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RESEARCH ARTICLE

Seroprevalence and Identification of Risk Factors Associated with Bovine Brucellosis in Faisalabad Division, Punjab, Pakistan

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ABSTRACT

Brucellosis is a major zoonotic disease, causing significant losses in livestock production, especially in low- and middle-income countries. In Pakistan, epidemiological information on bovine brucellosis is scattered, preventing effective disease control. In this study, the seroprevalence of bovine brucellosis was determined, and the associated risk factors were identified in Faisalabad Division, Punjab, Pakistan. The study was designed as a cross-sectional study, with a sample size of 1,000 collected from four districts: Faisalabad, Toba Tek Singh, Jhang, and Chiniot. Rose Bengal Plate Test (RBPT), Serum Agglutination Test (SAT), and indirect ELISA (iELISA) were used to detect anti-*Brucella* antibodies in serum. Epidemiological data were collected and analyzed using bivariate analysis and multivariable analysis. Seropositivity was 17.90% by RBPT, 16.30% by SAT and 14.70% by iELISA. Inter-test agreement was highest between RBPT & SAT (95.8%), followed by SAT & iELISA (93.4%) and RBPT & iELISA (92.4%). Multivariable analysis using generalized linear mixed-effects models identified larger herd size, older age (>8 years), male sex, pregnancy status, and repeat breeding history as significant independent risk factors for seropositivity ($P<0.05$). These findings confirm that bovine brucellosis is endemic in Faisalabad Division and highlight the need for active surveillance, strategic vaccination, and improved farm biosecurity.

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INTRODUCTION

In Pakistan, livestock is a key driver of the national economy, contributing 62.4 percent of agriculture's value addition and 14.6 percent to the overall GDP. The country has an estimated population of 61.96 million cattle and 49.1 million buffalo (Pakistan Economic Survey, 2025-26), placing it among the 4th largest global milk producers (Aranguiz & Spoelstra, 2025). Punjab, as the largest agricultural province of Pakistan, contributes a major share of livestock production. Faisalabad Division plays a central role as the largest contributor to Punjab's agricultural output and a major socio-economic hub. The region is a focal point of the "Comprehensive Agriculture Transformation Plan", which aims to shift toward high-value, export-oriented livestock systems. However, productivity is declining due to weak disease control and management (Agriculture Development Plan, 2023).

Among the diseases hindering this productivity, brucellosis stands out as a critical infectious zoonotic disease. Approximately 1.6-2.1 million new human cases

of brucellosis are reported annually across the globe (Laine *et al.*, 2023). Its persistent burden reflects gaps in surveillance, diagnostics, and coordinated control programs. Effective management requires integrated approaches that link animal and human health systems within a One Health framework (Moreno *et al.*, 2022).

Brucellosis is caused by Gram-negative bacteria of the genus *Brucella*. These facultative intracellular pathogens persist within host cells, which enables immune evasion and establishes chronic infection (Corbel, 2020; Jamil *et al.*, 2021). The disease is marked by intermittent bacterial shedding and diagnostic challenges, both of which hinder timely detection and effective control (Khurana *et al.*, 2021).

Transmission occurs primarily through exposure to infected reproductive materials, including aborted fetuses, placental tissues, uterine discharges, and through the consumption of raw milk of infected animals. Vertical transmission and the use of infected semen further contribute to disease spread (González-Espinoza *et al*., 2021). *Brucella* exhibits a strong affinity for the reproductive system of sexually mature animals. In females, infection leads to late-term abortion, retained placenta, and reduced fertility. In males, it causes epididymitis and orchitis, often resulting in permanent infertility (Ullah *et al.*, 2019). These reproductive losses translate into substantial economic damage through reduced herd productivity, decreased milk yield, and increased breeding inefficiencies (Deka *et al.*, 2018).

Accurate and timely diagnosis is central to brucellosis control. Although bacterial isolation remains the reference standard, it is labor-intensive, time-consuming, and requires high-containment biosafety facilities due to the risk of laboratory-acquired infections (Manheim & Lewis, 2022; Freire *et al.*, 2024). In field settings, screening tests such as the Milk Ring Test and the Rose Bengal Plate Test are widely used for rapid detection in large ruminants (Cadmus *et al.*, 2008). However, their limited sensitivity and specificity necessitate confirmation using more robust serological and molecular assays, such as ELISA and PCR, to reduce misclassification and improve epidemiological accuracy (Loubet *et al.*, 2024).

Despite its endemicity, brucellosis remains underreported in Pakistan. Limited diagnostic capacity, fragmented surveillance systems, and the absence of a comprehensive vaccination policy constrain effective disease control. Current reliance on passive surveillance captures only clinically apparent cases and likely underestimates the true burden of infection (Iqbal *et al.*, 2020; Ullah *et al.*, 2022).

There are a few fragmented studies on the epidemiology of brucellosis and its zoonotic implications in Pakistan (Ullah *et al.*, 2024; Shirwany *et al.*, 2026). However, division-level data is scarce in Punjab, which hosts most of the national bovine population. There is a need to establish comprehensive division-level seroprevalence data to define precise spatial zones for targeted vaccination. Thus, the present study aimed to estimate the seroprevalence of brucellosis and identify risk factors associated with the disease in bovine populations across the four districts of the Faisalabad Division, Punjab, Pakistan.

MATERIALS AND METHODS

Study area and target population: This study was conducted in Faisalabad division, one of the most important agroecological zones of Pakistan. It is located in the center of Punjab province of Pakistan. It comprises four districts, including Faisalabad (30.00°–31.50°N, 73.00°–74.00°E), Jhang (31.27°N, 72.33°E), Toba Tek Singh (30°33′–31°02′N, 72°08′–72°48′E) and Chiniot (31.72°N, 72.98°E) (Fig. 1). Faisalabad Division is one of the most livestock-dense regions in Pakistan, with approximately 3.069 million cattle and 4.679 million buffaloes (internal communication with the Livestock and Dairy Development Department, Govt. of Punjab, Pakistan).

