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RESEARCH ARTICLE

One Health Epidemiology, Associated Risk Factors and Molecular Characterization of *Toxoplasma gondii* at the Human–Small Ruminants–Cat Interface in Lahore, Pakistan

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ABSTRACT

Toxoplasma gondii (*T. gondii*) is a food-borne and zoonotic parasite that causes human and animal diseases worldwide. In this study, the epidemiology, factors associated with seropositivity, and molecular characteristics of *T. gondii* at the human–small ruminants–cat interface have been explored in Lahore, Pakistan, using the One Health perspective. Blood samples were taken from sheep (n=1000), goats (n=1000), and humans (n=200) and tested for anti-*T. gondii* IgG antibody levels using an indirect enzyme-linked immunosorbent assay (iELISA). A structured questionnaire was used to evaluate the potential risk factors, and selected biochemical parameters were analyzed to evaluate the physiological effects of infection. The presence of the parasite was assessed using microscopy, followed by PCR, sequencing, and phylogenetic analysis of positive samples from 200 cat fecal specimens. The overall seroprevalence of *T. gondii* was 23.0%, 28.0%, and 27.0% in sheep, goats, and human participants, respectively. Sex, age, reproductive status, and grazing system were associated with seropositivity in small ruminants; cat access to feed was associated with seropositivity in goats, while cat ownership was associated with seropositivity in humans. Biochemical changes in the serum were noted in seropositive small ruminants and humans. Microscopic examination detected *T. gondii*-like oocysts in 7 (3.5%) of feline fecal samples, with six confirmed via PCR and further analyzed by partial SAG2 gene sequencing. Phylogenetic analysis suggested limited genetic variation among the analysed feline-derived isolates. The results indicate that *T. gondii* is a ubiquitous parasite in humans, animals, and cats and highlight the importance of the human–small ruminants–cat interface in disease transmission. The present study contributes to the implementation of integrated One Health surveillance and control strategies to minimize the impact of toxoplasmosis and safeguard public and animal health.

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INTRODUCTION

Toxoplasma gondii (*T. gondii*) is an invasive intracellular protozoan parasite and is regarded as one of the most significant zoonoses affecting humans and animals. Almost all warm-blooded animals, including humans, livestock, wildlife, and pets, are infected with the parasite. About one-third of the world's population is

believed to be infected with *T. gondii,* and the infection rate varies from country to country depending on environmental pollution, dietary habits, climate, and socioeconomic status (Dubey, 2021). Owing to its wide host range and multiple modes of transmission, toxoplasmosis is an important public health and animal production problem worldwide (de Barros *et al*., 2022; Khairullah *et al*., 2024).

T. gondii has a life cycle that involves definitive hosts, which are mainly domestic cats, where sexual reproduction takes place, and oocysts are excreted in feces. Resistant oocysts may contaminate soil, water, and food, allowing transmission to livestock and humans. The intermediate hosts are infected when they ingest sporulated oocysts or tissue cysts, which are commonly found in undercooked meat (Dubey and Su, 2009). *T. gondii* infection is one of the most important foodborne parasite infections in the world as it is prevalent and is associated with high disease burden (Montoya and Liesenfeld, 2004).

The transmission of *T. gondii* exemplifies the interconnected nature of human, animal and environmental health. Domestic and stray cats contaminate the environment through oocyst shedding. At the same time, livestock become infected through contaminated feed, pasture or water and subsequently serve as a source of infection for humans through the food chain (Ubaid *et al*., 2025). Consequently, effective prevention and control require coordinated surveillance across multiple host species and their shared environment rather than investigations confined to a single population. This integrated perspective has become a central component of the One Health framework for toxoplasmosis research (İnci *et al*., 2023).

Small ruminants, particularly sheep and goats, play a major role in the prevalence of toxoplasmosis. These animals' infections are linked to reproductive losses, such as abortion, stillbirth, neonatal death and decreased productivity, which have a substantial financial impact on livestock producers (Ahaduzzaman and Hasan, 2022; Semango and Buza, 2024). Furthermore, humans can be infected by sheep and goats through raw and undercooked meat and other animal products. Several predispositions in small ruminant populations have been indicated, including age, sex, management practices, livestock hygiene and cat contact (Stelzer *et al*., 2019; Dubey *et al*., 2020).

