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Sero Prevalence and Molecular Characterization of Brucellosis in Goats

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ABSTRACT

The current study was conducted to identify the presence of brucellosis infection in goats in Punjab, Pakistan. A total of 61 animal herds suspected of brucellosis were randomly selected from Gujrat (n=44) and Jehlum (n=17) districts. Rose Bengal plate test (RBPT, screening test) and indirect enzyme-linked immunosorbent assay (i-ELISA) were performed from blood samples collected from these animals. Moreover, the samples were subjected to real-time polymerase chain reaction to identify various Brucella species in these goats. There observed a seropositivity of 5.9% (18/305) and 7.5% (23/305) using RBPT and i-ELISA, respectively. Moreover, PCR analysis further confirmed the presence of *B. abortus* in 4.2% samples. Gujrat district exhibited a higher seroprevalence compared to Jhelum region. These findings highlight the significant prevalence of brucellosis in goat emphasizing the need for further epidemiological investigations at individual animal and the herd levels to further surveillance of circulating Brucella spp. in goat, sheep, cattle and buffalo, as well as the zoonotic transmission of the pathogen. Combined blood analysis by RBPT and i-ELISA along with molecular detection by PCR confirm the presence of Brucella. The presence of anti-Brucella antibodies in the sera represents a significant risk to the consumers.

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INTRODUCTION

The dairy animals are considered as an important and foremost source of livelihood of people in Pakistan. However, the dairy animals face huge threats due to bacterial (Anwar et al. 2022; Du et al. 2022), viral (Hussain et al. 2022a; Jalees et al. 2022), parasitic (Asrar et al. 2022; Bhutta et al. 2022; Mahmood et al. 2022) and other zoonotic (Jamil et al. 2022; Rafique et al. 2022) diseases in the tropical and sub-tropical environmental conditions and diverse livestock operation systems. Brucellosis is caused by a facultative intracellular gram-negative bacterium belonging to genus Brucella. The disease poses a substantial threat to the livestock species, wildlife and humans across the globe (Hussain et al. 2022b). Although the developed countries have achieved effective prophylactic and control measures, the disease still remains endemic in many regions with notably high prevalence rates reported among humans in the Middle East and Central Asia (Abubakar et al. 2010). The Brucella infection causes specific clinical manifestations including fever, abortion, infertility in females and orchitis in male animals. Likewise, in humans, the disease is called as Mediterranean fever, Malta fever or undulant fever and is a common zoonotic endemic infection in the countries of Mediterranean, Middle East, Central Asia and America and Sub-Saharan Africa that cause severe acute headaches, fever, chills, myalgia, nausea, vomiting, diarrhea and ultimately weight loss (Chaidarun and Hutchinson 2022; Khan et al. 2020a). The genus Brucella encompasses 12 recognized species, each exhibiting varying host preferences, pathogenicity and epidemiological characteristics (Khan et al. 2020a). *Brucella melitensis* primarily affects goats and sheep

while *B. abortus* is prevalent in cattle and buffaloes, *B. canis* in dogs and *B. suis* in pigs with documented instances of cross-infection between animal species (Khan et al. 2019). The impact of brucellosis in livestock is profound that cause significant economic losses attributed to reduced fertility, abortion and diminished quality of livestock products.

The livestock sector holds immense significance in the economy of the country with sheep and goats accounting for populations of 32.3 million and 84.7 million, respectively and serve as a vital sources of meat, milk and wool in Pakistan (Pakistan Economic Survey 2023). However, brucellosis poses a substantial economic burden by affecting the health and production of sheep, goats, cattle and buffaloes. Despite its significance in severity, brucellosis still remains a neglected disease in Pakistan with under diagnosis and varying prevalence rates observed at different livestock settings across the country (Iqbal et al. 2020; Khan et al. 2020a; Hussain et al. 2022b). Moreover, *B. melitensis* is also known as the most pathogenic Brucella species for humans in Pakistan that is the primary cause of zoonotic brucellosis and is implicated, although less frequently, in acute febrile illness in clinically indistinguishable disease (Saddique et al. 2019; Yousaf et al. 2016).