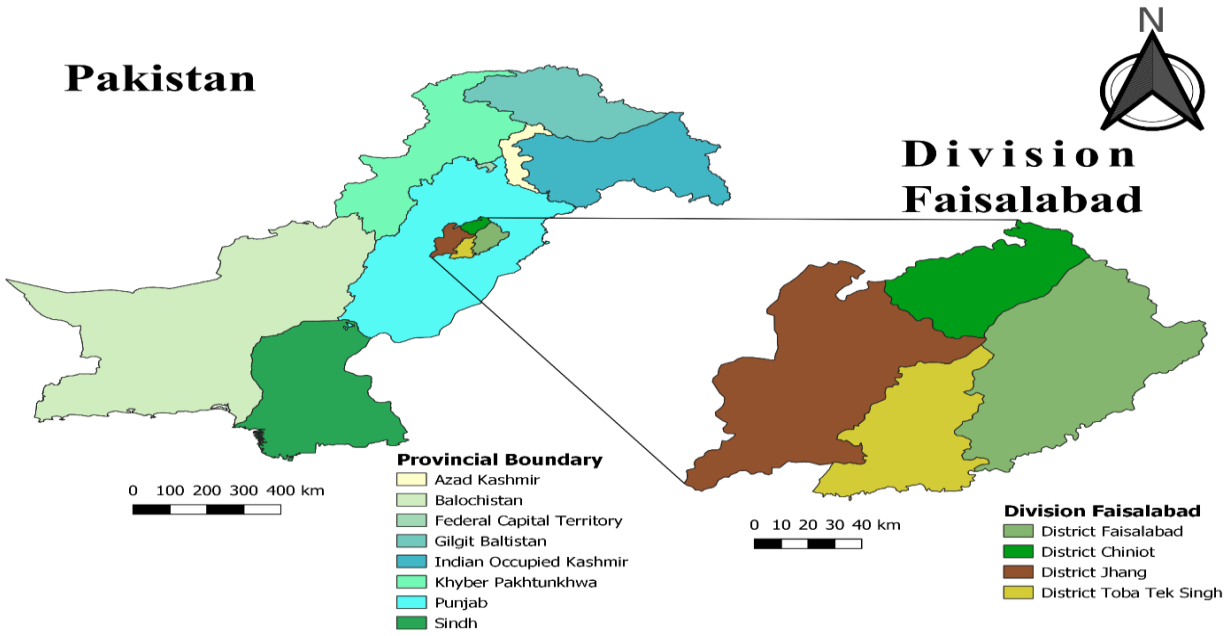

Fig. 1: Map of the study area (developed using software QGIS; version 3.24.2).

Study design and duration: A cross-sectional study was carried out between November 2024 and October 2025 to estimate the seroprevalence and identify the risk factors associated with bovine brucellosis in large ruminants in Division Faisalabad, Punjab, Pakistan.

Sample size estimation: A stratified sampling technique was used to collect samples from the study area. The study area was stratified into four strata, which represented constituent districts of the Faisalabad Division. Sample sizes for each stratum were calculated using the formula described by Pourhoseingholi *et al.* (2013) as follows:

$$n = \frac{(1.96)^2.\ \mathrm{p}.\ (1-\mathrm{p})}{e^2}$$

Where, n=No. of samples; Z=1.96 (95% level of confidence); *e*=level of precision (5%) and p=Expected prevalence (fixed at 20%): based on previously reported animal-level seroprevalence (20.4% in cattle) within the Punjab province by Khan *et al.* (2021b). The calculated sample size for each stratum was 245, with an overall sample size of 980. To ensure adequate representation and to account for potential sample loss, 250 serum samples were collected from each district, yielding a total of 1000 serum samples from the study area. During data collection through farmer interviews, it was confirmed that no animal in the study had received vaccination against brucellosis, precluding vaccine-induced seropositivity as a source of false-positive results.

Sampling: Technical and logistic assistance of District Disease Diagnostic Labs, Livestock and Dairy Development Department (L&DDD), Govt. of Punjab, Pakistan was used to collect the sera samples of target population of study area. Approximately 3mL of blood was aseptically collected by drawing it in the jugular vein of each animal and transferred to gel clot activator tubes to harvest the serum samples. All the serum samples were kept at -20ºC until laboratory examination.

Epidemiological data collection: The descriptive epidemiological data regarding the associated risk factors (including breed, age, gender, abortion history, milking status, pregnancy status, herd size, and farming system) were collected using a well-designed questionnaire.

Sero-detection of brucellosis using different techniques
Rose Bengal Plate test: All sera samples were screened for brucellosis using the Rose Bengal Plate Test following WOAH guidelines (Anonymous, 2022). For each sample, a total of 30µL of RBPT antigen (VRI-Lahore, Pakistan) was mixed with 30µL of serum on a clean glass slide and spread over a circular area. The slide was gently rotated for 4 minutes at room temperature (25℃) to allow antigen–antibody reaction. Results were observed under adequate lighting, and a visible agglutination indicated a positive serological result for brucellosis.

Serum Agglutination test: SAT was performed following Nasir *et al.* (2005) with minor modifications using 96-well microtiter plates. Briefly, serum samples were subjected to two-fold serial dilutions in phosphate-buffered saline containing 10mM EDTA (pH 7.2), and an equal volume of concentrated *Brucella antigen* (VRI, Lahore, Pakistan) diluted 1:10 in phenol saline was added to each well. Plates were incubated at 37°C for 20 hours and read visually. Titres ≥1:40 with 50% agglutination were considered positive, 1:20 as suspicious, and 1:10 as negative.

Indirect ELISA: anti-*Brucella* antibodies in all serum samples were tested using a commercial indirect multispecies ELISA kit (ID Screen Brucellosis Serum Indirect Multispecies, BRUS-MS-10P; IDvet, France) as per the manufacturer's instructions. Measurement of optical density at 450nM with an ELISA plate reader (Labodam, UK) was performed after the assay was completed. The IDSoft data-analysis software (Version 5.20.7; ID.vet France) was used to analyze the results, according to the interpretation criteria of the manufacturer.

