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Seroprevalence of brucellosis in cows and buffaloes

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Abstract
Brucellosis poses a significant zoonotic risk to humans and animals, and is caused by the Gram-negative bacteria of the *Brucella* species. In cows and buffaloes, infection is typically attributed to *Brucella abortus,* often resulting in abortion during late pregnancy and posing a subsequent risk of sterility in females. The current study aimed to assess the seroprevalence of brucellosis in cows and buffaloes in district Kasur, Pakistan. A total of 400 blood samples were randomly collected from cows and buffaloes in the district. The Rose Bengal Plate Test (RBPT) was initially employed for screening the blood serum samples of each animal, with Indirect ELISA serving as a confirmatory test. Higher seroprevalence rates were observed in females compared to those in males in both tests. Sex-wise, higher incidence of brucellosis in cows was observed, with a prevalence of (7%)/(5%) in females compared to that (2%)/(1%) in males, as detected by the RBPT and iELISA tests, respectively. Similarly, buffaloes exhibited a higher prevalence of brucellosis in females, with rates (10%)/ (8%) compared to (3%)/(1%) in males, via RBPT and iELISA, respectively. A total of 200 cow and 200 buffalo samples were analyzed, that revealed a seroprevalence of (4.5%)/(3%) in cows and (6.5%)/(4.5%) in buffaloes, respectively, using RBPT and iELISA. Overall, out of a total of 400 serum samples, 22 from cows and buffaloes were tested positive through RBPT, while 15 samples from both species were seropositive via iELISA. The overall seroprevalence of brucellosis in the district was found to be 5.5%/3.75% through RBPT and iELISA, respectively. This study highlights a significant challenge for the livestock being rared in district Kasur.

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Introduction

Livestock holds a significant importance in the livelihoods of rural communities. It is estimated that over 60% of people reside in pastoral regions and largely depend on livestock as their primary occupation. According to the Government of Pakistan (GOP), about 8 million countryside people are connected with the livestock sector. Livestock is the main source of income of 35-40% of the population in the rural areas of Pakistan. In Pakistan, the dairy sector plays a crucial role in the economy, with dairy

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products holding greater value than that of the cotton and wheat sectors. The country's economic system relies heavily on agriculture, which contributes approximately 26% to the gross domestic product (GDP) of Pakistan. Livestock, as a subsector of agriculture, accounts for approximately 11.9% of Pakistan's GDP (Rehman et al., 2015; Rehman et al., 2017; Jamal et al., 2022).

Brucellosis, a highly zoonotic bacterial infection caused by *Brucella abortus*, affects livestock, humans, and wild animals worldwide (McDermott et al., 2013; Mansour, 2022; Awais et al., 2024). Although livestock productivity has a considerable impact on the country's economy, various diseases such as, trypanosomiasis, foot and mouth disease, brucellosis, etc. adversely affect the productivity (Khan et al., 2018, Imtiaz et al., 2018, Hussain et al., 2020; Mansour, 2022). In cows, brucellosis is caused by *Brucella abortus*, in goats by *Brucella melitensis*, in pigs by *Brucella suis* and in sheep by instigation of *Brucella ovis* (Ali et al., 2013; Ali et al., 2017). Brucellosis is widely spread particularly in underdeveloped countries due to lack of aseptic measures (Farouk et al., 2017; Awais et al., 2024).

Healthy animals sharing water reservoirs with diseased animals is a significant factor contributing to the transmission of infection (Tukana and Gummow, 2017). In livestock, the infection shows delivery of unhealthy newborns, reappearance of breeding disorder, and feebleness due to joint inflammation in animals (Jajere et al., 2016). It instigates key propagative complications such as feticide and fragile progeny in fertile organisms (Shahzad et al., 2017). In cows and buffaloes, hazard aspects contain gender, breed, age and bad ranch supervision systems (Tasiame et al., 2016; Zeng et al., 2017). Mixed farming poses numerous risk factors for animals of different species sharing the same grassland, leading to the transmission of *Brucella abortus* to bovines and ruminants (Khan et al., 2021).