Nevertheless, there is a dearth of data of seroprevalence specifically focusing on brucellosis in goats in Pakistan and the limited previous studies have reported an overall sero prevalence of 6.25% in cattle and buffaloes (Abubakar et al. 2010). Additionally, in the Punjab region, sero-prevalence rates of 1.47% and 1.94% have been documented in sheep and goat, respectively (Nasir et al. 2000; Saeed et al. 2019). Hence, the current study aimed to explore the seroprevalence and molecular identification of brucellosis in goat in Punjab; investigating 61 herds of only two selected districts of Gujrat and Jhelum.

MATERIALS AND METHODS

Study area and collection of blood samples

This study was conducted at Gujrat and Jhelum districts of Punjab province, Pakistan. Gujrat (32.6618°N, 73.9360 °E) is geographically bounded by the Chenab River in the East and South-East and Mirpur in the North-East and North-West. While Jhelum (32.9425°N, 73.7257°E) is located along the Jhelum river in the Punjab province. The predominant goat breeds in these areas are Beetal and Teddy, since the districts offer ample free land and fodder for grazing of goat herds. The goat herds with a history of abortion in females and swelling of testicles in males were selected. A total of 305 animals were randomly sampled from a total of 44 herds from Gujrat (218 samples) and 17 herds from Jhelum (87 samples) districts.

Ethical approval for animal experimentation

The blood samples were collected as per standard operation procedures from the animals of the farmers those are willing to participate in the study. No animals were killed or harmed during restraining process for blood collection. This study was reviewed and approved by the ethical committee of the College of Veterinary and Animal Sciences, Jhang, sub-campus of University of Veterinary and Animal Sciences, Lahore regarding the welfare and use of animals in the current research study (CVAS/ERC-CS-1976, Dated: 16-11-2021).

Blood collection and analysis

Approximately, 5 mL blood was collected from the jugular vein of each goat in EDTA free blood collection vacutainers having gel containing clot activating factors. The collected samples were kept in the icebox at 4°C and immediately shifted to the laboratory. After centrifugation at 5000 rpm for 5 minutes, the serum was separated and collected in sterile 1.5 mL Eppendorf tubes. The serum samples were then stored at -20°C for further analysis (Ayub et al. 2018; Malik et al. 2018; Qureshi and Ali 2016). Blood samples were analyzed to determine the antibodies against Brucella species using screenings test of Rose Bengal Plate Test (RBPT) and locally prepared antigens by Veterinary Research Institute, Lahore (Pakistan). While all the serum samples were screened by indirect Enzyme-Linked Immunosorbent Assay (i-ELISA) by using commercially available kits (IDVet Innovative Diagnostics, Grabels, France) according to instructions of the manufacturers.

Molecular detection and confirmation of Brucella spp.

All the collected sera samples were properly labeled and sent to the Friedrich-Loeffler-Institute, Jena (Germany) for confirmation of brucellosis. For molecular studies, DNA was extracted from all the serum samples using commercially available DNA extraction kit (QIAamp DNA Mini Kit, Qiagen, Germany) as described earlier (Ali et al. 2014a; 2014b). Real-time PCR technique was used for confirmation of DNA of different brucella species such as genus Brucella and other species (*B. abortus* and *B. melitensis*) by using specific primers. The primers used for *Brucella species* were: (Forward: GCT CGG TTG CCA ATA TCA ATG C, Reverse: GGG TAA AGC GTC GCC AGA AG and probe: 6-FAM-AAA TCT TCC ACC TTG CCC TTG CCA TCA-MGB), *B. Abortus* (Forward: GCG GCT TTT CTA CGG TAT TC, Reverse: CAT GCG CTA TGA TCT GGT TAC G and probe; Hex-CGC TCA TGC TCG CCA GAC TTC AAT G-BHQ1-3) and for *B. Melitensis* (Forward: AAC AAG CGG CAC CCC TAA AA, Reverse: CAT GCG CTA TGA TCT GGT TAC G and Probe; Cy5-CAG GAG TGT TTC GGC TCA GAA TAA TCC ACA) with primer sequences (5′-3′) as previously described (Probert et al. 2004).

