regions, it can be said that *T. gondii* infection varies more with the environmental factors than with the age of the subject. Studies conducted in the central province of Northeast India (Borkakoty *et al.*, 2016) and Sri Lanka (Iddawela *et al.*, 2017) suggest that the prevalence of IgG is higher in hilly regions. In a study aimed to gather the presently available intercontinental data on *T. gondii* prevalence to analyze global trends, it was observed that the global prevalence of *T. gondii* infection varies significantly with no certain inter- and intra-continental trends which may be because of a complex interaction between evolving environmental and human factors (Molan *et al.*, 2019). Van der Colf *et al.* (2020) suggested that risk factors and seroprevalence of *T. gondii* lack a significant association. Their study also concluded that there is no association

between consumption of unwashed vegetables or fruits and seropositivity of *T. gondii* infection since 3.45% of the subjects who denied of consuming unwashed vegetables and fruits were found seropositive. Molan *et al.* (2020) also did not identify any risk factor associated with the seroprevalence of *T. gondii* infection.

It has been reported that immune status that develop in response to acute *T. gondii* infection and that during pregnancy are antagonistic to each other (Borges *et al.*, 2018). The pathogen-associated molecular markers present in *T. gondii* such as profilin, GIPLs (Hunter and Sibley 2012) and HSP70 (Ibrahim *et al.*, 2014) interact with Toll-like receptors (TLRs-2, 4 and 11) present on the host cells. It also has cyclophilin 18 which can bind to CCR5 resulting in the activation of neutrophils, dendritic cells and macrophages and this leads to the production of IL-12 which in turn stimulates natural killer (NK) cells to produce IFN- γ (Borges *et al.*, 2018). The combined action of IFN- γ and IL-12 results in the differentiation of Th1 cells which again secrete IFN- γ and favor the activation of M1 macrophage. These macrophages produce inducible nitric oxide synthase (iNOS) which in turn produces nitric oxide (NO) that is known to inhibit essential enzymes of the mitochondria and nucleus of the parasite that results in killing of tachyzoite stage of the parasite (Pittman and Knoll 2015). Nonetheless, excessive Th1 response results in tissue damage which is the reason behind pathology (Borges *et al.*, 2018). It has been studied that Th2 cells and Treg cells are mainly associated with reducing inflammation during toxoplasmosis. Progesterone, which is an important maternal hormone is secreted by the breasts, endometrium, ovaries and feto-placental unit. It regulates certain immune cells so as to maintain pregnancy (Borges *et al.*, 2018). It induces stimulation of progesterone-receptor-positive lymphocytes which in turn produce progesterone-induced blocking factor (PIBF). It is the PIBF that downregulates activity of NK cells and favours the expansion of Th2 cells. Treg cells play a key role in immune regulation, development of the

The primary method of determining toxoplasmosis is the detection of *Toxoplasma*-specific antibodies, the most common of which are enzyme immunoassays and immunofluorescence assay for IgG and IgM. However, determining the time of infection during pregnancy is of utmost importance in order to prevent foetal infection. Tests for the detection of immunoglobulins (IgM and IgG) produced against *T. gondii* are most commonly used to determine whether a pregnant woman has acquired acute infection (Khan and Khan 2018). If there is a low IgG titre and a negative IgM test in the first 24 weeks of gestation, then the infection is essentially acquired prior to gestation. Usually, detection of anti-*Toxoplasma*-specific IgM antibodies is a precise indicator of a recent infection nevertheless; false-positive IgM antibody test results have also been reported in earlier studies that is why anti-*Toxoplasma* IgG avidity test is more reliable for the diagnosis of a recent infection (Gras *et al.*, 2004; Iqbal and Khalid 2007). It helps to differentiate between recent and past infections as the net antigen binding force of the antibody strengthens gradually by antigen driven B-cell selection. The antibody-antigen complex is dissociated by protein denaturing agents, including urea. The ratios of antibody titration curves of urea treated and untreated samples are used to determine the avidity result (Iqbal and Khalid 2007). In our study, we found that only 3 (1.85%) out of the 162 seropositive subjects had low avidity IgG while the remaining 159 (98.15%) had high avidity antibodies indicating towards past infection. We also found that mother-to-child-transmission (MTCT) of *T. gondii* took place in women who were diagnosed in the third trimester. However, the third subject in our study was diagnosed in the first trimester and was immediately advised to take spiramycin which is known to prevent congenital transmission of the infection. Fortunately, she delivered a normal baby who was tested negative for anti-*Toxoplasma* IgM and IgG. Studying the avidity of IgG in pregnant women in whom seroconversion has taken place during gestation showed that high avidity test results were associated with *T. gondii* infections acquired for at least 3-5 months.

In order to offer the pregnant women early therapy or other interventions to check congenital infection, the early diagnosis in the first trimester is of great importance (Emna *et al.*, 2006; Reis *et al.*, 2008; Khan and Khan 2018). Moreover, the avidity test in women during the first 16 weeks of gestation decreased the need of unnecessary treatment with spiramycin and other drugs thereby reducing the cost of the treatment as earlier observed by Siddiqui *et al.* (2014b). Hence, the *Toxoplasma*-specific antibody status of a woman before or during the first trimester of pregnancy is desirable to know. In case of toxoplasmosis, routine serological tests are highly sensitive; nevertheless, specificity varies depending on the test used. It has been documented in the earlier studies that VIDAS avidity test gives highly sensitive and specific results which make it easier to detect a recently acquired infection in IgM positive cases (Petersen *et al.*, 2005).

Toxoplasma gondii is known to cause devastating effects if transmitted transplacentally to the foetus; therefore, its timely diagnosis during gestation is of great importance. As it is asymptomatic in immunocompetent individuals, its diagnosis in pregnant women becomes difficult, thereby increasing the chances of its transmission to the foetus. It is therefore, necessary to educate women of childbearing age about the risks associated with undercooked meat and soil contact. They also need to be made aware of the safe handling of cat litter during pregnancy. In our study, most common cause of pregnancy wastage was spontaneous abortions while pregnancy loss due to congenital anomalies was rarely obtained.

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Authors' Contributions

K. Khadija conceived and carried out all the experiments and prepared the manuscript; K. Wajihullah reviewed the manuscript; K. Tamkeen helped in collecting blood samples; G. Naaz prepared the table and graphs. N. Ansari and A. Sana helped in the preparation of manuscript.

Ethical approval

The protocol was ethically approved by the Institutional Ethics Committee, Faculty of Medicine, Jawaharlal Nehru Medical College. All experiments were carried out in compliance with the relevant laws and guidelines, in accordance with the ethical standards of the Declaration of Helsinki. All the participants provided written informed consent to participate in the study and to have their information obtained from treating physician.

Conflict of interest

All authors declare that there is no competing interest.

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