Statistical analysis: All analyses were performed in R (version 4.3.1) using RStudio (version 2025.05.1+513). Seroprevalence was estimated at the overall, species, and district levels with 95% confidence intervals. Inter-test agreement among RBPT, SAT, and iELISA was assessed using Cohen's kappa for pairwise comparisons and Fleiss' kappa for all three tests simultaneously. McNemar's chi-square test was applied to evaluate statistically significant differences in positivity rates between test pairs. Diagnostic performance of RBPT and SAT was evaluated against iELISA as the reference standard, with sensitivity, specificity, PPV, NPV, and overall accuracy calculated accordingly. Associations between seropositivity and potential risk factors were examined at the bivariate level using chi-square or Fisher's exact tests with crude odds ratios.

To account for the hierarchical structure of the data, in which multiple animals were sampled within the same herd, multivariable analysis was performed using two generalized linear mixed-effects models (GLMMs) with a binomial error distribution and logit link function. These models were implemented via the glmer() function in the lme4 package. A random intercept for herd was included in both models to explicitly partition within-herd correlation from fixed-effect estimates, and to account for unmeasured herd-level heterogeneity in disease risk.

Fixed effects in both models were specified as a priori based on either an association with seropositivity at $P \leq 0.25$ or consistent identification as biologically relevant risk factors in the published brucellosis literature, irrespective of univariable significance. Breed was excluded from the overall population model because breed classifications differed substantially between cattle and buffalo, limiting cross-species biological interpretability and risking unstable parameter estimates in a combined model. Interaction terms between biologically plausible variable pairs (herd size × farming system; age × species) were tested using likelihood ratio tests and retained only if significant ($P<0.05$).

Two separate GLMM models were developed. The first Model was fitted to the full study population and included species, sex, age category, herd size, and farming system as candidate fixed effects with herd as a random intercept. The second Model was fitted exclusively to female animals to permit the inclusion of reproductive

covariates (*viz.* pregnancy status, abortion history, and repeat breeding history), which are biologically inapplicable to male animals and would otherwise generate substantial structural missingness in a combined-sex model. The female model retained the same farm-management covariates as Model 1, with species, age, herd size, and farming system included as fixed effects alongside the reproductive variables.

The intraclass correlation coefficient (ICC) was calculated from the random-effect variance component to quantify the proportion of total outcome variability attributable to between-herd clustering. ICC values greater than 0.05 were considered indicative of meaningful herd-level clustering and supporting the use of mixed-effects modeling. Marginal R^2 (variance explained by fixed effects alone) and conditional R^2 (variance explained by both fixed and random effects) were computed. Model fit was compared using the Akaike Information Criterion (AIC), with likelihood ratio tests used to assess the significance of the random intercept and of the overall fixed-effects structure against null (intercept-only random-effects) models. Simulation-based scaled residuals were generated using the DHARMa package to assess residual uniformity, dispersion, and the presence of outliers. Discriminative ability was evaluated using the area under the receiver operating characteristic curve (AUC-ROC) with 95% CIs. Multicollinearity among fixed effects was assessed using generalized variance inflation factors (GVIF), and values of GVIF^(1/(2·Df))<1.5 were considered acceptable. Results are reported as adjusted odds ratios (aOR) with 95% CIs.

RESULTS

Overall seroprevalence of brucellosis: The RBPT showed an overall seropositivity of 17.90% (95%CI=15.64-20.40; n=179/1000), the SAT detected 16.30% (95%CI=14.14-18.72; n=163/1000) overall seropositivity, whereas the iELISA revealed an overall seropositivity of 14.70% (95%CI=12.64-17.03; n=147/1000). Species-wise analysis showed higher seropositivity in cattle than buffaloes across all assays. In cattle, RBPT detected 21.70% positives (95%CI=18.33-25.49; n=110/507), SAT detected 18.93% (95%CI=15.76-22.57; n=96/507), and iELISA detected 18.74% (95%CI=15.58-22.36; n=95/507). In buffaloes, RBPT showed 13.99% seropositivity (95%CI=11.21-17.34; n=69/493): SAT showed 13.60% (95%CI=10.84-16.90; n=67/493): and iELISA showed 10.55% (95%CI=8.13-13.57; n=52/493) (Fig. 2).

District and herd level seroprevalence of bovine brucellosis: Chiniot district had significantly higher seroprevalence of bovine brucellosis (25.20%; n=63/250), followed by districts Jhang (12.40%; n=31/250), Toba Tek Singh (11.20%; n=28/250), and Faisalabad (10.00%; n=25/250). A herd was considered positive for brucellosis if at least one animal tested seropositive by the iELISA. A total of 62 out of 201 targeted herds were found positive with herd level prevalence of 30.84% in Division Faisalabad. Significant difference was observed in herd-level prevalence between different districts of the study area, with the highest herd-level seroprevalence (54.35%; n=25/46) recorded in Chiniot, and the lowest one in district Faisalabad (20.63%; n=13/63) (Table 1).

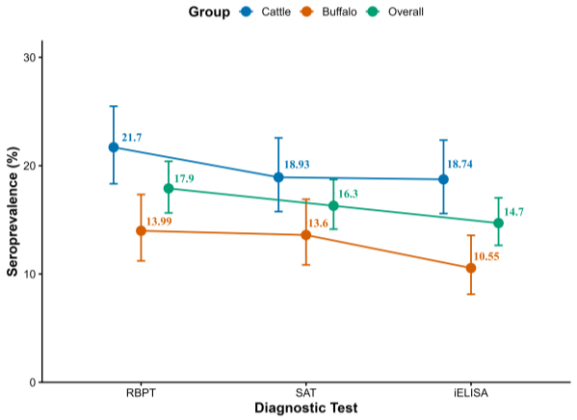

Fig. 2: Seroprevalence of bovine brucellosis in division Faisalabad using RBPT, SAT, and iELISA. The points and values represent seroprevalence, and error bars represent the 95% confidence intervals. The connecting lines in the point range plot are used only to help visualize the trend in seroprevalence across diagnostic tests within each group.

