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Serological identification of *Toxoplasma gondii* in Goats and their Female Possessors in Kurram District of Khyber Pakhtunkhwa

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SAS, ZK, MI and SZS presented the concept and planned methodology. NS and MS performed formal analysis and investigation. AQ curated data. NS wrote the manuscript. CS, MA, MI, AA, VS, TBE, FM and MN reviewed and edited the manuscript. ZK supervised the study. All authors read and approved the final version of the manuscript.

Key words
Female possessors, Goats, *Toxoplasma gondii*, Toxoplasmosis, Protozoan parasite

ABSTRACT

Toxoplasma gondii is a protozoan parasite that causes toxoplasmosis in animals. The present study was conducted to investigate the percentage of toxoplasmosis in goats and their female possessors in the Kurram district Pakistan. A total of 200 blood samples (100 each from goat and their female possessors) were collected randomly from three different regions Malana, Malikhel, and Parachinar of Kurram Pakistan, and were screened for *Toxoplasma gondii* infection through latex agglutination test and ELISA. Out of 100 goats, 52 were seropositive for toxoplasmosis showing an overall percentage of 52%. The percentage of *Toxoplasma gondii* was higher in female goats (53.75%) as compared to male goats (45%), and the highest percentage (57.14%) was observed in goats aged more than 2 years. The overall percentage of human toxoplasmosis was 70%. Out of a total of 100 serum samples of female possessors tested 60% were positive for IgM, 10% were positive for IgG and 30 were negative for both IgG and IgM as assessed through ELISA. The high rate of infection in females was observed in age group 46-55 years. The results of the current study reveal that infected goats might be a potential risk for toxoplasmosis in female possessors of Kurram, Khyber Pakhtunkhwa, Pakistan.

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INTRODUCTION

Toxoplasmosis is a zoonotic disease caused by a facultative protozoan *Toxoplasma gondii* (Majidiani *et al*., 2016), which remains a significant public health problem (Chen *et al*., 2022). Cats are the definitive hosts while all warm-blooded animals are the intermediate hosts of *T. gondii*. These are the only animals in which oocysts develop. Infectious oocysts excreted by cats can survive in warm and moist soil for more than one year (Hill and Dubey, 2002). The disease is transmitted to intermediate hosts by the ingestion of oocysts, present in the contaminated food and water. Similarly, transmission can also occur from mother to fetus in pregnant females. The prevalence of *T. gondii* in goats and sheep is very high due to the continuous contamination of pastures by *T. gondii* oocysts that make this parasite a common infectious agent among these animals (Rafique *et al*., 2022). Toxoplasmosis is responsible for abortions, stillbirth, and neonatal losses in livestock animals (Buxton *et al*., 2007). If humans or domestic animals ingest the infected oocysts, the parasite undergoes asexual reproduction characterized by rapidly dividing tachyzoites followed by slowly dividing bradyzoites stage, which encyst in the heart, brain, and other tissues and remain there for the host lifetime (Webster and McConkey, 2010). Mutton and milk obtained from sheep and goats contain the highest amount of *T. gondii* cysts (Webster, 2010). Toxoplasmosis consequences lead to a decreased reproductive ratio of the animals which results in economic losses in the livestock animals (Jittapalapong *et al*., 2005).

In humans, toxoplasmosis exists in two forms i.e., acute, and latent. Acute stage toxoplasmosis infection is asymptomatic in immunocompetent individuals (Lindström *et al*., 2006), but often shows flu-like symptoms, fever, headache, and body aches or no illness, however, these symptoms fade away within a few weeks leading to the latent stage of the disease. The latent stage can re-attack the immunocompromised individuals (pregnant females or HIV-infected persons) and greatly affect the pregnant female which leads to abortion, stillbirth, intellectual disability, blindness, and finally death (Negash *et al*., 2007). Infection leads to severe complications in immunocompromised patients including those infected with the human immunodeficiency virus (HIV) and those receiving suppressive chemotherapy following neoplastic disease and bone marrow or heart transplants (Campos *et al*., 2014). Pyrimethamine and sulphadiazine are two drugs widely used for the treatment of toxoplasmosis. These drugs are effective only in the acute stage of infection. Certain other drugs e.g., diaminodiphenyl sulphone, clindamycin, atovaquone, and spiramycin are used to treat toxoplasmosis in latent cases (Dunay *et al*., 2018).