The epidemiology of *T. gondii* infection in small ruminants varies considerably across geographical regions owing to differences in climatic conditions, husbandry practices, animal management systems and environmental contamination with feline-derived oocysts. Serological investigations have reported variable prevalence in sheep and goats from Algeria, Iraq, Iran, India, Benin and China, indicating that local management practices and the presence of domestic cats strongly influence infection dynamics (Zhao *et al*., 2011; Bachan *et al*., 2018; Abdallah *et al*., 2019; Al Hamada *et al*., 2019; Izadyar *et al*., 2019; Tonouhewa *et al*., 2019). Similar observations have also been reported from Pakistan, where differences in farming systems and animal husbandry have been associated with variations in seroprevalence among small-ruminant populations (Ahmed *et al*., 2016; Hanif and Tasawar, 2016).

Molecular techniques have greatly enhanced the understanding of the genetic diversity and abundance of *T. gondii*. Among the molecular markers available for *Toxoplasma gondii*, the surface antigen 2 (*SAG2*) gene is widely used for genetic characterization, genotype differentiation and phylogenetic analysis because it enables comparison of parasite isolates from different hosts and geographical regions (Fernández-Escobar *et al*., 2022; Fadel *et al*., 2024). Previous studies from Pakistan have provided valuable host-specific evidence on *T. gondii* exposure and its zoonotic relevance; however, these reports also provide an important basis for broader investigations linking serological findings with molecular characterization across human, livestock, and feline sources (Qamar and Alsayeqh, 2023; İnci *et al*., 2023).

Human toxoplasmosis has also been investigated in several countries, particularly among pregnant women and other high-risk populations, where infection has been associated with adverse pregnancy outcomes and other public health concerns. Serological studies from Saudi Arabia, India, Egypt, Iran, Sudan and Pakistan have demonstrated considerable variation in prevalence, reflecting regional differences in exposure risk, food consumption habits and environmental conditions (Khan *et al*., 2011; Alghamdi *et al*., 2016; Borkakoty *et al*., 2016; Mandour *et al*., 2017; Naeini *et al*., 2019; Taha *et al*., 2019). In addition, increasing evidence indicates that *T. gondii* infection may be associated with neurological and psychiatric disorders, further emphasizing its importance as a neglected zoonotic pathogen requiring integrated surveillance within a One Health perspective (Chen *et al*., 2019; İnci *et al*., 2023).

Despite the availability of numerous serological studies on individual host species, integrated investigations simultaneously evaluating humans, livestock and cats within the same epidemiological setting remain limited in Pakistan. Furthermore, studies combining serological, epidemiological, biochemical and molecular characterization with phylogenetic analysis are scarce, leaving important gaps in understanding transmission dynamics at the human–small ruminants–cat interface. Therefore, the present study was designed to investigate the occurrence of *T. gondii* infection in humans, sheep, goats and cats in Lahore, Pakistan. Specifically, the study aimed to determine seroprevalence in humans and small ruminants, identify associated risk factors, evaluate selected serum biochemical parameters, molecularly characterize feline-derived *T. gondii* isolates using PCR and *SAG2* gene sequencing, and assess their phylogenetic relationships with reference sequences available in GenBank.

MATERIALS AND METHODS

Study design and sample collection: A cross-sectional survey was conducted in Lahore, a metropolitan city in the province of Punjab, Pakistan. Lahore is in the northeast region of Punjab, where a subtropical climate could be conducive for the environment and spread of *T. gondii* oocysts. Blood was collected from sheep (1000), goat (1000), and human (200), and fecal samples were collected from cats (200). Information regarding potential risk factors associated with seropositivity, including sex, age, reproductive status, herd management practices, hygienic conditions, cat ownership, and cat access to feed and water sources, was recorded using a structured questionnaire.

The sample sizes for humans, sheep, goats, and cats were determined based on the objectives of the study, accessibility of the target populations, expected prevalence reported in previous studies, and logistical feasibility during the sampling period. The selected sample numbers were considered sufficient to provide representative estimates of *T. gondii* occurrence in the study area.

Human participants were recruited from the general population of Lahore. Written informed consent was obtained from all participants prior to sample collection. Fecal samples were collected from cats presented from different areas of Lahore. Information regarding ownership status (owned or stray), access to outdoor environments, and general management conditions was recorded whenever available. Cats were selected based on sample availability and owner consent (where applicable). The study was conducted in accordance with the guidelines of the Institutional Ethical Review Committee of the University of Veterinary and Animal Sciences (UVAS), Lahore, Pakistan (Approval No. ERC-3355).

Serological analysis: Blood samples were allowed to clot at room temperature for 1–2 h, and sera were separated by centrifugation at 2300×g for 10 min. Serum samples were screened for anti-*Toxoplasma gondii* IgG antibodies using a commercial indirect enzyme-linked immunosorbent assay (iELISA) kit (Vircell Microbiologists, Spain). According to the manufacturer, the assay has high diagnostic sensitivity and specificity for detecting anti-*T. gondii* IgG antibodies. Samples were interpreted as positive or negative according to the manufacturer's recommended cut-off criteria based on optical density values. The assay has been widely used for epidemiological investigations of toxoplasmosis. Optical density values were measured using a microplate reader (RT-6000, Devrim Limited, UK).