Different techniques are used for the investigation of brucellosis in animals like serological, molecular and culture procedures. Serological methods are very effective for investigating the incidence of brucellosis (Kumar et al., 2018). So, various procedures accessible for identification of brucella are Rose Bengal Test (RBT), Milk Ring Test (MRT), Enzyme-Linked Immunosorbent Assay (ELISA) and complement fixation test (CFT) (Islam et al., 2013; Ducrotoy et al., 2018). No treatment of the disease is available throughout the world. Control is possible through vaccination that is a very helpful method to stop the transmission of brucellosis (Kauffman et al., 2016). Thus, the major objective of the present investigation was to appraise the seroprevalence of brucellosis in cows and buffaloes using the state-of-the-art techniques, RBPT and iELISA.

Materials and Methods

Study area

The current research was conducted in district Kasur, Punjab, Pakistan, covering four tehsils, Kasur, Chunian, Pattoki, and Kot Radha Kishan.

Blood sample collection and preservation

A total of 400 blood samples were randomly collected from cows and buffaloes equally across four tehsils (100 samples from each tehsil; 50 male and 50 female; 10 samples from each farm) in district Kasur. Fifty blood samples each from cows and buffaloes were collected randomly from each tehsil (**Table 1**). Before the collection of blood samples, a questionnaire was filled by interviewing the farm supervisor in order to observe the risk factors. Prior to blood sample collection, meetings were organized with farmers to gather data regarding risk factors. After completing the questionnaire, approximately 5 mL of blood were drawn from each animal, using a 10 mL disposable syringe via venipuncture. The blood was then promptly transferred to plain tubes without anticoagulant for further processing. The collected blood samples were kept in an icebox to avoid degradation and shifted to the laboratory of The University of Lahore for further analysis. The serum was separated from the collected blood samples by centrifugation and kept at -20 °C for further analysis.

Serum analysis

The serological analysis of the collected serum was conducted using Rose Bengal Plate Test (RBPT), and a confirmatory test was performed using the indirect ELISA. The RBPT was used as a primary screening tool and the indirect ELISA was used as a confirmatory test (Khan et al., 2017).

Table 1: Sample collection from different farms across tehsils in district Kasur

Tehsil	Farms	Samples collected from cows			Samples collected from buffaloes		
		Male	Female	Total	Male	Female	Total
Kasur	A	5	5	10	5	5	10
	B	5	5	10	5	5	10
	C	5	5	10	5	5	10
	D	5	5	10	5	5	10
	E	5	5	10	5	5	10
Chunian	F	5	5	10	5	5	10
	G	5	5	10	5	5	10
	H	5	5	10	5	5	10
	I	5	5	10	5	5	10
	J	5	5	10	5	5	10
Pattoki	K	5	5	10	5	5	10
	L	5	5	10	5	5	10
	M	5	5	10	5	5	10
	N	5	5	10	5	5	10
	O	5	5	10	5	5	10
Kot Radha Kishan	P	5	5	10	5	5	10
	Q	5	5	10	5	5	10
	R	5	5	10	5	5	10
	S	5	5	10	5	5	10
	T	5	5	10	5	5	10

Rose Bengal Plate Test (RBPT)

Rose Bengal Plate Test was applied for diagnosing the *Brucella abortus*. *Brucella abortus* stained antigen for RBPT was used at room temperature. A 30 µL drop of *B. abortus* stained antigen was taken by a micropipette and placed on a glass slide. Then a drop (30 µL) of the serum was taken by a micropipette and added on that slide. The antigen and serum were mixed in a circular movement with the help of a glass rod. Both antigen and serum were rocked at room temperature for 2-4 minutes and the slides were checked on a colony counter, and a sign of agglutination was recorded as positive (**Figure 1a**) and sign without agglutination on the slide was recorded as negative (**Figure 1b**) (Teng et al., 2017).

