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Microscopic and ELISA Based Detection of Toxoplasma and its Associated Risk Factors in Pregnant Women of District Mardan

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Toxoplasmosis caused by the protozoan parasite Toxoplasma gondii, the most prevalent disease worldwide. The infections in human are extremely dangerous if they happen during pregnancy because they may lead to miscarriage or congenital abnormalities, which predominantly affect the central nervous system. The main objective of current study was to identify the risk factors for toxoplasmosis and its prevalence among pregnant women in Mardan. The blood serum was tested using the ELISA and the latex agglutination test. Through interviews, sociodemographic and prospective risk factor data were gathered using a standardized questionnaire. Our findings, obtained from ELISA and latex agglutination tests, showed considerably higher toxoplasmosis rates of 23% and 11%, respectively. The geographic distribution of toxoplasma infection in district Mardan revealed a high prevalence in Shah Baig village through ELISA (28%) and latex test (12%) followed by Ibrahim Khan Killi (24%) and (16%), while lower cases were recorded through ELISA (20%) in Sheikh Yousaf Killi and Saleem Khan Killi. Compared to working women in the community (5.85 percent), the infection was more prevalent (18%) in non-occupational women/house wives. Moreover, it was also observed that abortion occurred to the woman who have been clinically diagnosed positive by ELISA and it reported 20% by ELISA and 11.4% by latex agglutination test. It was concluded that T. gondii infection were more prevalent in pregnant women of rural areas of Mardan as compared to the urban centers. Women and children were more vulnerable if they frequently interact with domestic animals and play in contaminated environment.

Keywords: Abortion; Diagnosis; Mardan; Prevalence; Toxoplasmosis.

Almost one-third of the world's population suffers from toxoplasmosis [1]. The primary causes of this disease include eating raw meat contaminated with *Toxoplasma gondii* oocysts, drinking water contaminated with feces that have been released, and genetic infection [2]. Prenatal infection is the most dangerous consequence of toxoplasmosis in pregnant women, with a global incidence rate of over 200,000 infections each year [3]. If a mother becomes infected with *T. gondii* in maternity, particularly during the initial phase, the parasite is suspected of slipping through into the perinatal boundaries and triggering miscarriage or unexpected abortion in the mother and fetus [4]. There are numerous factors that can lead to abortion, including genetics, anatomical anomalies,

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endocrine disorders, rheumatic syndromes, and infections [5]. The main cause of spontaneous abortion is the infection caused by *T. gondii* [6, 7]. Previous researchers reported a link between toxoplasmosis and spontaneous abortion.

Toxoplasmosis can be diagnosed using either serological techniques to identify particular antigens in specimens or the molecular approach to isolate parasitic genome from the samples [8, 9]. Traditional epidemiological techniques, including ELISA, indirect immune fluorescent tests, and immunoblotting, can identify toxoplasmosis immunological response of immunoglobulin M and immunoglobulin G responses. The S-F dyeing check, as well as testing for particular IgA and IgM, such as additional immunoglobulin, are accessible for clarification of preliminary serological testing [10, 11]. The indirect haemagglutination test approach, which uses solubilized *Toxoplasma* antigen, renal excretion antigens, transgenic antibodies, or refined genotypes of *T. gondii*, is the most widely used immunological technique for detecting particular antibodies in blood serum [12]. These analyses provide a lot of false detection findings, particularly for IgA and IgM antibodies, which makes diagnosing recurrent and inherited infections difficult [13]. The advent of extremely sensitive and repeatable techniques using antiserum has been intensively explored in addition to enhancing toxoplasmosis diagnosis. Prepared Monoclonal antibodies that identify parasitic variants can be employed as other antibodies attached to styrene sheets for the extraction of pathogen specific ligands in *T. gondii* extracts, inside a variant of the traditional Immunoassay. This method has recently been used to detect specific antibodies to SAG1 antigenic as well as other *T. gondii* antigens in blood samples of pregnant women [14].