Agreement between different serological tests: 116 serum samples were positive across all three tests, with the full distribution of concordant and discordant results shown in Table 2 and Fig. 3. Kappa statistics indicated substantial to almost perfect inter-test agreement: RBPT vs. SAT (κ=0.852, 95.8%), SAT vs. iELISA (κ=0.748, 93.4%), and RBPT vs. iELISA (κ=0.722, 92.4%), with an overall agreement of 93.9% (κ=0.775). McNemar's test revealed that RBPT detected significantly more positive cases than both SAT (P=0.02) and iELISA (P<0.001), while detection rates between SAT and iELISA did not differ significantly (P=0.06).

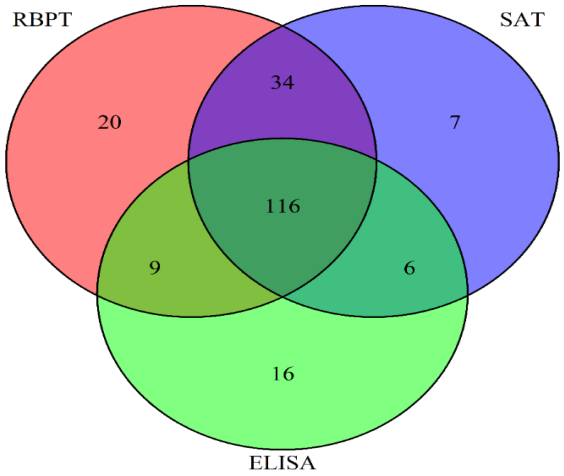

Fig. 3: Venn diagram showing a summary of serological test results (RBPT, SAT, and iELISA) for serum samples.

Diagnostic performance of RBPT and SAT: Both RBPT and SAT demonstrated high diagnostic performance against iELISA as the reference test (Table 3). RBPT achieved a sensitivity of 85.03%, specificity of 93.67%, and overall accuracy of 92.4%, while SAT showed comparable sensitivity (82.99%) with slightly higher specificity (95.19%) and accuracy (93.4%). NPV was high for both tests (RBPT: 97.32%; SAT: 97.01%), reflecting their reliability in ruling out negative cases. Overall, SAT showed a marginal advantage over RBPT in specificity, PPV, and diagnostic accuracy.

Table 1: District and Herd-level seropositivity of bovine brucellosis

	Positive/ Total (n/N)	Seroprevalence % (95% CI)	Odds Ratio (95% CI)	χ^2 p-value	Fisher exact p-value
District-Level					
Faisalabad	25/250	10.00 (6.86-14.34)	0.33 (0.19-0.56)	<0.001	<0.001
Toba Tek Singh	28/250	11.20 (7.86-15.71)	0.38 (0.22-0.62)		
Jhang	31/250	12.40 (8.87-17.06)	0.42 (0.25-0.69)		
Chiniot	63/250	25.20 (20.22-30.93)	Ref.		
Herd-Level					
Division Faisalabad (Overall)	62/201	30.84(24.87-37.54)			
District-Wise Herd Level					
Faisalabad	13/63	20.63 (12.48-32.17)	0.22 (0.09-0.51)	0.001	0.002
Toba Tek Singh	10/42	23.81 (13.48-38.53)	0.27 (0.10-0.66)		
Jhang	14/50	28.00 (17.47-41.66)	0.33 (0.14-0.77)		
Chiniot	25/46	54.35 (40.18-67.84)	Ref.		

The differences were considered significant at P<0.05. Fisher's Exact test p-value is reported and considered applicable in cases where expected cell counts are <5.

Table 2: Agreement between RBPT, SAT, and iELISA using Kappa Statistics

Comparison	Observed Agreement (%)	95% CI of observed agreement (%)	Kappa (κ)	95% CI of kappa	SE	P value	Level of Agreement
RBPT vs SAT	95.8	94.37-96.88	0.852	0.808-0.895	0.022	<0.001	Almost perfect
SAT vs iELISA	93.4	91.69-94.78	0.748	0.690-0.806	0.029	<0.001	Substantial
iELISA vs RBPT	92.4	90.59-93.88	0.722	0.663-0.781	0.029	<0.001	Substantial
Overall (Fleiss' κ)	93.87	92.24-95.22	0.775	0.739-0.811	0.018	<0.001	Substantial

Table 3: Diagnostic Performance of RBPT and SAT compared to iELISA

		iELISA			Accuracy % (95%CI)	Sensitivity % (95%CI)	Specificity % (95%CI)	PPV % (95%CI)	NPV % (95%CI)
		Positive	Negative	Total					
RBPT	Positive	125	54	179	92.4 (91-94)	85.03 (78-90)	93.67 (92-95)	69.83 (63-76)	97.32 (96-98)
	Negative	22	799	821					
	Total	147	853	1000					
SAT	Positive	122	41	163	93.4 (92-95)	82.99 (76-89)	95.19 (94-97)	74.85 (67-81)	97.01 (96-98)
	Negative	25	812	837					
	Total	147	853	1000					

Bivariate analysis of risk factors of brucellosis: Among demographic and management variables (Table 4): In buffalo, age ≥8 years was significantly associated with higher seropositivity compared to younger animals (P=0.013), while no significant age association was observed in cattle or the overall population. Male cattle showed significantly higher seroprevalence than females (P=0.030), with the association remaining significant across the overall bovine population (P=0.039). Breed was a highly significant risk factor in cattle (P<0.001), with Friesian and Sahiwal animals showing markedly higher seroprevalence than crossbreds. Herd size was significantly associated with seropositivity in both species (P<0.001), with prevalence increasing consistently with herd size. Farming system was also significant (P<0.001), with cattle-only systems recording the highest seroprevalence (28.57%).