Serum biochemical analysis: Selected serum biochemical parameters were determined using a clinical chemistry analyzer (1600DR, Metrolab, Argentina). Total protein, albumin, blood urea nitrogen (BUN), and cholesterol concentrations were measured using commercial diagnostic kits (MTD Diagnostics Srl, Italy). Globulin concentration was calculated by subtracting albumin from total protein. Biochemical profiles of seropositive individuals and animals were evaluated and compared with established reference intervals to assess the potential impact of *T. gondii* infection on host health and physiological status.

Microscopic examination of cat fecal samples: Fecal samples were processed using standard flotation and sedimentation techniques to detect *T. gondii*-like oocysts. Suspected oocysts were examined microscopically using a compound microscope. Positive samples were subsequently sporulated in a 2.5% potassium dichromate solution at room temperature. Oocyst enumeration was performed using the McMaster counting chamber technique. Sporulated oocysts were then recovered and processed for molecular analysis.

DNA extraction and PCR amplification: Genomic DNA was extracted from microscopically positive feline fecal samples using the QIAamp Fast DNA Stool Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. DNA concentration and purity were assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA).

Initial molecular screening was performed using the TOX4 (5′-CGCTGCAGGGAGGAAGACGAAAGTTG-3′) and TOX5 (5′-CGCTGCAGACACAGTGCATCTGGATT-3′) primers, which target the highly repetitive 529-bp element of *T. gondii*, as described by Hanafiah *et al*. (2018). PCR products were separated by electrophoresis on 1.5% agarose gel stained with ethidium bromide and visualized using a gel documentation system (Bio-Rad Laboratories, USA).

Samples positive for the repetitive 529-bp PCR assay were subsequently amplified using *SAG2*-specific primers (forward: 5′-CCTGGTGTCTCTTCAAGCGT-3′; reverse: 5′-AAAGGAGAATGAGCGCACGA-3′) for molecular characterization and phylogenetic analysis (Hafeez *et al.,* 2022). Amplified products were analyzed by agarose gel electrophoresis. Positive amplicons were purified and subjected to bidirectional Sanger sequencing.

Sequencing and phylogenetic analysis: PCR-positive SAG2 amplicons were purified using a commercial PCR purification kit according to the manufacturer's instructions and were subjected to bidirectional Sanger sequencing. Chromatograms were inspected and edited using BioEdit version 7.2.5, and consensus sequences were generated prior to sequence analysis. Sequence identity was confirmed using the Basic Local Alignment Search Tool (BLAST) available through the National Center for Biotechnology Information (NCBI) database.

Six partial SAG2 gene sequences obtained from feline-derived *Toxoplasma gondii* isolates were included in the phylogenetic analysis. Representative *T. gondii* reference sequences were retrieved from GenBank and aligned using the MUSCLE algorithm implemented in MEGA version 12. Evolutionary relationships were inferred using the Maximum Likelihood method under the Tamura–Nei nucleotide substitution model. The robustness of the inferred phylogeny was assessed by bootstrap analysis with 1000 replicates. Molecular characterization and phylogenetic interpretation were performed following previously described approaches for SAG2-based genotyping of *T. gondii* isolates (Arefkhah *et al*., 2020).

Statistical analysis: Descriptive analysis was performed using SPSS Statistics version 26.0 (IBM Corp., Armonk, NY, USA). Seroprevalence was calculated as the proportion of positive samples among the total samples tested and is presented with corresponding 95% Wilson confidence intervals (95% CIs). Pearson's χ^2 test was used to compare overall seroprevalence among host species. For risk-factor analyses, crude odds ratios (ORs), 95% CIs, and corresponding P-values were obtained using univariable logistic regression with the Wald test. Fisher's exact test was used when expected cell counts were <5. Statistical significance was considered at $P<0.05$.

RESULTS

Seroprevalence of *Toxoplasma gondii* in humans, goats and sheep: Serological screening using indirect ELISA revealed the presence of anti-*T. gondii* IgG antibodies in

humans, goats, and sheep from Lahore, Pakistan. Overall seroprevalence was 27.0% (54/200) in humans, 28.0% (280/1000) in goats, and 23.0% (230/1000) in sheep (Table 1). Pearson's Chi-square analysis demonstrated a significant association between host species and *T. gondii* seropositivity (χ^2=6.77, df=2, P=0.034).