Briefly, the PCR reaction contained a mixture of 5µl DNA template, 15µl multiplex PCR master mix (2× TaqMan™), 0.1µM of each probe and 0.2µM of specific primer. The PCR conditions included the initial process of denaturation at 95°C for 7 min followed by 50 cycles of PCR; each composing of denaturation at 95°C for 25sec, annealing at 57°C for 60sec according to earlier protocols with slight modifications (Dupain et al. 2016; Urbinati et al. 2016). The results were analyzed and visually inspected with the help of graphical plots and the cycle threshold (CT) values obtained in each cycle for each sample (Urbinati et al. 2015). All the CT values lower that 38 were considered as positive for confirmation of target DNA. *B. abortus* S-99 (ATCC 23448) and *B. melitensis* 16M (ATCC 23456) strains were used as positive controls.

Statistical Analysis

The collected data were subjected to SAS 9.1 statistical software. For epidemiological investigations, the data on various epidemiological factors were analyzed using Chi-square analysis.

RESULTS

In total, 5.9% (18/305) and 7.5% (23/305) seropositivity was observed using RBPT and i-ELISA, respectively. Only 4.2% sera were found positive using RT-PCR and identified as *B. abortus*. In district Gujrat, 7.4%, 7.9% and 4.6% sera were found positive using RBPT, i-ELISA and RT-PCR, respectively. While in the district Jhelum, 2.4%, 5.8% and 2.4% female animals were detected positive using RBPT, i-ELISA and RT-PCR, respectively. Only 1 sample of male animal from district Jhelum was found positive using both i-ELISA and PCR. Hence, the higher prevalence of brucellosis was observed in the Gujrat district as compared to Jhelum (Table).

DISCUSSION

Brucellosis is a widespread zoonotic and contagious disease and occurs in many parts of the world including Pakistan and poses a significant threat to the health of both animals and humans (Hussain et al. 2022b). Different farm animals and wildlife species including cattle, buffalo, sheep, goats, camels, horses, dogs, bison, African buffalo and Alpine ibex have been identified as carriers of different Brucella species highlighting their role in the spread of the disease (Godfroid et al. 2013; Godfroid 2017; Musa et al. 2008; Jamil et al. 2019; Rabah et al. 2022). The humans acquire the infection either through direct exposure to the infected animals or by consuming unpasteurized milk and dairy products contaminated with the pathogen (Saddique et al. 2019).

Unfortunately, the lack of diagnostic facilities and limited resources in the developing countries hinder large-scale efforts of disease surveillance. In Pakistan, most of the laboratories rely on fast and economical serum agglutination tests to diagnose brucellosis (Hassan et al. 2022). Although, these tests provide valuable information, however, the definitive diagnosis often requires time-consuming procedures that carry risks for infection of laboratory personnels (Khan et al. 2020b). To overcome these challenges, it is therefore recommended to screen-out the disease using serological tests such as RBPT, SAT and ELISA along with molecular detection of Brucella DNA especially in the developing countries (Abnaroodheleh, et al. 2023; Godfroid et al. 2010; Legesse et al. 2023).

In a recent study, the researchers investigated the sero prevalence of brucellosis at the herd level in 789 goats and revealed a high prevalence (17.36%) of brucellosis in all three districts with an average herd size of over 250 goats (Teshome et al. 2022). In another study conducted near Pakistan Afghanistan border, the seropositivity was found to be 0.99%; 18 / 1821 (1196 from sheep and 625 from goats) for anti-Brucella antibodies (*B. abortus* and *B. melitensis*) by RBPT and indirect ELISA (Jamil et al. 2020). Moreover, seroprevalence was found to be 3.4% and 0.8% in male and female goat, respectively by RBPT in four districts of central Punjab, Pakistan (Saeed et al. 2020). These findings could be compared with data from India where brucellosis is also common in different livestock species, wherein a study revealed 1.73% and 1.04% of brucella in goats by RBPT and i-ELISA tests, respectively (Meena et al. 2023). Additionally, similar reports from neighboring areas of districts Jhelum and Gujrat including Rawat, Islamabad and Kherimurat also showed increasing trends in small ruminant brucellosis with sero-prevalence rates of 8.6% by RBPT and 9.4% by milk ring test in sheep and goats (Ali et al. 2015). While, among the 61 animals tested for brucellosis in our study, which had a history of abortion and were suspected for infection, a higher prevalence was observed in the goats from Gujrat (39.5%) compared to Jhelum district (33.3%). The variations in the rate of infection of both districts may be attributed to the difference in the climatic conditions of the two districts and the management and husbandry practices of rearing small and large ruminants together. Similarly, a high prevalence rate of brucellosis in goats with a history of abortion have also been reported in India (Saikia et al. 2019), China (Zhou et al. 2021), Uganda (Miller et al. 2016) and Iran (Adabi et al. 2022). Moreover the infection often leads to high abortion rates and other reproductive disorders like metritis, still births or birth of weak kids (Bhandi et al. 2019).