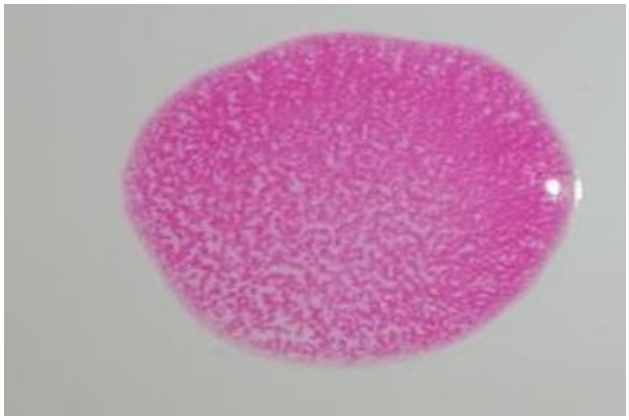

Figure 1a. Agglutination in RBPT

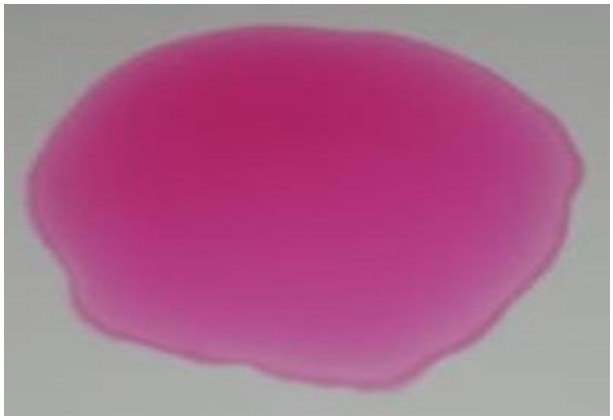

Figure 1b. No agglutination in RBPT

Indirect ELISA (iELISA)

The contents of the ELISA Kit were stored at 4-8 °C until use, while all kit chemicals and serum samples were maintained at 21 °C prior to the commencement of the experiment. First, 190 µL of dilution buffer-2 were added to in each of all wells in 96 well plates. An aliquot of 10 µL of negative/ positive control was appended into every well (A1 and B1 wells and C1 and D1wells). Then, all the protocol was performed by the protocol of BRERM and optical density of the wells was read out at 450 nm using the Bio-Rad ELIZA reader machine (BRERM) (**Figure 2**) as described elsewhere (Naik et al., 2017; Khan et al., 2017).

Figure 2. Yellowish colour shows positive iELISA for *Brucella abortus*

Results

Overall seroincidence of brucellosis in cows and buffaloes in district Kasur via RBPT

With the aim of assessing overall seroincidence of brucellosis in cows and buffaloes, 400 serum samples of both animal species (200 serum samples of cows and 200 of buffaloes) were screened and analyzed using the RBPT as shown in **Table 2**.

Overall iELISA-based seroprevalence of brucellosis in cows and buffaloes from district Kasur

Overall seroprevalence of brucellosis in both species was calculated via indirect enzyme linked immunosorbent assay (iELISA). The results showed 3% and 4.5% seroprevalence in cows and buffaloes, respectively, while overall seroprevalence in both was 3.75% (**Table 3; Figure 4**).