Toxoplasmosis infection is high in regions where people consume undercooked meat and unwashed vegetables or fruits (Almuzaini, 2023; Rafique *et al*., 2022), and among people who are in direct contact with cats dogs, or other domestic animals.

The seroprevalence of *T. gondii* is about 25%, 30%, 50%, and 60% in Japan, the USA, Finland, and Poland respectively and some countries have seroprevalence of even 80% (Remington *et al*., 2004). In Pakistan, the prevalence rate is high varying between 38% in Khyber Pakhtunkhwa, 48% in Azad Kashmir, and 63% in Punjab (Shah *et al*., 2013). However, currently, there is no data available about the *T. gondii* in Kurram district, previously known as Federally Administrated Tribal Areas (FATA) Pakistan. This study was carried out to determine the percentage of *T. gondii* in goats and their female possessors in Kurram.

MATERIALS AND METHODS

Samples collection

This study was carried out to determine the percentage of *T. gondii* in goats and their female possessors in Parachinar Kurram, at serological and molecular levels. A total of 200 blood samples (100 each) were collected from goats and their female possessors by simple random sampling method. Blood samples (5 mL) were collected from goats and their possessors in EDTA tubes. The blood was centrifuged at 4000 rpm for 10 min for the extraction of serum. The obtained serum was separated and transferred to a 1.5 mL Eppendorf tube and was stored at -20°C for serological analysis.

Serological analysis

ELISA was performed for the detection of IgG and IgM antibodies to *T. gondii* in serum samples of female possessors.

Detection of IgG antibodies

For the determination of IgG antibodies in serum samples of female possessors, a toxoplasma IgG Immunoassay test kit (Biocheck, USA) was used. Quantification was carried out according to the manufacturer's instructions. Briefly, 5 µL serum and 200 µL of the sample diluent were mixed in wells and incubated at 37°C for 30 min followed by the addition of 100 µL of enzyme conjugate. The samples were again incubated at 37°C for 30 min. Then 100 µL of TMB reagent was added into each well and the reaction was stopped by the addition of 100 µL stop solution (1 N HCl). The optical density (OD) was read at 450 nm with an ELISA reader (DAS, Italy plated ELISA). A calibration curve was obtained using IgG as a positive control. Distilled water was used

as a negative control. The results were calculated using the following formula:

Cut off calibrator *toxoplasma* IgG index value = 1.0

The serum was considered to be positive if OD > cut off the calibrator index value; The serum was considered to be negative if OD < cut off calibrator index value.

Detection of IgM antibodies

IgM antibody in serum samples was determined using a *toxoplasma* IgM Immunoassay test kit (BioCheck, USA) according to the manufacturer's instructions. The experimental procedure is the same as described for IgG quantification. However, the IgM antibody was used as a positive control. The results were calculated using the following formula.

Cut off Calibrator *toxoplasma* IgM index = 1.0

The serum was considered to be positive if OD > cut off the calibrator index value; The serum was considered to be negative if OD < cut off calibrator index value.

Latex agglutination test

To detect *T. gondii* antibodies in serum samples of goats, a toxoplasmosis latex test kit (Antec Diagnostic Products UK) was used. Serum samples were diluted with normal saline (0.9% NaCl) and 50 µL of the diluted serum was transferred onto the slide. 25 µL of latex reagent was added to a drop of serum and mixed well and the presence or absence of agglutination was observed after incubating the mixture for 4 min at room temperature. A negative reaction with no agglutination indicated the absence of toxoplasma antibodies while a positive reaction with agglutination indicated the presence of *T. gondii* antibodies equal to or greater than 4 IU/mL which reflects either a past infection or an evolving infection.

Statistical analysis

The results were analyzed by using chi-square and Fisher exact tests through the statistical software SPPS version 20. All analyses were carried out in triplicate. Mean±SD was calculated and $p \leq 0.05$ was considered statistically significant.