Since, *B. melitensis* is the primary pathogen responsible for brucellosis in sheep and goat, however, *B. abortus* can also cause infection in these animals particularly when raised with large animals. The diseased animals not only suffer but also serve as potential sources of infection for healthy animals, perpetuating the transmission cycle. Moreover, animals without apparent signs of illness can also act as carriers, potentially shedding brucella into the environment and posing ongoing risk factors by sharing pastures, mixing animal types and uncontrolled movement contribute to the spread of infection beyond the preferred hosts (Wareth et al. 2015). In the study area, *B. abortus* was also found to be involved in goat brucellosis, aligning with several previous reports that highlight its endemicity in Pakistan (Abubakar et al. 2012; Ali et al. 2015; Fatima et al. 2016; Rehman et al. 2017; Saddique et al. 2019). Interestingly in the study, *B. melitensis* infection evidence was not found, contrary to previous reports. However, the majority of studies conducted in Pakistan consistently point-out to *B. abortus* as the endemic and primary pathogen

Table: Sero prevalence and molecular identification of *Brucella* DNA from goats.

District	Sex	No. of samples	Brucella positive sera			Molecular identification	
			RBPT No. (%)	I-ELISA No. (%)	rt-PCR No. (%)	DNA of Brucella spp.	CT-values
Gujrat	Female	215	16 / 215 (7.4%)	17 / 215 (7.9%)	10 / 215 (4.6%)	*B. abortus*	30, 28, 29, 28, 28, 30, 30, 32, 28, 30
Jhelum	Female	85	2 / 85 (2.4%)	5 / 85 (5.8%)	2 / 85 (2.4%)	*B. abortus*	29, 25
	Male	05	0 / 5 (0%)	1 / 5 (20%)	1 / 5 (20%)	*B. abortus*	28
	Total	305	18 / 305 (5.9%)	23 / 305 (7.5%)	13 / 305 (4.2%)		

causing brucellosis in both domestic animals and humans (Ali et al. 2015; Jamil et al. 2019; Saddique et al. 2019). Given the potential risk of human brucellosis from brucella circulating in the herds of small and large ruminants, it was therefore crucial to determine the prevalence of brucellosis and to identify the associated risk factors to understand the epidemiological status of the disease.

Conclusions

In conclusion, brucellosis continues to be a persistent and concerning infection within animal herds in Pakistan, with *B. abortus* being identified as the primary causative agent of caprine brucellosis. The combined analysis of blood samples by RBPT (5.9%) and i-ELISA (7.5%) along with molecular detection by PCR (4.2%) confirmed the presence of Brucella in goats of Punjab. The detection of anti-Brucella antibodies in animal sera represents a significant risk to the consumers, highlighting the importance of this issue to be addressed promptly. To effectively combat brucellosis in developing countries like Pakistan, urgent prophylactic measures are quite necessary including the elimination of positive sero-reactors as well as development and implementation of robust control strategies tailored specifically to brucellosis. In this regard, equally important are the education programs aimed at enhancing the awareness regarding the control and prevention of zoonotic diseases. By emphasizing these crucial aspects elimination of positive sero-reactors, strategic control measures and farmer education make significant progress in mitigating the impact of brucellosis. Moreover, it is imperative for governments, stakeholders and communities to collaborate closely in implementing comprehensive approaches to effectively combat the infection.

DECLARATION

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Conflict of interest: The authors affirm that they have no conflict of interest to disclose.

Data Availability: All the data is included in this manuscript.

Ethics Statement: This research was carried out according to following the guidelines regarding use of animal for research purpose.

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