Regarding reproductive variables (Table 5): pregnancy was significantly associated with seropositivity in buffalo (P=0.0001) and the overall population (P=0.0003), but not in cattle alone. Abortion history was significant in cattle (P=0.045) but not in buffalo or the overall population. Repeat breeding history was a significant risk factor in buffalo (P=0.028) and the overall bovine population (P=0.007).

Multivariable analysis using GLMM: A total of 1,000 animals from 201 herds were included in Model 1 (overall population), of which 147 (14.7%) were iELISA seropositive. The female-only subgroup (Model 2) comprised 952 animals, of which 135 (14.2%) were seropositive. The large ICC values of both models (Model 1=0.4571; Model 2=0.4866) indicated that an animal's herd membership was a dominant determinant of its serostatus and confirmed that a mixed-effects framework was required.

Both models showed a significant improvement over their respective null (herd-only) models. In Model 1, the likelihood ratio test (LRT) yielded χ^2=52.22 (df=9, P<0.001), and in Model 2, χ^2=62.07 (df=11, P<0.001). This confirmed that the fixed-effects structure explained meaningful variation beyond herd clustering alone. The AIC dropped from 733.63 to 699.41 (Model 1) and from 686.42 to 646.36 (Model 2), which further supported model improvement. Marginal R^2 was 0.215 and 0.239 for Models 1 and 2, respectively, indicating that the fixed effects alone explained approximately 21–24% of variance. Conditional R^2 rose to 0.574 and 0.609 when herd-level random effects were included. Multicollinearity was acceptable in both models (all GVIF1/(2·Df) values were below 1.16). No interaction terms (herd size × farming system; age × species) were statistically significant (all P>0.38), and both were excluded from the final specifications. Simulation-based residual diagnostics (DHARMa) indicated adequate model fit, with no evidence of over-dispersion (Model 1 dispersion ratio=1.05, Model 2 dispersion ratio=1.04; both P>0.60) and no excess outliers (P>0.45). Both models demonstrated excellent discriminative ability, indicated by AUC-ROC=0.919 (95%CI=0.899–0.939) for Model 1 and AUC-ROC=0.928 (95%CI=0.909–0.947) for Model 2 (Figure 4). Adjusted odds ratios with 95% confidence intervals for all predictors are presented in Table 6 and displayed graphically in Figure 5.

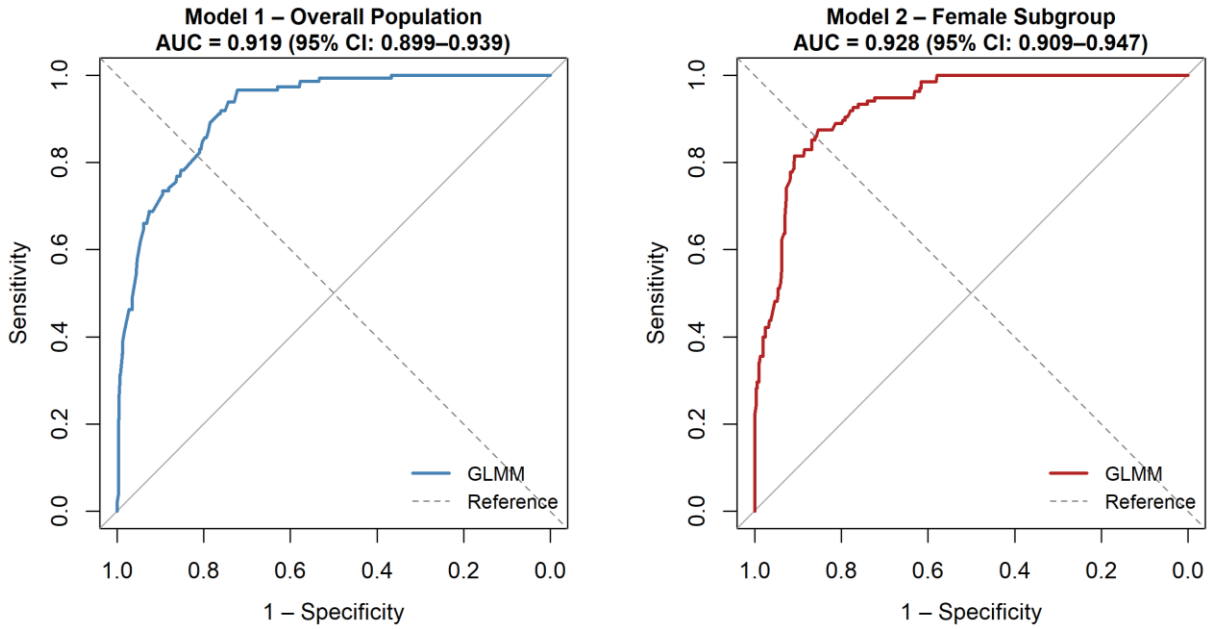

Fig. 4: ROC curves for the generalized linear mixed-effects models.