RESULTS

In the current study, both goats and their female possessors (100 each) were selected to detect *T. gondii* antibodies in their blood samples using a latex agglutination test and ELISA, respectively. In this study, different clinical findings were noted in the goats and female possessors. Most of the positive animals have possessed fever, anorexia, dyspnea, abortion, and neurological signs whereas in female possessors the most common signs recorded were flu, fever, anorexia, and muscle aches. In some of the positive female possessors has also abortion history in early pregnancy.

Toxoplasma gondii *in female possessors of goats*

Blood samples were collected from goats and their female possessors from three regions including Malana, Malikhel, and the city area in Parachinar Kurram. A questionnaire was also designed to obtain relevant information from the female possessors. A total of 100 blood samples were collected from female possessors of goats. The overall percentage of human toxoplasmosis was 70% during the present study (Table I).

Table I. Seroprevalence of IgG and IgM antibodies of *T. gondii* in female possessors in different areas of Parachinar.

Location	No of samples	IgM +ve	IgG +ve	IgM/IgG –ve	+ve percentage (%)
Malana	40	28	5	7	82.5
Malikhel	40	24	3	13	67.5
City	20	8	2	10	50
Total	100	60	10	30	70

Table II. Age-wise frequency of *T. gondii* infection in female possessors.

Group	Age (years)	Frequency	Seroposi-tivity	Percentage (%)
I	16-25	26	18	69.23
II	26-35	40	29	78.57
III	36 45	20	12	60
IV	46-55	14	11	72.5

Out of a total of 100 serum samples of female possessors tested 60 were positive for IgM, 10 were positive for IgG and 30 were negative for both IgG and IgM. Toxoplasmosis was more prevalent in Malana (95%) compared to Malikhel (67%) and Parachinar main city (50%) (Table I).

For the age-wise percentage of toxoplasmosis, the female possessors were divided into four age groups: Group I (16-25 years), Group II (26-35 years), Group III (36-45 years) and Group IV (46-55 years). The corresponding frequencies of these groups were 26% for the group having aged 16-25 years, 40% for the group having possessors of age 26 to 35 years, 20% for the 36-45 years aged group, and 14% for the 46-55 years age group. The results indicate that the high percentage is found in the age group 46-55 years

(78.57 %) as shown in Table II. The high rate of infection in the age group 46-55 years was due to close interaction with domestic animals and agriculture fields.

Percent identification of T. gondii *in goats*

A total of 100 blood samples of goats from three different localities of Parachinar were examined for the presence of *T. gondii* antibodies using a latex agglutination kit. *T. gondii* antibodies were detected in 52 (52%) out of 100 goats (Table III). Of these 100 goats, 80 were female and 20 were male goats.

Table III. Sex-wise frequency of *T. gondii* in goats.

Sex	Frequency	Positive	Percentage (%)
Female	80	43	53.75
Male	20	9	45 .00
Total	100	52	52

The percentage of *T. gondii* was higher in female than male goats (Table III). All goats were divided into three age groups (<1 year, 1-2 years, and ≥2 years). The percentage varied in different age groups of goats ranging from 50% to 57.14%. Out of 43 examined goats whose age was less than one year 22 (51.16%) were detected seropositive for *T. gondii* infection.

The *T. gondii* infection was found in 18 (50%) out of 36 examined goats aged 1-2 years. The highest percentage (57.14%) was observed in goats of age more than 2 years, where 12 out of 21 goats were seropositive for *T. gondii* (Table IV).

Table IV. Age-wise prevelance of *T. gondii* in goats.

Age	No of goats	LAT +ve	Prevalence (%)
< 1 year	36	18	50.00
1-2 years	43	22	51.16
≥ 2 years	21	12	57.14

DISCUSSION

Since its discovery 100 years ago, *T. gondii* has become one of the most successful and adaptable parasites on earth mainly because of its worldwide distribution, broad host range, and its ability to maintain a gentle co-existence with the hosts. Although *T. gondii* causes acute and chronic diseases in healthy individuals, its exceptionally high infection rates show that it is a serious threat to human health (Carruthers, 2002). Toxoplasmosis can be diagnosed by different methods like immunological or serological testing, isolation in tissue culture, histological identification, and recovery of the parasite DNA by polymerase chain reaction (Kompalic-Cristo *et al*., 2004). Serological tests, like the Sabin Feldman Dye test, complement fixation test, indirect hemagglutination test, direct agglutination test, immunofluorescence assay, Western blot test, and ELISA are the most widely used, yet they have the greatest limitations as they often provide ambiguous results (Skiest, 2002).