The development of an accurate early detection method is essential for the successful treatment of toxoplasmosis. Identifying the pathogen is the most consistent toxoplasmosis diagnostic technique, although it is expensive, time-consuming, and overly sensitive. When comparing to indirect immunoassay tests, which is utilized as a standard tests, the Immunoassay for identifying pathogenic organisms' antibodies was shown to be extremely sensitive and specifically in detecting Toxoplasma antibodies [15]. Immunoglobulin G (IgG) are commonly used to identify *Toxoplasma* infections, although IgG antibodies can last a lifetime in immune-competent *T. gondii* people. As a result, the test is unreliable when used to distinguish between contemporary and distal infections. Furthermore, it may be inconsistent in individuals who have preexisting illnesses that cause reduced immunogenicity or who are on immune suppression treatment [16]. Antibodies for IgM class in toxoplasmosis patients were found to rise at three months and last for till six months. The high level of particular IgM might last for few years in certain cases. The appearance of the IgM does not imply that an illness has occurred recently [17]. Specific antibody detection revealed a poor prognostic validity for initial *T. gondii* infections [18]. In past years, there has been a strong push to diagnosis newly acquired infections. Two emerging techniques of this sort are the serum IgG antigen test and real-time PCR. The IgG avidity test, for instance, can be used in conjunction with other serological assays as a confirming test. The IgG avidity test can detect previous infection, but the tenacity of low avidity IgG reduces its ability to distinguish between severe infections. Similarly, *T. gondii* generates soluble antigens that enter the tissues and circulatory system of the host, *T. gondii* infection symptoms are vague and substandard for diagnosis. Mainstream *T. gondii* infection diagnosis relies on toxicity tests and immunological testing for detecting or distinguishing parasitic strains [19, 20].

In the present study, we used ELISA and latex agglutination test methods to identify Toxoplasma antibodies in pregnant women in district Mardan. The application of these techniques are favored for screening Toxoplasma infection because of their high sensitivity and specificity, simpler methodology, and lower cost. The recent study was developed as a strategic slant to the prevention of congenital toxoplasmosis and determine the associated risk factor among the pregnant women.

MATERIAL AND METHODS

Study area

The current study was carried out between December 1, 2020, and April 30, 2021, in District Mardan, which is situated at 34° 11' 54" North and 72° 2' 45" East (coordinates) in Khyber

Pakhtunkhwa, Pakistan, at a height of 314 meters above sea level. The three administrative divisions of Mardan are Tehsil Mardan, Takht Bhai, and Katlang. The agriculture business is well-known in the Mardan district, and most residents in the rural areas work as farmers. The temperature is temperate; the winters are bitterly cold, while the summers are scorching. The warmest months are May, June, July, August, and September, with temperatures reaching 43.5 °C in June. The coldest months are December and January, with low temperatures ranging from 0.5 to 1°C. The following settlements were chosen for sampling in the district of Mardan:

Sampling

The informed consents were obtained from women whom samples were collected. Age, residence location, pregnancy stage, previous abortion information, animals contact (cats and others), and exposure to contaminated soils were all assessed using a structured questionnaire. A total of one hundred blood samples from pregnant women as well as one hundred randomly from the domestic cats were collected in sterile polythene bottles, labeled with date of collection, gender and location similarly the 5ml blood samples were collected in sterilized vacutainer, tag and labeled. For further examination, these samples were kept at -200 C in the Parasitology Laboratories of Abdul Wali Khan University Mardan Department of Zoology and College of Veterinary Sciences and Animal Husbandry (CVS & AH). Using the method, the prevalence of Toxoplasma gondii infection in cats was determined.

Similarly, cats were selected as sampling group from which fecal sample were collected during the study period in the mentioned areas of district Mardan. Cats were randomly divided into four subgroups namely W, X, Y& Z respectively. Subgroup W included Male cats from Shah Baig Killi, subgroup X included cats from Sheikh Yousaf Killi and Subgroup Y included cats from Saleem Khan Killi and subgroup Z included cats from Ibrahim Khan Killi. The sampling was carried out during December 01, 2020 to April 30 2021 from the women community in four villages of district Mardan as well as the fecal samples from the cat inhabited in their houses in that particular villages of Mardan.

Prevalence (%) = No. of Infested Animals / Total No. of Animals Examined x 100

Sporulation of Oocysts

The fecal sample added with 25% potassium dichromate solution and mixed thoroughly in the Petri dishes, placed in incubator at 26 ^{0}C with 80% humidity for three days. The samples examined after 24h, 48hrs and 72h under 60 x and 100 x magnifications by inverted microscope to observe the oocysts sporulation. The sporulated oocysts identified and their images were saved.

Blood Serum

One hundred blood samples of each 5 ml in sterilized vacutainer from the pregnant women manually rotated in hand to dissolve smoothly. These samples further placed in the centrifuge machine at 4000 rpm for 5-10 min. The supernatant collected through micropipette and placed in the Eppendorf tube .The serum labeled and stored at -20^{0}C in refrigerator for onward process.