Table 4: Risk Factors Associated with Bovine Brucellosis in Faisalabad Division

Variable	Categories	Positive/Total (n/N)	Seroprevalence (95% CI)	Odds Ratio (OR)	95%CI OR		χ^2 P-value	Fisher exact P-value
					Lower	Upper		
Age (years)	Cattle							
	≤ 4	28/167	16.77 (11.86-23.17)	0.83	0.48	1.4	0.712	0.693
	> 4 but ≤ 8	56/287	19.51 (15.34-24.49)	Ref				
	> 8	11/53	20.75 (12.00-33.46)	1.08	0.47	2.31		
	Buffalo							
	≤ 4	7/104	6.73 (3.30-13.25)	0.73	0.26	1.8	0.013	0.019
	> 4 but ≤ 8	25/277	9.03 (6.19-12.98)	Ref				
	> 8	20/112	17.86 (11.87-25.98)	2.19	1.09	4.32		
	Overall (Cattle + Buffalo)							
	≤ 4	35/271	12.92 (9.43-17.43)	0.88	0.56	1.38	0.23	0.23
	> 4 but ≤ 8	81/564	14.36 (11.71-17.50)	Ref				
	> 8	31/165	18.79 (13.56-25.43)	1.38	0.84	2.21		
Gender	Cattle							
	Female	87/485	17.94 (14.78-21.60)	Ref			0.030	0.046
	Male	8/22	36.36 (19.73-57.05)	2.61	1.06	6.42		
	Buffalo							
	Female	48/467	10.28 (7.84-13.36)	Ref			0.409	0.340
	Male	4/26	15.38 (6.15-33.53)	1.59	0.52	4.8		
	Overall (Cattle + Buffalo)							
	Female	135/952	14.18 (12.11-16.54)	Ref			0.039	0.056
	Male	12/48	25.00 (14.92-38.78)	2.02	1.02	3.97		
Breed	Cattle							
	Sahiwal	34/100	34 (25.46-43.72)	4.44	2.41	8.27	<0.001	<0.001
	Friesian	30/82	36.59 (26.98-47.39)	4.97	2.62	9.52		
	Crossbred	27/261	10.34 (7.21-14.63)	Ref				
	Non-descript	4/64	6.25 (2.46-15.00)	0.58	0.14	1.75		
	Buffalo							
	Nili Ravi	45/382	11.78 (8.92-15.40)	Ref			0.098	0.115
	Non-descript	7/111	6.31 (3.09-12.45)	0.5	0.22	1.15		
Herd Size	Cattle							
	Up to 10	4/96	4.17 (1.63-10.23)	0.09	0.02	0.25	<0.001	<0.001
	11 to 30	35/241	14.52 (10.63-19.53)	0.35	0.21	0.57		
	> 30	56/170	32.94 (26.32-40.32)	Ref				
	Buffalo							
	Up to 10	6/188	3.19 (1.47-6.79)	0.17	0.05	0.47	<0.001	<0.001
	11 to 30	28/193	14.51 (10.23-20.17)	0.89	0.45	1.80		
	> 30	18/112	16.07 (10.41-23.98)	Ref				
	Overall (Cattle + Buffalo)							
	Up to 10	10/284	3.52 (1.92-6.36)	0.1	0.05	0.21	<0.001	<0.001
	11 to 30	63/434	14.52 (11.51-18.14)	0.48	0.32	0.71		
	> 30	74/282	26.24 (21.45-31.67)	Ref				
Farming System	Overall							
	Cattle Only	48/168	28.57 (22.03-35.94)	2.75	1.23	6.79	<0.001	<0.001
	Buffalo Only	9/71	12.68 (6.54-22.21)	Ref				
	Cattle & Buffalo	53/580	9.14 (6.98-11.75)	0.69	0.32	1.68		
	Cattle/Buffalo & Sheep/Goat	37/181	20.44 (15.02-26.96)	1.77	0.78	4.42		

The differences between variables were considered significant at P<0.05. Fisher's Exact test p-value is reported and considered applicable in cases where expected cell counts are <5.

Table 5: Association of Pregnancy and Reproductive Disorders with Bovine Brucellosis in Faisalabad Division

Variable	Categories	Positive/Total (n/N)	Seroprevalence (95% CI)	Odds Ratio (OR)	95%CI OR Lower	95%CI OR Lower	95%CI OR	Fisher exact p-value
Pregnancy	Cattle							
	Yes	28/119	23.53 (16.81-31.90)	1.6	0.96	2.66	0.067	0.074
	No	59/366	16.12 (12.71-20.24)	Ref				
	Buffalo							
	Yes	26/141	18.44 (12.91-25.65)	3.12	1.7	5.73	0.0001	0.0003
	No	22/326	6.75 (4.50-10.01)	Ref				
	Overall (Cattle + Buffalo)							
	Yes	54/260	20.77 (16.28-26.11)	1.98	1.35	2.89	0.0003	0.0005
	No	81/692	11.71 (9.52-14.31)	Ref				
Abortion history	Cattle							
	Yes	25/101	24.75 (17.36-33.99)	1.71	1.01	2.89	0.045	0.057
	No	62/384	16.15 (12.80-20.16)	Ref				
	Buffalo							
	Yes	6/80	7.50 (3.48-15.41)	0.67	0.27	1.62	0.369	0.426
	No	42/387	10.85 (8.13-14.35)	Ref				
	Overall (Cattle + Buffalo)							
	Yes	31/181	17.13 (12.34-23.28)	1.33	0.85	2.05	0.207	0.236
	No	104/771	13.49 (11.26-16.08)	Ref				
Repeat breeding history	Cattle							
	Yes	32/141	22.70 (16.56-30.28)	1.54	0.95	2.51	0.080	0.090
	No	55/344	15.99 (12.49-20.23)	Ref				
	Buffalo							
	Yes	21/140	15.00 (10.02-21.84)	1.96	1.07	3.60	0.028	0.032
	No	27/327	8.26 (5.74-11.75)	Ref				
	Overall (Cattle + Buffalo)							
	Yes	53/281	18.86 (14.72-23.84)	1.67	1.14	2.44	0.007	0.011
	No	82/671	12.22 (9.96-14.92)	Ref				

The differences between variables were considered significant at P<0.05. Fisher's Exact test p-value is reported and considered applicable in cases where expected cell counts are <5.