In Pakistan, the goat population accounts for an important part of the national economy because of its important contribution to the animal population. It gives high-quality food in the form of mutton and milk and contributes major parts in the improvement of close industries by giving crude materials such as skin, hides, and horns (Rahumatullah *et al*., 2012). Goats play an important part in the financial state of rural people. The high rate of fertility and short gestation period make the goat special for selection. In addition, goats are comparatively hard animals, adapted to adverse climatic conditions and resistant to diseases (Kumar *et al*., 2010). In the form of extreme environments, diseases, and poor management the goats are facing great challenges. Among different infectious diseases toxoplasmosis is one of the most important diseases that represent a serious risk to the goat population (Sharif *et al*., 2015).

Toxoplasmosis is found in most parts of the world but there are very few reports on toxoplasmosis in Pakistan. The prevalence of toxoplasmosis in Pakistan has been reported in school-going children, pregnant females, cattle, and in some high and low-risk groups in different areas. The infection rates of toxoplasmosis are different in different regions of Pakistan. In Pakistan, the prevalence of toxoplasmosis in goats also varies. But so far, no research data is available on the prevalence of *T. gondii* in goats and their female possessors in Parachinar Kurram.

Considerable variations in the prevalence of *T. gondii* antibodies in different animal groups have been reported in different areas. Comparing the seroprevalence of *T. gondii* between goats and their female possessors it was observed that the seropositivity is greater in female possessors (70%) as compared to goats (52%). This high percentage of female possessors depicts that goats are not the only source through which females can get infected rather there are many other sources as well through which females can acquire this infection. The seroprevalence varies depending on various factors, such as animal species, feeding habits of the animals, outdoor access of animals, the degree of intensive farming, and the presence of cats in the farm or homes. The overall percentage of *T. gondii* antibodies in goats was 52% while in their female possessors, it was 70%. In our study, the seroprevalence

rate in female possessors is high as compared to Malakand of Khyber Pakhtunkhwa (65.7%) (Khan *et al*., 2014), while in Kohat (Khan *et al*., 2011), the seroprevalence in pregnant females was 14.4%. In these two regions ELISA technique was used for *T. gondii* detection, while in Southern Punjab (Tasawar *et al*., 2012), District Swabi (Faisal *et al*., 2014), and Lahore (Ahmad *et al*., 2012), regions of Pakistan the prevalence rates were 29.45%, 19.25% and 11.33%, respectively in females as assessed by Latex agglutination test. The low seroprevalence rate in these regions may be due to the less sensitive diagnostic method used in these studies. The seroprevalence rate against *T. gondii* also varies worldwide, being reported to be 6.7% in the Korean (Shin *et al*., 2009), 12.3% in the China (Xiao *et al*., 2010), 23.9% in Nigeria (Kamani *et al*., 2009), 46% in Tanzania (Swai and Schoonman, 2009), 47% in rural areas of France (Fromont *et al*., 2009), and 24.4% in women from the North of Portugal in their childbearing years (Lopes *et al*., 2012), which are lower than in our study. Thus, our results and the data available from developed countries and other regions of the world show variable seropositivity in different geographical regions that reflect variations in climate characteristics, and cultural and dietary habits of the population. Moreover, some experimental factors including sampling size, and distinct immune assays adopted for diagnosis may also account for these differences.

In the present study, the female goats showed a higher percentage (53.75%) than male goats (45%). It is because female animals are more susceptible to protozoan parasites than males. After all, the hormonal differences between males and females play an essential part in deciding susceptibility to parasitic infection. It is broadly recognized that numerous hormones specifically impact the immune system including the sex-related hormones (Roberts *et al*., 2001), apart from the fact that during pregnancy immunity is broken down in females (Lobo *et al*., 2017). The relationship between age and toxoplasmosis in goats revealed that *T. gondii* has the highest percentage (57.14%) in the age group ≥2 years and the lowest percentage (50%) in the age group <1 year. The prevalence increases as the age of animals increases. A progressive increase of *T. gondii* infection with age suggests continuous exposure to the parasite in the environment as earlier reported (Tasawar *et al*., 2011).