Biochemical alterations associated with toxoplasmosis: Biochemical profiling revealed alterations in serum metabolites among seropositive hosts (Table 2). In goats, seropositive animals exhibited lower concentrations of total protein, albumin, globulin, blood urea nitrogen (BUN) and cholesterol compared with established reference intervals. In sheep, albumin concentrations were reduced, whereas globulin, BUN and cholesterol values were elevated relative to reference ranges. In humans, seropositive individuals exhibited increased total protein, globulin and BUN concentrations accompanied by decreased albumin and cholesterol levels. These findings suggest possible metabolic and physiological alterations associated with *T. gondii* infection.

Table 1: Seroprevalence of *Toxoplasma gondii* in humans, goats and sheep from Lahore, Pakistan, with corresponding 95% Wilson confidence interval

Area	Species	Sample size (n)	Seropositive	Prevalence (%)	95% CI
Lahore	Human	200	54	27.0	21.3-33.5
	Goat	1000	280	28.0	25.3-30.9
	Sheep	1000	230	23.0	20.5-25.7

Table 2: Serum biochemical parameters observed in seropositive humans, goats and sheep

Species	Condition	Total protein (g/dL)	Albumin (g/dL)	Globulin (g/dL)	BUN (mg/dL)	Cholesterol (mg/dL)
Human	Reference interval	6–8	3.5–5.0	2.6–3.5	5–20	200–240
Human	Seropositive	9.13	2.05	7.08	25.00	145.00
Goat	Reference interval	6–7.5	2.4–3.0	2.7-4.1	8–20	52–76
Goat	Seropositive	4.32	1.80	2.52	7.00	43.00
Sheep	Reference interval	6–7.5	2.4–3.0	3.5-5.7	8–20	52–76
Sheep	Seropositive	8.85	1.30	7.55	26.00	84.00

Risk factors associated with *Toxoplasma gondii* seropositivity: Questionnaire-based analysis identified several host-related and management-related factors associated with *T. gondii* infection (Table 3). In goats, higher seroprevalence was observed in females (34.00%) than in males (10.00%), in pregnant animals (35.03%) than in non-pregnant animals (16.67%), and in animals older than two years (33.33%) compared with younger age groups. Outdoor grazing and cat access to feed were also associated with higher seroprevalence rates.

A similar trend was observed in sheep, where females (28.27%) showed higher seroprevalence than males (7.20%), pregnant animals (29.63%) showed higher prevalence than non-pregnant animals (8.33%), and animals older than two years showed the highest prevalence (30.00%). In humans, seroprevalence was highest among participants aged 40 years or older (33.33%), pregnant women (34.00%), and cat owners (36.67%). In cats, higher prevalence was observed among females, older cats, cats with street access, cats in contact with other cats, and those kept under poor hygienic conditions.

Microscopic detection of *Toxoplasma gondii* oocysts in cats: Microscopic examination of 200 feline fecal samples identified *T. gondii*-like oocysts in 7 samples, corresponding to an overall prevalence of 3.5% (7/200). The observed oocysts were spherical to subspherical, measuring approximately 10–14 µm in diameter (Fig. 1).

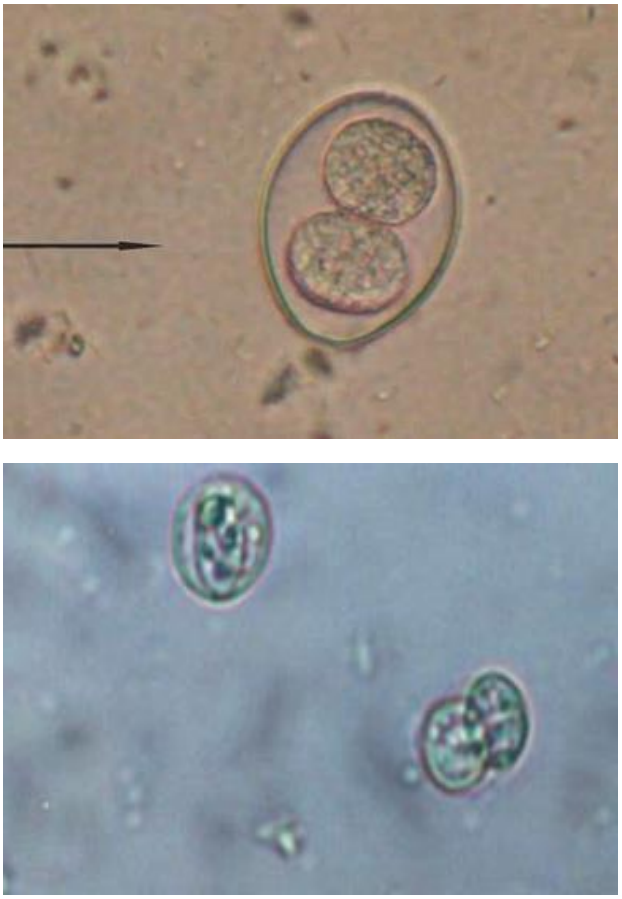

Fig. 1: Sporulated *Toxoplasma gondii* oocysts observed in feline fecal samples under a compound microscope (40×). Arrows indicate spherical to subspherical oocysts.