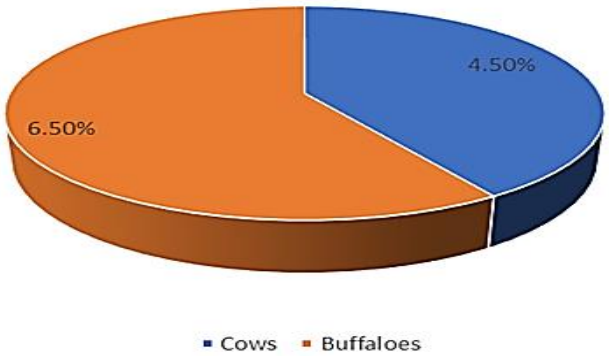

Figure 3. Seroprevalence (%) via RBPT of brucellosis in cows and buffaloes

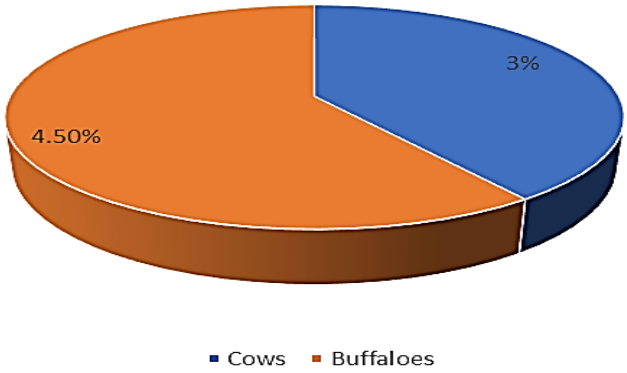

Figure 4. Seroprevalence of brucellosis in cows and buffaloes via iELISA

Table 2. Overall RBPT-based sero-prevalence (%) of brucellosis in cows and buffaloes from district Kasur

Animals	Hosts Tested	RBPT +ve	RBPT -ve	Prev (%)
Cows	200	9	191	4.5
Buffaloes	200	13	187	6.5
Total	400	22	378	5.5

Table 3. Overall iELISA-based seroprevalence (%) of brucellosis in cows and buffaloes from district Kasur

Animals	RBPT (+ve)	iELISA (+ve)	iELISA (-ve)	Perv (%)
Cows	9	6	3	3.00
Buffaloes	13	9	4	4.50
Overall	22	15	7	3.75

RBPT sample (200 for both test subjects); iELISA samples (cows =194; buffaloes =191)

Sex-wise seroprevalence of brucellosis in cows from district Kasur

RBPT indicated 7% incidence in females and 2% in males, while iELISA showed 5% incidence in females and 1% in males as shown in **Figure 5**.

Sex-wise seroprevalence of brucellosis in buffaloes from district Kasur

With respect to sex-wise prevalence of brucellosis in buffaloes, RBPT showed 10% incidence in

females and iELISA presented 8% incidence in females, while 3% incidence was recorded in males by RBPT and 1% incidence in males via iELISA (**Figure 6**).

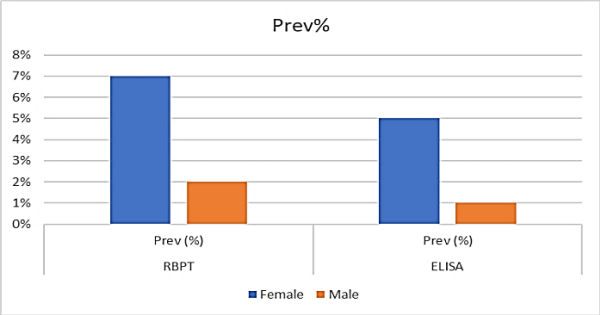

Figure 5. Sex-wise prevalence in cows via RBPT and iELISA

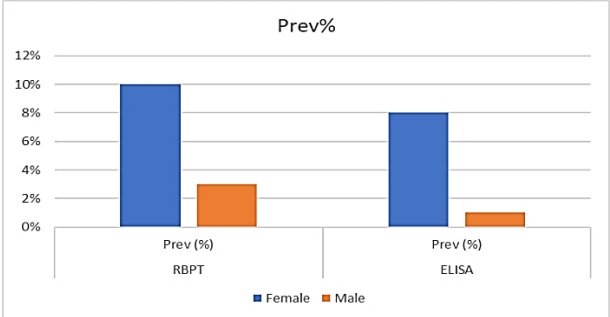

Figure 6. Sex-wise prevalence in buffaloes via RBPT and iELISA

Seroprevalence of brucellosis in cows in four tehsils of district Kasur using RBPT

Using RBPT, it was observed that seroprevalence of brucellosis was 6%, 4%, 2% and 6% in tehsils Kasur, Chunian, Pattoki and Kot Radha Kishan, respectively (**Figure 7**).