ELISA Technique

The ELISA tests of the collected serum sample carried out as per protocol of the ELISA kit to assess the qualitative and quantitative results. Initially, the temperature in the water bath was set at 37°C, and without removing the plates from the bag, all of the reagents thawed to room temperature before use. The constituents mixed by shaking well. Then the plates taken out of their packaging and the subjected to determine the number of wells to under four different controls: two for the cutoff serum and one each for the negative and positive sera. The remaining wells returned to the pouch and sealed, as they were not required for the test. After that, each well was filled with 100 µL of serum diluents, then fill the matching wells with a 5 µL of each sample, 5 µL of positive control, 5 µL of negative control, and 5 µL of cut-off control. We followed manual protocol for the test, and all the plates vigorously shaken for two minutes in a plate shaker to ensure a homogeneous mixing of the chemicals. To create a homogeneous mixture of the reagents, the plates were subjected to plate shaker for extra 2 minutes. The homogenized samples, 105 µL of each diluents of each sample dispensed into the wells by using the pipette. Then covered with a sealing-sheet and heated for 45 minutes at 37 °C. The seal was removed, aspirate

the liquid from all wells, and wash each well five times with 0.3 mL of washing solution. A 100 µL of IgG conjugate solution mixed into each well right away, covered with a sealing sheet, and heated at 37 °C for 30 minutes in the water bath. Then, the seal was removed aspirate the liquid from all wells, and washed each well five times with 0.3 mL of washing solution to remove any leftover liquid. Finally, a 100 µL of substrate solution added into each well and incubated for 20 minutes at room temperature, followed by 50 µl of stopping solution into each well. The readings were calculated after 1 hour by using a spectrophotometer at 450/620 nm.

Risk factors

The questionnaire developed to obtain the information from the women community of the four villages of the research areas to determine the threat and different factors responsible for spreading of the disease.

Determination of sensitivity and specificity

The sensitivity and specificity of both diagnostic techniques will be determined as per procedure adopted by Pelloux *et al*. (1996) and Remington *et al*. (2004) [9, 10].

Statistical analysis

To calculate the *P* value, data was examined statistically using suitable statistics (Chi-square test), and univariate and multivariate ANOVA. The data was analyzed using SPSS, and the *P*-value was calculated, with a value of $P<0.05$ deemed significant.

RESULTS

Seroprevalence detection of *Toxoplasma gondii* in human population varies greatly among different countries, geographical areas and among different groups living in the same area in the world and particularly in Pakistan. One-hundred serum sample of the pregnant women were tested through ELISA and latex agglutination test and found prevalence rate were 23% and 12% respectively. Similarly, the Oocysts of *T. gondii* was observed in the feces of cats 14% respectively.

Overall prevalence and area prevalence

Overall prevalence and area prevalence revealed stable results in different replica. Through ELISA and latex agglutination test the prevalence of *T. gondii* in district Mardan was found 23% (ELISA) and 12% (latex agglutination) (Table 1). While, high prevalence was recorded in Shah Baig village through ELISA (28%) and latex test (12%) followed by Ibrahim Khan Killi (24%) and (16%) and lowest was recorded through ELISA (20%) in Sheikh Yousaf Killi and Saleem Khan Killi, respectively. However, difference were noted in latex agglutination test (8-12%), respectively (Table 2).

By age and occupation prevalence

High prevalence was recorded in age group ranges above 30 years through ELISA (33.33%) and latex agglutination (13.33%) and lowest prevalence was observed in age group ranges from 20 -30 years through both techniques (7.5%) and (10%) respectively (Table 2). The *T. gondii* infection was recorded highest prevalence