Molecular confirmation of *Toxoplasma gondii*: All microscopically positive samples were analyzed. Initial screening was performed using PCR targeting the highly repetitive 529-bp element of *T. gondii*. Six of the seven microscopy-positive samples were confirmed by PCR, yielding the expected 529-bp amplicon (Fig. 2). These six PCR-positive samples were subsequently amplified using SAG2-specific primers for sequencing and phylogenetic analysis (Fig. 3).

Phylogenetic analysis of feline-derived *Toxoplasma gondii* isolates: Six PCR-positive isolates were successfully sequenced, and the partial SAG2 gene sequences were subjected to phylogenetic analysis. BLAST analysis confirmed high sequence similarity with published *Toxoplasma gondii* sequences available in GenBank. A Maximum Likelihood phylogenetic tree was constructed in MEGA version 12 using the Tamura–Nei nucleotide substitution model with 1000 bootstrap replicates (Fig. 4). The analysis included the six study isolates (GenBank accession numbers PZ766476–

PZ766481) together with representative *T. gondii* reference sequences retrieved from GenBank.

The phylogenetic tree demonstrated that all study isolates clustered within the *T. gondii* lineage. Isolates PZ766478 and PZ766480 formed a closely related cluster with 82% bootstrap support, indicating moderate confidence in their evolutionary relationship. The remaining isolates (PZ766476, PZ766477, PZ766479, and PZ766481) occupied separate branches within the phylogeny, reflecting limited sequence variation among the feline-derived isolates. Overall, the phylogenetic analysis confirmed the genetic identity of the study isolates as *T. gondii* and indicated low genetic diversity among the analyzed partial SAG2 gene sequences.

Table 3: Univariable analysis of risk factors associated with *Toxoplasma gondii* infection in goats, sheep, cats, and humans.

Host	Factor	Category	Positive/ Total	Prevalence (%)	OR	95% CI	P-value
Goat	Gender	Male	25/250	10.00	Ref	—	<0.001
		Female	255/750	34.00	4.64	2.99–7.18	
	Age	< 1 year	70/350	20.00	Ref	—	<0.001
		1–2 years	110/350	31.43	1.83	1.27–2.63	
		> 2 years	100/300	33.33	2.00	1.37–2.91	
	Reproductive status	Pregnant	248/708	35.03	2.70	1.18–6.16	0.019
		Non-pregnant	7/42	16.67	Ref	—	
	Cat access to feed	Yes	230/700	32.86	2.44	1.72–3.46	<0.001
		No	50/300	16.67	Ref	—	
	Grazing system	Indoor	90/400	22.50	Ref	—	0.002
		Outdoor	190/600	31.67	1.60	1.19–2.16	
Sheep	Gender	Male	18/250	7.20	Ref	—	<0.001
		Female	212/750	28.27	5.08	3.02–8.56	
	Age	< 1 year	65/350	18.57	Ref	—	0.002
		1–2 years	75/350	21.43	1.20	0.83–1.73	
		> 2 years	90/300	30.00	1.88	1.30–2.71	
	Reproductive status	Pregnant	208/702	29.63	4.63	1.64-13.06	0.004
		Non-pregnant	4/48	8.33	Ref	—	
	Cat access to feed	Yes	170/700	24.29	1.28	0.92–1.79	0.14
		No	60/300	20.00	Ref	—	
	Grazing system	Indoor	60/400	15.00	Ref	—	<0.001
		Outdoor	170/600	28.33	2.25	1.60–3.18	
Cat	Gender	Male	2/80	2.50	Ref	—	0.70†
		Female	5/120	4.17	1.69	0.32–8.92	
	Age	< 1 year	1/40	2.50	Ref	—	0.70†
		1–3 years	2/80	2.50	1.00	0.09–11.37	
		> 3 years	4/80	5.00	2.05	0.22–19.00	
	Street access	Yes	5/120	4.17	1.69	0.32–8.92	0.70†
		No	2/80	2.50	Ref	—	
	Contact with other cats	Yes	7/180	3.89	Ref	—	1.00†
		No	0/20	0.00	Undefined	—	
	Cleanliness	Good	2/120	1.67	Ref	—	0.12†
		Poor	5/80	6.25	3.94	0.74–20.9	
Human	Age	18–25 years	13/60	21.67	Ref	—	0.352
		26–40 years	21/80	26.25	1.29	0.58–2.84	
		> 40 years	20/60	33.33	1.81	0.80–4.09	
	Reproductive status	Pregnant	34/100	34.00	2.06	1.09–3.91	0.027
		Non-pregnant	20/100	20.00	Ref	—	
	Cat owner	Yes	44/120	36.67	4.05	1.87–8.79	<0.001
		No	10/80	12.50	Ref	—	
	Knowledge of toxoplasmosis	Yes	15/50	30.00	Ref	—	0.581
		No	39/150	26.00	0.82	0.40–1.66	