Table 6: Adjusted odds ratios (aOR) from GLMM for iELISA seropositivity for bovine brucellosis in Division Faisalabad

Variables	Model 1: Overall population (n=1,000)			Model 2: Females only (n=952)		
	aOR	95% CI	P value	aOR	95% CI	P value
Species						
Buffalo	Ref.			Ref.		
Cattle	1.28	0.63–2.60	0.500	1.28	0.60–2.71	0.523
Sex						
Female	Ref.			—	—	—
Male	8.10	2.64–24.88	< 0.001	—	—	—
Age category						
≤ 4 years	Ref.			Ref.		
> 4 to ≤ 8 years	2.35	1.19–4.63	0.014	1.95	0.95–3.99	0.067
> 8 years	4.56	1.90–10.92	< 0.001	4.12	1.65–10.27	0.002
Herd size						
≤ 10 animals	Ref.			Ref.		
10 to 30 animals	4.19	1.56–11.21	0.004	3.51	1.22–10.07	0.020
> 30 animals	14.05	4.27–46.22	< 0.001	13.00	3.71–45.53	< 0.001
Farming system						
Buffalo only	Ref.			Ref.		
Cattle only	2.95	0.54–16.10	0.212	3.70	0.56–24.60	0.175
Cattle and buffalo	0.75	0.17–3.32	0.707	0.97	0.18–5.14	0.968
Cattle/buffalo and small ruminants	0.97	0.20–4.71	0.970	1.28	0.22–7.58	0.784
Reproductive factors (females only)						
Pregnancy status (Ref. = Not pregnant)	—	—	—	2.56	1.50–4.38	< 0.001
Abortion history (Ref. = No)	—	—	—	1.13	0.60–2.11	0.707
Repeat breeding history (Ref. = No)	—	—	—	2.26	1.35–3.78	0.002

aOR = adjusted odds ratio; CI = confidence interval. Dashes (—) indicate the variable was not included in that model. Significance is considered at P<0.05.

Generalized Linear Mixed-Effects Model 1: Male sex was the strongest individual-level predictor of seropositivity (aOR=8.10; P<0.001). Older animals were also at significantly higher risk, and those in the age group >4 to ≤8 years were approximately 2.4 times more likely to be seropositive compared with the youngest age group (aOR=2.35; P=0.014), and odds rose further in animals older than 8 years (aOR=4.56; P<0.001). A positive association was also observed between herd size and brucellosis seropositivity, with larger herds showing a proportionally greater odds. Animals in medium-sized herds (10 to 30 head) had four times the odds of seropositivity relative to small herds (aOR=4.19; P=0.004), while animals in large herds (>30 head) had over 14 times the odds (aOR=14.05; P<0.001). Species (cattle vs. buffalo) and farming system type were not independently significant after accounting for herd clustering and other covariates.

Generalized Linear Mixed-Effects Model 2: The female-only model replicated the herd size gradient observed in Model 1. Medium herds showed 3.5-fold higher odds (aOR=3.51; P=0.020) and large herds 13-fold higher odds (aOR=13.00; P<0.001) compared with small herds. Among age groups, only animals older than 8 years retained significance (aOR=4.12; P=0.002). Two reproductive

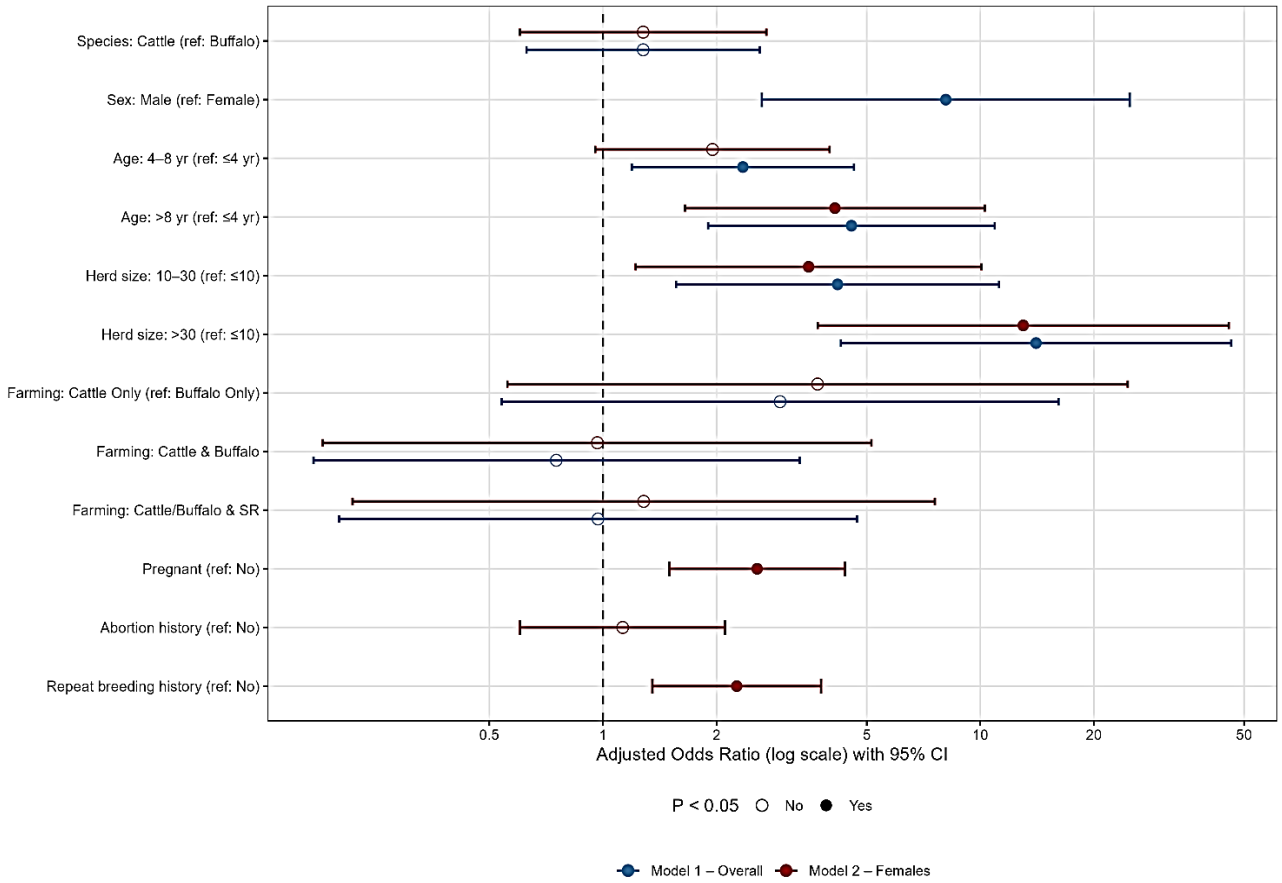

Fig. 5: Forest plot of aOR with 95% confidence intervals from the GLMM models.

risk factors were independently associated with seropositivity. Pregnant females were 2.6 times more likely to be seropositive than non-pregnant animals (aOR=2.56; P<0.001). A history of repeat breeding also conferred elevated risk (aOR=2.26; P=0.002). Abortion history did not reach significance (aOR=1.13; P=0.707). Species and farming system were again non-significant.