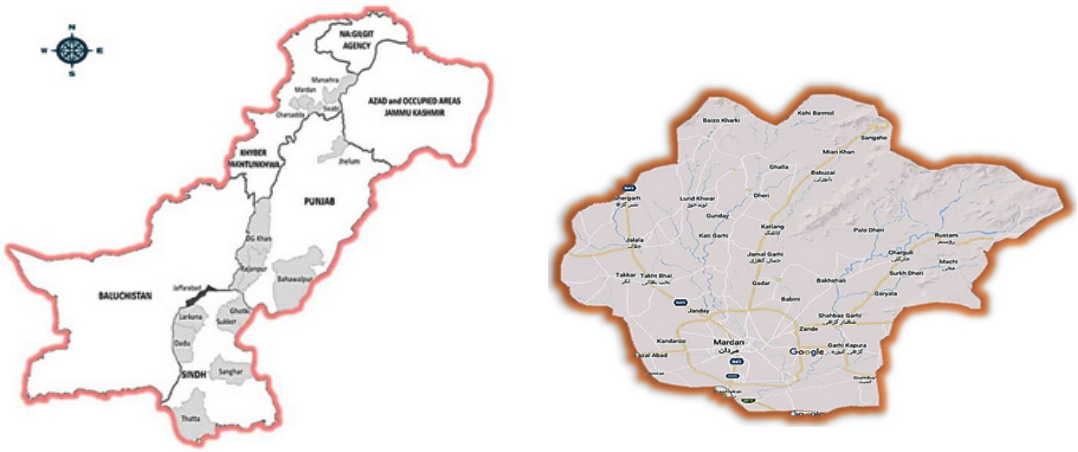

Fig. 1. Geographical map of Pakistan (A), map of the study area District Mardan (B)

18.0% in the non-occupation women of the community in rural areas of district Mardan where is low infection rate was noted in the occupational category (5.85%) of the women of that community. Similarly, it was re-asset by latex agglutination test in occupational (7.2%) and non-occupational women was (5.8%) respectively (Table 2).

Table 1. Comparative detection of T. gondii through ELISA and microscopy in Mardan by taking samples from pregnant women and cats

Variables	Total Women samples	ELISA test	P-Value	Total cats samples	Microscopy	P- Value
1. Shah BaigKilli	25		0.891	25		0.332
	+	7		+	4	
	–	18		–	21	
2.Sheikh Yousaf Killi	25			25		
	+	5		+	3	
	–	20		–	22	
3.Ibrahim Khan Killi	25			25		
	+	6		+	3	
	–	19		–	22	
4. Saleem Khan Killi	25			25		
	+	5		+	4	
	–	20		–	21	
Total	100			100		
+	+	23		+	14	
–	–	77		–	86	

Table 2. Comparative detection of *T. gondii* infection in women (pregnant) in MMC hospital Mardan through ELISA and latex agglutination test

Variables	Total samples	ELISA	P-Value	Latex test	P-Value
Areas			0.891		0.859
Shah Baig Killi	25	7		3	
Sheikh Yousaf Killi	25	5		2	
Ibrahim Khan Killi					
Saleem Khan Killi	25	6		4	
Subtotal	25	5		3	
	100	23		12	
Age group					
20-30yrs.	40	3	0.002	4	0.615
>30yrs	60	20		8	
Occupation					
Yes	17	8	0.009	2	0.973
No	83	15		10	
Abortion history					
Yes	35	7	0.6	3	0.438
No	65	16		9	
Abortion Trimester					
1st trimester					
2nd trimester	20	10	0.324	5	0.727
	15	5		3	

Table 3. Risk factor associated with toxoplasma positivity in mardan region

Variables	Total	ELISA		Latex test	
Interaction with cats					
Yes	70	17	0.64	8	0.788
No	30	6		4	
Interaction with other animals					
Yes	60	12	0.382	7	0.9
No	40	11		5	
Exposure to soil					
Yes	68	14	0.403	3	0
No	32	9		9	
Consumption of undercooked mutton					
Yes	45	9	0.519	7	0.322
No	55	14		5	

Sensitivity of latex test=11/23 100=43.82%
Specificity of latex test=76/77 100=98.70%

By abortion and trimester prevalence

During the study it was observed that abortion was occurred to those women who have clinically diagnosed positive by ELISA infected antibody *T. gondii* and it was reported (20%) by ELISA and (11.4%) by latex agglutination test. Contrary to this, a lower rate of abortion was observed in the *T. gondii* negative patients of the community (Table 2). Highest rate of abortion was recorded in the first trimester.

Associated risk factors

During the current study it was observed through questioner that contact with cat, and other animals like dog, sheep, uncooked meat and exposure to soil were the main factors in contamination and help in the transmission of the disease to the women population in the urban as well as rural areas. (Table 3).

DISCUSSION

The *Toxoplasma* infection are present throughout the world within different level 15% - 77% depending upon the risk factor and transmission resources [21, 22]. In our study, the *T. gondii* detection in pregnant women community was 23% in Mardan. Where abortion in the 1st trimester was 62.8% in the rural community. Toxoplasmosis seroprevalence was found to be 30% in 600 persons in an age group of 7–50 years [23]. In another report [21] revealed that the incidence of *T. gondii* infection was increases with age group within the community as well in the rural areas. However, the effect on the age was studied in the current research work. Using ELISA methods, a substantial difference in toxoplasmosis was discovered between urban and rural regions in the current study. A similar results were observed by Baril *et al*. (1999) [24]; however, discovered greater seroprevalence in metropolitan regions. It is important to figure out what kind of infection you are dealing with.