For cats, positivity refers to microscopic detection of *T. gondii*-like oocysts in fecal samples. † P-values marked with a dagger were obtained using Fisher's exact test because of small, expected cell counts. For all other comparisons, crude odds ratios (ORs), 95% confidence intervals (CIs), and P-values were obtained from univariable logistic regression using the Wald test. The reference category was assigned an OR of 1.00. Where one category contained zero positive cases, the conventional OR could not be estimated and was reported as undefined. Statistical significance was considered at P<0.05

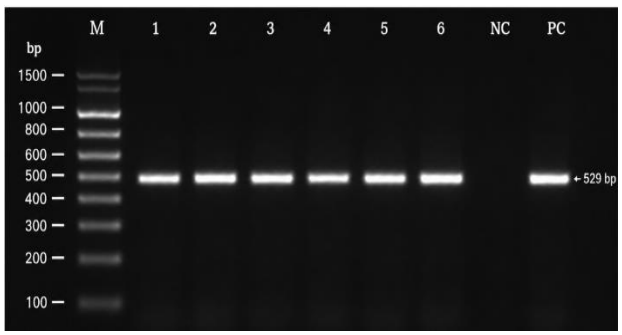

Fig. 2: Agarose gel electrophoresis showing amplification of the 529-bp repetitive element of *Toxoplasma gondii* using TOX4/TOX5 primers. M: 100-bp DNA ladder (molecular weight marker); lanes 1–6: PCR-positive feline samples; PC: positive control; NC: negative control.

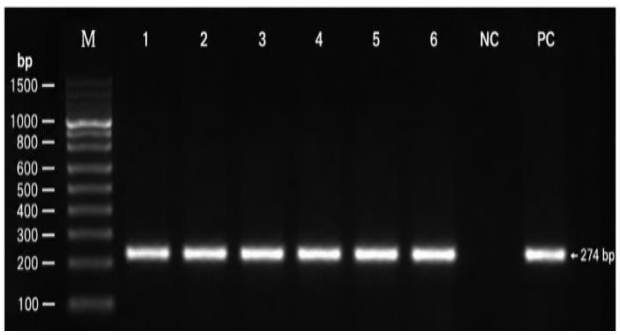

Fig. 3: Agarose gel electrophoresis showing amplification of the partial SAG2 gene of *Toxoplasma gondii*. M: 100-bp DNA ladder (molecular weight marker); lanes 1–6: SAG2-positive feline samples; PC: positive control; NC: negative control.

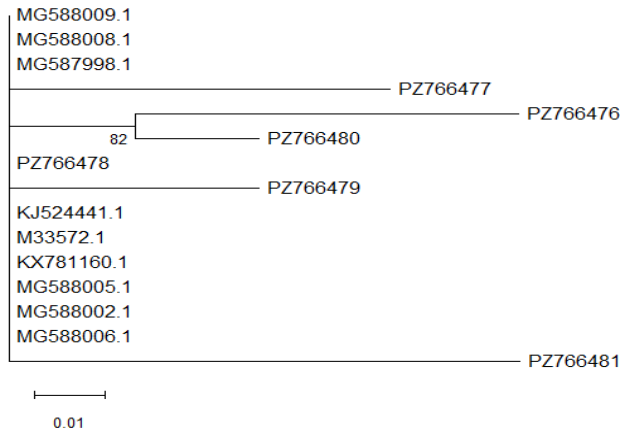

Fig. 4: Maximum Likelihood phylogenetic tree based on partial SAG2 gene sequences of six feline-derived *Toxoplasma gondii* isolates (PZ766476–PZ766481) and representative GenBank reference sequences. Bootstrap values (1000 replicates) are indicated at the internal nodes, and the scale bar represents 0.01 nucleotide substitutions per site.