T. gondii infection is high in regions where people eat undercooked meat, and unwashed vegetables, and have contact with cats and dogs or other domestic animals or have direct contact with the soil. In Pakistan, the *T. gondii* infection is common among food animals and these infections seem to be confined largely among cattle. The ever-increasing preference for eating meat among Pakistanis gives the probability that infected meat could be a source of infection that cannot be ruled out.

The present study revealed a higher percentage in Malana and Malikhel *T. gondii* than in Parachinar city, where health and living standards are comparatively good which may account for a lower percentage. Secondly majority of the females of Malana and Malikhel work in their fields and bring fodder for their cattle and vegetables for cooking which also increases the chance of being infected by oocysts. Moreover, the difference in the environmental condition of these areas may also increase the *T. gondii* infection in Malana and Malikhel. The temperature in summer in these areas is lower and humidity is high which may favor the transmission of infection. As majority of the population in Parachinar are farmers living in mud houses, which need maintenance (mud coating) twice a year. So, due to close contact with soil, the infection may be transmitted, and the percentage is higher in Malana and Malikhel as compared to Parachinar main city where health and living standards are comparatively better. Also, most of the females of Malana and Malikhel work in their fields and bring fodder for their cattle and vegetables for cooking which also increases the chance of being infected by oocysts.

In the present study 62.79 % of females have *T. gondii* antibodies in their serum that have contact with cats and 56.25% of females have *T. gondii* antibodies in their serum who have contact with dogs at their homes. Cats are the only known source of oocysts present in the environment and are very important in the life cycle of *T. gondii*. Other causes of *T. gondii* infection in females from the Kurram region could be the ingestion of *T. gondii* oocysts in undercooked meat during food preparation, drinking the milk of infected animals, and due contact with soil. The later seems to be the main mode of transmission of toxoplasma infection. The study has confirmed the zoonotic importance of toxoplasmosis because the incidence was 70% in the female possessors and 52 % in their goats.

CONCLUSION

In conclusion, our study was conducted in the Kurram District of Khyber Pakhtunkhwa which reveals a high seroprevalence of *T. gondii* in goats and their female possessors. Notably, goats aged over 2 years exhibited a higher likelihood have being seropositive for toxoplasmosis, underscoring the significance of age as a contributing factor to infection. Furthermore, our investigation identified additional risk factors for

T. gondii transmission, including contact with other domestic animals such as cattle, dogs, and cats, as well as exposure to contaminated soil. The presence of infected goats in the region raises concerns about the potential for human toxoplasmosis transmission. This underscores the importance of implementing measures to mitigate this risk, such as promoting proper hygiene practices, safe food handling, and thorough cooking of meat to prevent infection. Additionally, the consumption of raw goat or sheep milk should be discouraged to reduce the likelihood of exposure to the parasite. One of the key recommendations arising from our study is the necessity for routine serological testing in pregnant women due to the high percentage of toxoplasmosis among the female possessors of goats. Timely detection and management of toxoplasmosis during pregnancy are crucial to prevent potential adverse outcomes for both the mother and the fetus. Considering our findings, the local health authorities, government agencies, and non-governmental organizations must collaborate to develop and implement effective strategies for the control and prevention of toxoplasmosis in the Kurram District. This study provides valuable data that can serve as a foundation for evidence-based policies and interventions aimed at safeguarding the health and well-being of both the human population and the livestock in the region. By raising awareness and implementing preventive measures, one can reduce the burden of toxoplasmosis and promote a healthier environment for the community.

IRB approval

This study was approved by an institutional review board of the University of Peshawar for laboratory animal/ experimental animals.

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Ethics approval and consent to participate

Ethical approval with the number "No:21/FLES/62 was obtained from the ethical committee of the University.

Statement of conflict of interest

The authors have declared no conflict of interest.

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