Seroprevalence of brucellosis in cows in four tehsils of district Kasur using iELISA

Using iELISA, seroprevalence of brucellosis was 4% ,4%, 2% and 2% in tehsils Kasur, Chunian, Pattoki and Kot Radha Kishan, respectively, as shown in **Figure 8**.

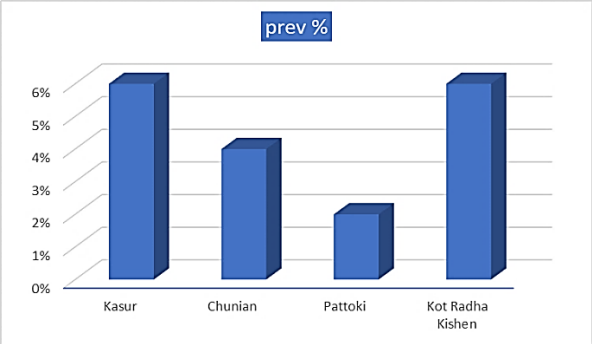

Figure 7. Tehsil-wise incidence of brucellosis in cows via RBPT

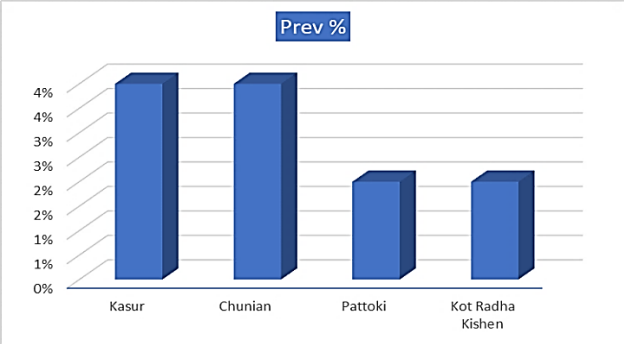

Figure 8. Tehsil-wise incidence of brucellosis in cows via iELISA

Seroprevalence of brucellosis in buffaloes in four tehsils of district Kasur using RBPT

Using RBPT, seroprevalence of brucellosis in buffaloes was 8%, 4%, 6% and 8% in tehsils Kasur, Chunian, Pattoki and Kot Radha Kishan, respectively (**Figure 9**).

Seroprevalence of brucellosis in buffaloes in four tehsils of district Kasur using iELISA

The iELISA test showed 6%, 4%, 6% and 2% seroprevalence of brucellosis in buffaloes in tehsils Kasur, Chunian, Pattoki and Kot Radha Kishan, respectively (**Figure 10**).

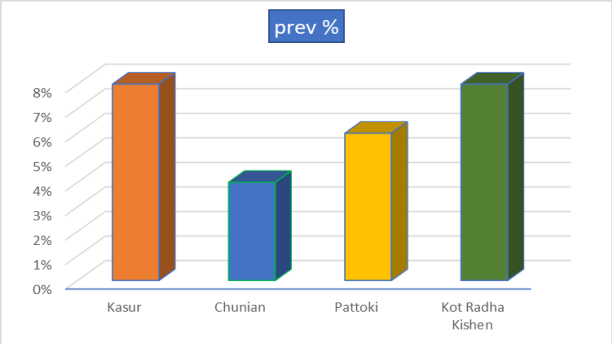

Figure 9. Tehsil-wise incidence of brucellosis in buffaloes via RBPT

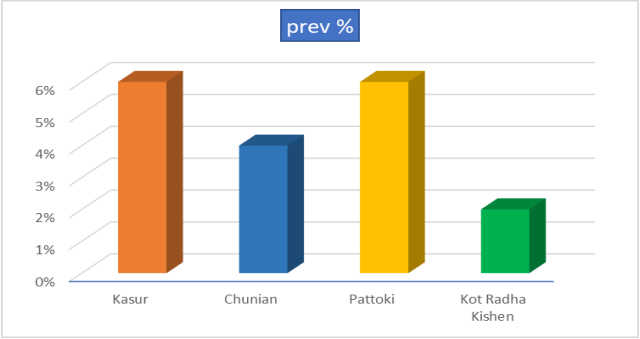

Figure 10. Estimated tehsil-wise incidence in buffaloes via iELISA

Risk factors associated with prevalence of brucellosis in cows from district Kasur