DISCUSSION

The present study provides an integrated assessment of *T. gondii* infection in humans, small ruminants and cats in Lahore, Pakistan, from a One Health perspective. By combining serological, epidemiological, biochemical and molecular approaches, the study highlights the interconnected role of humans, livestock and feline hosts in the local epidemiology of toxoplasmosis. The detection of exposure in humans, sheep and goats, together with molecular confirmation of *T. gondii* in feline fecal samples, supports the importance of shared host environments and cat-associated contamination routes in parasite circulation. One Health perspectives increasingly emphasize an integrated surveillance system for zoonotic pathogens, such as *T. gondii*, especially in settings where humans, livestock, and domestic or stray cats have frequent contact (Aguirre *et al*., 2019; İnci *et al*., 2023). Such multidisciplinary approaches can improve understanding of transmission pathways and facilitate coordinated prevention and control strategies, particularly in endemic regions (Dubey, 2021; İnci *et al.,* 2023).

The overall seroprevalence observed in humans, goats, and sheep is 27%, 28%, and 23%, respectively, indicating substantial exposure to *T. gondii* in the study area. The prevalence observed in humans is comparable to reports from Upper Myanmar (23.5%) (Thái *et al*., 2019), pregnant women in Iran (27.5%) (Khademi *et al*., 2019), and previous studies from Pakistan (Ahmad *et al*., 2019). The differences in prevalence between geographical regions may be explained by variation in husbandry practices, grazing systems, environmental contamination with oocysts, climatic factors, and the number of definitive hosts. Because sheep and goats are important food-producing animals, their exposure to *T. gondii* has important implications for both livestock productivity and food safety, particularly through the consumption of raw or undercooked meat and animal products (Dubey *et al*., 2020; Stelzer *et al*., 2019). These findings support the need for continuous surveillance of food-producing animals as a part of integrated zoonotic disease control programs.

In the present study, several host- and management-related factors were significantly associated with seropositivity. Higher seroprevalence was observed in pregnant animals than in non-pregnant animals, and older animals showed greater exposure than younger animals. Similar observations have been reported previously in small ruminants and may be explained by cumulative environmental exposure over time, pregnancy-associated immunosuppression, lactation stress, physiological conditions and hormonal fluctuations (Stelzer *et al.,* 2019; Ahaduzzaman and Hasan, 2022; Khalife *et al.,* 2022). Outdoor grazing was associated with higher seroprevalence in both small-ruminant species, whereas cat access to feed was significantly associated with seropositivity in goats but not in sheep, suggesting that feline-derived oocyst contamination may contribute to transmission. In humans, higher seroprevalence among cat owners than among non-cat owners further supports the possible role of behavioral and environmental exposure in infection risk. Taken together, these findings emphasize the importance of the human–small ruminants–cat interface and indicate that several associated factors may be reduced through improved farm management, hygienic practices, and public awareness.

The present study also examined selected serum biochemical parameters in *T. gondii*-seropositive humans, sheep, and goats. Seropositive goats showed lower concentrations of total protein, albumin, globulin, blood urea nitrogen (BUN), and cholesterol, whereas seropositive sheep showed reduced albumin and increased total protein, globulin, BUN, and cholesterol. In humans, higher total protein, globulin, and BUN were observed, along with low albumin and cholesterol. Similar biochemical changes have been reported in previous studies (Al-Hussary and Al-Zuhairy, 2010; Al-Kaysi *et al*., 2010; Mahboub *et al*., 2013). These changes may reflect inflammatory or immune-metabolic responses, altered protein metabolism, hepatic involvement, or changes in lipid utilization associated with parasitic infection. Lower albumin concentrations observed in seropositive hosts may reflect reduced protein synthesis or increased protein loss. The lower cholesterol concentrations observed in goats and humans may be associated with altered host lipid utilization during infection, including the dependence of *T. gondii* on host-derived lipids for intracellular replication (Portugal *et al*., 2008). In addition, inflammatory mediators produced during infection may also influence lipid and protein metabolism, thereby contributing to the biochemical patterns observed (Khovidhunkit *et al*., 2004). However, these findings should be interpreted cautiously because the present study describes biochemical patterns associated with seropositivity rather than direct causal effects of infection.

Cats are the definitive hosts of *T. gondii* and play a central role in environmental contamination through oocyst shedding. In the present study, microscopy detected *T. gondii*-like oocysts in feline fecal samples, suggesting a potential route for environmental contamination in the study area. Similar findings have been reported in Pakistan (Nabi *et al*., 2018), China (Yang and Liang, 2015), and Iran (Khodaverdi and Razmi, 2019), while higher prevalence rates have been reported in Indonesia (Hanafiah *et al*., 2018) and lower prevalence rates in Europe, North America, and Kenya (Njuguna *et al*., 2017). Although oocyst shedding was detected in only a small proportion of

cats, its epidemiological importance remains considerable because a single infected cat can shed millions of oocysts, which may contaminate soil, water, pasture, and feed resources (Dubey, 2010; Shapiro *et al*., 2019). Therefore, even limited feline shedding may contribute to exposure to livestock and humans through shared environmental routes.