Seroprevalence was found to be changed according to the education level in the current study. In the low literate people, the highest prevalence was (18.0%). Water for the municipal network in Aydýn, is gathered from open springs in processing pools near a few settlements. The presence of oocysts in chlorinated arrangement water may explain the high seroprevalence among general network water consumers, according to recent publications. For individuals whose water sources are the same, a research on oocysts in home and outdoor life is essential. The oocysts type of *T. gondii* appears to be the main cause of water contamination. The study of Bowie *et al*. (1997) [25] investigated a toxoplasmosis epidemic in the western Canadian province of British Columbia, concluding that chloraminated; unfiltered surface water supply was the possible source of the massive community-wide disease.

The relationship of the domestic cat and human infection is difficult to calculate by questioners in the community. The oocysts are commonly found in soil contaminated with cat feces, and soil contact was the major route of disease transmission to the female population. They frequently buried in soil with cat feces, and soil contact is ubiquitous and difficult to avoid [26]. In the current study, cleaning the cat litter box, having inadequate hand hygiene, consuming raw veggies outside of the house were all discovered to be toxoplasmosis risk factors. These variables were investigated in the current investigation, but no link was discovered [22, 24, 27]. In this study, the link different variables, *Toxoplasma*, its risk factors in pregnant women in mardan region was investigated. According to the present statistics, pregnant women above the age of 30 were more likely to be positive than those under the age of 30. According to previous research, the prevalence of *Toxoplasma* infection rises with age [28]. There was no evidence of a link that the presence of *T.gondii* was related to the social and demographic variables. Using LAT, Geelaye *et al.* (2015) discovered comparable forms on social and demographic characteristics in the toxoplasmosis in the pregnant women of Ethiopia [29].

The practice of latex test and the ELISA test, a substantial rise in the associated risk variables and *T. gondii* positivity in the women was found in the current study as compared to those who had no contact with cats. Cats were the major source of infection to women as well human population through excreting the oocysts with its feces reported in different studies [26, 28, 30, 31]. Excreted oocysts usually survived in very harsh season for year and more in the environment [32]. As a result, soil exposure might be regarded a possible source of infection for humans, particularly pregnant women. Based on ELISA results, it was found that pregnant women who had contact with soil had much higher rates of infection than those who had none. This conclusion was consistent with the results of several studies undertaken in China, France, Iran, Indonesia, the Philippines, and Saudi Arabia, which revealed that exposure to soil poses a significant risk to pregnant women [24, 33-37].

The reliability of the diagnosis approach combining the two assays to obtain precise and concise data on parasite prevalence during acute and chronic infection was highlighted by the current study, which found a significant difference in the diagnosis of toxoplasmosis in pregnant women and cats using either specific diagnosis approach based on ELISA or latex-agglutination test or both of the methods. The recent research revealed that *T. gondii* infection is most common in cats and the pregnant women in Mardan's rural and urban areas. Finally, *T. gondii* detection by ELISA and latex agglutination assays is reliable and helpful for infection control and prevention.

CONCLUSION

It is concluded from the current study that *T. gondii* infection were more prevalent in pregnant women of rural areas of four village of Mardan as compare to the urban areas. The women and children were more at risk whose contact were usually with cats, contaminated soil and exposure to the livestock. The present results will definitely help in future research and the control of congenital toxoplasmosis, it is necessary to conduct studies showing the prevalence of T. gondii in neonates in order to implement a routine antenatal screening program to manage congenital toxoplasmosis, and further research is required to facilitate the development of more affordable preventive methods.

Conflict of interest

The authors declare no conflict of interest.

Funding

The authors have no financial conflict of interest to declare.

Author's contributions

HM, SS, and MH conceived and designed the experiments. HM, IR, and SS performed the experiments. SA, IR and SA analyzed the data and interpretation. SA and MH contributed reagents/ materials/analysis tools. HM, SA, SS and MH wrote the paper. All authors have read and agreed to publish this manuscript.

Statement of Informed Consent

Not applicable.

Ethics of Human and Animal Experimentation

Research grants and Experimentation Ethics Committee of the Department of Zoology Abdul Wali Khan University Mardan on the use of Animal samples approved the experimental protocol. Moreover, the study was carried out

in strict compliance with the National Research Council guidelines on the care and use of the laboratory.

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