The present study assessed the risk factors associated with the prevalence of brucellosis in cows in district Kasur. Farmers with higher education levels exhibited lower risks compared to those with lower education levels, likely due to awareness of brucellosis. Additionally, sharing of animals in farms was associated with higher risks of brucellosis as compared to not sharing animal farms, likely due to increased contact with other animals. Similarly cleaned water reservoirs have shown the lower risks of brucellosis than that of the contaminated water reservoirs. Induction of new species in farms showed the higher risks of brucellosis than those farms in which new species were not introduced. Mutual browsing with other farm animal species exhibited the higher risks than the no-mutual browsing with other farm animal species due to increase in number of animals (**Table 4**).

Table 4. Risk factors associated with prevalence (Prev) of brucellosis in cows in district Kasur

Risk factors	Hosts examined	Positive samples	Prev (%)
Education status of farmers	125	1	0.80
Non-education status of farmers	75	5	6.67
Sharing of animals	105	4	3.81
Non-sharing of animals	95	2	2.11
Cleaned water reservoirs	103	1	0.97
Non-cleaned water reservoirs	97	5	5.15
Introduced new species in farms	115	4	3.48
Nonintroduced new species in farms	85	2	2.35
Mutual browsing with other farm animal species	153	5	3.27
No-mutual browsing with other farm animal species	47	1	2.13

Risk factors associated with prevalence of brucellosis in buffaloes from district Kasur

Education status of farmers revealed lower risks than those from the non-education status of farmers due to much awareness regarding infectious brucellosis in buffaloes. Sharing of animals demonstrated the higher risks of brucellosis in buffaloes than that of the non-sharing of animals, because of close contact with other animals. Cleaned water reservoirs were associated with lower risks of brucellosis in buffaloes as compared to those that were not using cleaned water. Introduction of new species in farms was linked to higher risks of brucellosis compared to farms where no new species was introduced, as new species can act as carrier of *Brucella* spp. Mutual browsing with other farm animal species indicated higher risks than that by non-mutual browsing, likely due to increased number of animals and farmers sharing common grazing areas, leading to an elevated risk of brucellosis in buffaloes (**Table 5**).

Table 5. Risk factors associated with prevalence of brucellosis in buffaloes from district Kasur

Risk factors	Hosts examined	Positive samples	Prev (%)
Education status of farmers	103	3	2.91
Non-education status of farmers	97	6	6.19
Sharing of animals	133	7	5.26
Non-sharing of animals	67	2	2.99
Cleaned water reservoirs	110	3	2.73
Non-cleaned water reservoirs	90	6	6.67
Introduced new species in farms	117	7	5.98
Non-introduced new species in farms	83	2	2.41
Mutual browsing with other farm animal species	120	6	5.00
No-mutual browsing with other farm animal species	80	3	3.75

Discussion

In the present study, higher seroprevalence of brucellosis was recorded in females than that in males; in females it was (7%), and (5%), and in males (2%) and (1%) detected via RBPT and iELISA, respectively. Sex-wise prevalence in cows can be compared with other prevalent countries. In Gujarat India, lower sex-wise incidence in males (18.81%)/(9.90%) than that in females (20.71%)/(14.47%), was recorded via the RBPT and iELISA tests, respectively (Shrimali et al., 2019). Another study in other districts of Punjab, Pakistan recorded greater prevalence in females (26.99%)/(25.57%) than that in males (17.52%)/(17.52%) through RBPT and c-ELISA (Shahzad et al., 2017). The results recorded by Munir et al. (2011) agree with those of the present study, e.g., they recorded 7.9 % prevalence of brucellosis in females and 1.6% in males through RBPT, while iELISA showed higher predominance in females (13.2%) than that in males (1.3%) in Punjab, Pakistan.