Molecular confirmation was performed using PCR targeting the repetitive 529-bp element of *T. gondii* followed by sequencing of the *SAG2* gene. The microscopic results were corroborated by molecular detection of *T. gondii* DNA in feline feces. Six of the seven microscopy-positive feline fecal samples were confirmed by PCR. This lower confirmation rate may be explained by false-positive microscopic identification of morphologically similar coccidian oocysts, low oocyst burden, PCR inhibitors in fecal material, or partial DNA degradation during sample processing and storage. Similar discrepancies between microscopy and PCR have been reported previously in studies of feline toxoplasmosis. Feline *T. gondii*-like oocysts or molecular evidence have also been reported in Pakistan (Nabi *et al.,* 2018), China (Yang and Liang, 2015), Iran (Khodaverdi and Razmi, 2019), Indonesia (Hanafiah *et al.,* 2018), Kenya (Njuguna *et al.,* 2017), and Turkey (Karakavuk *et al.,* 2021), although prevalence estimates vary with cat population and diagnostic method. Variations between studies could be attributed to differences in sample sizes, environmental conditions, cat management practices, geographical location and diagnostic sensitivity.

Partial *SAG2 s*equences confirmed the identity of all six feline-derived isolates as *T. gondii* and showed limited sequence variation among the analysed isolates. Recent molecular studies have demonstrated the use of the *SAG2* gene for the molecular characterization and phylogenetic comparison of *T. gondii* isolates (Nzelu *et al.,* 2021; Hafeez *et al.,* 2022). The phylogenetic analysis indicated that the study isolates clustered closely together, with PZ766478 and PZ766480 showing the closest relationship, while PZ766476 appeared relatively more divergent. These findings should be interpreted as SAG2-based variation among the six analysed feline-derived isolates, rather than broad genetic diversity of *T. gondii* circulating in Lahore and are consistent with previous molecular epidemiological studies that showed similar genetic differences between geographically distant parasites (Lass *et al*., 2019; Arefkhah *et al*., 2020). Nevertheless, molecular characterization using sequence-based approaches provides valuable information for understanding local parasite population circulation and supports the value of molecular surveillance within a One Health framework (Arefkhah *et al.,* 2020; Dubey, 2021).

Overall, the serological, epidemiological, biochemical, microscopic and molecular findings support the role of cats as an important component in the maintenance and transmission of *T. gondii* in the study area. The concurrent detection of exposure in humans and small ruminants, together with PCR-confirmed feline fecal infection, further reinforces the importance of coordinated control measures and the connection between *T. gondii* transmission and the need for a One Health perspective in disease control. These include improved farm biosecurity, restriction of cat access to livestock feed and water sources, public education on food hygiene and zoonotic transmission, responsible cat management and integrated surveillance programs. Such measures may help reduce environmental contamination, limit transmission to livestock and humans, and decrease the public health and economic burden of toxoplasmosis.

This study has some limitations. Environmental samples such as soil, water, pasture, and feed were not directly collected; therefore, environmental contamination was inferred indirectly from feline fecal oocyst detection. In addition, molecular characterization was based on six PCR-positive feline-derived isolates and partial SAG2 sequencing, which limits broader conclusions regarding genetic diversity. The biochemical findings should also be interpreted as associations with seropositivity rather than causal effects of infection.

Conclusions: The results of this study showed that human and animal populations in Lahore, Pakistan, are substantially exposed to *T. gondii*, highlighting the public and animal health significance of toxoplasmosis in the area. The combined use of serological, epidemiological, biochemical, molecular, and phylogenetic approaches confirmed the circulation of parasites and provided insights into several host- and management-related factors associated with infection. Molecular identification of feline isolates suggested limited genetic variation among the analysed *T. gondii* isolates while supporting the potential role of cats in environmental contamination. These findings highlight the urgent need for integrated One Health surveillance and coordinated control strategies to reduce the burden of toxoplasmosis, minimize economic losses in livestock production, and protect both animal and public health.

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Institutional Review Board Statement: The study was conducted in accordance with the ethical principles of the University of Veterinary and Animal Sciences (UVAS), Lahore, Pakistan, and was approved by the Institutional Ethical Review Committee (ERC) (Approval No. ERC-3355).

Informed Consent Statement: Written informed consent was obtained from all human participants prior to sample collection.

Data Availability Statement: The data supporting the findings of this study are available within the article. Additional data are available from the corresponding author upon reasonable request.

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