In the present research, sex-wise prevalence of brucellosis in buffalo's females was (10%)/(8%), while in males it was observed as 3%/1% via RBPT and iELISA, respectively. Similar results have been reported in other districts of Punjab, Pakistan, in which higher prevalence of brucellosis had been reported in females (41.81%)/(36.96%) than that in males (6.66%)/(6.66%), respectively (Shahzad et al., 2017). Another previous study in Punjab, Pakistan showed similar findings by recording 5.62% prevalence in females and 4.64% in males via the RBPT and iELISA (Jamil et al., 2020). Munir et al. (2011) also recorded similar findings, e.g., higher incidences through RBPT in females revealed 7.9% prevalence than 1.6% in males, while iELISA showed higher predominance in females as 13.2% and only 1.3% in males in Punjab, Pakistan. The findings of Jamil et al. (2020) were also similar to those recorded in the present investigation as they reported higher prevalence (5.6%)/(4.7%) in buffaloes than that in cows (2.2%)/(1.9%) through RBPT and iELISA, respectively, in Punjab, Pakistan.

Using RBPT, Ismail et al. (2018) documented overall incidence as 4.4% in cows (13/250) and 5.2% in buffaloes (11/250) in district Rajanpur. However, the iELISA showed 1.2% overall prevalence of brucellosis in cows and 2% in buffaloes in the same district. Another study exhibited lower prevalence in cows (5.06%) than that in buffaloes (7.74%) (Abubakar et al., 2010). Various studies have shown that different factors such as non-education status, sharing of animals, mutual browsing, non-cleaned water reservoirs and introduction of new species in farms cause higher risks of brucellosis in cows and buffaloes (Matope et al., 2010; Godfroid et al., 2013; Khurana et al., 2021; Mansour, 2022; Awais et al., 2024).

Conclusion

The study conducted in the four tehsils of district Kasur, Pakistan, revealed overall brucellosis seroprevalence of 5.5% and 3.75% detected through RBPT and iELISA, respectively. This highlights a significant challenge to both local livestock farmers, as brucellosis poses threats not only to animal health, but also to economic stability and public health. Various risk factors, including lack of education, animal sharing, mutual browsing, uncleaned water reservoirs, and introduction of new species in farms, contribute to higher risks of brucellosis in both cows and buffaloes at the agricultural and economic scale.

Author(s), Editor(s) and Publisher's declarations

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Supplementary material
No supplementary material is included with this manuscript.

Conflict of interest
The authors declare no conflict of interest.

Source of funding
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Contribution of authors
Planning and conduction of experiment: TA, SH, SA, AI. Research supervision: SH, SA. Data acquisition, analysis, and interpretation: TA, SH. Write-up of initial draft: TA, SH, SA, AI. Review of final draft: TA, SH, SA, AI.

Ethical approval
Ethical approval to work on animals was obtained from the Institute (IMBB) Ethical Review Committee vide Ref. # IMBB/BBBC/20/245), The University of Lahore, Lahore, Pakistan. A formal permission for obtaining blood samples from animals at different farms was also obtained from the respective farm managers.

Handling of bio-hazardous materials
The authors certify that all experimental materials were handled with care during collection and experimental procedures. After completion of the experiment, all materials were properly discarded to minimize/eliminate any types of bio-contamination(s).

Availability of primary data and materials
As per editorial policy, experimental materials, primary data, or software codes are not submitted to the publisher. These are available with the corresponding author and/or with other author(s) as declared by the corresponding author of this manuscript.

Authors' consent
All contributors have critically read this manuscript and agreed to publish in IJAaEB.

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It is declared that we the authors did not use any AI tools or AI-assisted services in the preparation, analysis, or creation of this manuscript submitted for publication in the International Journal of Applied and Experimental Biology (IJAaEB).

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