DISCUSSION

Brucellosis represents a major veterinary public health concern, particularly in low- and middle-income countries (Holt *et al.*, 2021), where it causes substantial economic losses in livestock due to reproductive wastage, reduced production, and medication cost. This study presents a comprehensive seroprevalence survey of bovine brucellosis in Faisalabad Division, Punjab, Pakistan. The results give invaluable epidemiological data on disease burden, species selectivity, variation at the district level, and other risk factors, which have significant implications for disease control and policy formulation.

The iELISA revealed that the seroprevalence of bovine brucellosis in the target population was 14.70%. These results are higher than the 11.22% and 7.5% seroprevalence of bovine brucellosis previously reported in Punjab (Awais *et al.*, 2024; Hussain *et al.*, 2025), yet consistent with the broader range documented across Pakistan, with variation attributable to differences in herd size, farming system, and breed composition rather than true geographical differences in disease burden. Internationally, our estimate exceeds the 2.4% and 5.10% seroprevalence reported in Ethiopia and Indonesia, respectively (Asgedom *et al.*, 2016; Yanti *et al.*, 2021) but remains lower than the 28.9% and 55% recorded in Rwanda and Albania, respectively (Fero *et al.*, 2020; Ntivuguruzwa *et al.*, 2022), reflecting the global spectrum of brucellosis prevalence shaped by the adequacy of local disease control infrastructure. The herd-level prevalence of bovine brucellosis in Faisalabad Division was 30.84%, with the highest prevalence in herds of district Chiniot, followed by Jhang, Toba Tek Singh, and Faisalabad. The herds having at least one positive animal for *Brucella* antibodies were considered positive (Boukary *et al.*, 2013). Previously, no herd-level prevalence had been reported in the study area. This herd level prevalence was consistent with 32.83% of herd level prevalence reported in district Multan by Awais *et al*. (2024). However, a previous study in district Gujranwala by Khan *et al.* (2021b) reported a high 58.7% herd-level prevalence of bovine brucellosis.

Consistently higher seroprevalence in cattle than buffalo across all three assays is consistent with findings from comparable studies in Punjab's settings (Awais *et al.*, 2024; Khan *et al.*, 2025), though it contrasts with reports of higher buffalo prevalence in smallholder systems (Jamil *et al.*, 2020; Hussain *et al.*, 2025), where greater reliance on natural breeding and higher repeat breeding rates in buffaloes likely drive transmission. The elevated cattle seroprevalence may reflect a combination of environmental and breed-specific factors; exotic and crossbred cattle on organized dairy farms are not only reared under intensive, high-density conditions but also

generally possess a higher natural susceptibility to infection compared to local indigenous breeds (Akhtar *et al.*, 2019). District Chiniot recorded the highest seroprevalence (25.20%), likely reflecting its position at the intersection of major livestock trading routes and the predominance of smallholder mixed farming systems characterized by natural breeding, absent vaccination, and poor biosecurity, as demonstrated by Arif *et al.* (2019) and Saeed *et al.* (2020). These district-level differences reinforce the inadequacy of a uniform national control policy and highlight the need for district-stratified, targeted intervention.

The higher positivity rates of RBPT and SAT relative to iELISA reflect the inherently higher sensitivity but lower specificity of agglutination-based screening assays, which target smooth lipopolysaccharide antigens shared with cross-reacting Gram-negative organisms, including *Yersinia enterocolitica* O:9 and *Escherichia coli* (Andrade *et al.*, 2024). iELISA employs defined antigen preparations enabling greater discrimination between *Brucella*-specific and cross-reacting antibodies, validating its selection as the reference standard and supported by its documented superior sensitivity and specificity (Paweska *et al.*, 2002; Getachew *et al.*, 2016). While bacterial isolation remains the true gold standard, its requirement for BSL-3 containment makes it operationally impractical in field settings (Freire *et al.*, 2024), and iELISA represents the most feasible and analytically robust reference comparator available (Alamian *et al.*, 2023). Kappa statistics indicated almost perfect agreement between RBPT and SAT ($\kappa = 0.852$), and substantial agreement between SAT and iELISA ($\kappa = 0.748$) and RBPT and iELISA ($\kappa = 0.722$), consistent with the Landis and Koch (1977) classification, confirming that RBPT is appropriate as an initial screening tool followed by iELISA confirmation.

The large intraclass correlation coefficients observed in both models (ICC=0.457 in Model 1; ICC=0.487 in Model 2) confirm that herd membership was a dominant determinant of individual serostatus, with approximately 46–49% of total outcome variability attributable to unmeasured between-herd heterogeneity. This magnitude of clustering substantially exceeds the conventional threshold of 0.05 recommended for justifying a mixed-effects framework (Audigé, 2005), and is consistent with ICC estimates reported for brucellosis serotiter data in the veterinary epidemiology literature, which have been documented to reach as high as 0.46 in herd-clustered studies (Otte & Gumm, 1997). A comparable herd-level ICC of 0.40 was similarly reported in a livestock brucellosis study employing GLMM in Kenya (Kairu-Wanyoike *et al.*, 2019), underscoring that within-herd clustering of this magnitude is a well-established feature of brucellosis epidemiology in endemic settings.

Herd size was the strongest and most consistent structural risk factor across both models, with a clear dose-response gradient from small to large herds. This finding is consistent across Pakistani settings, regardless of production system, as documented by Arif *et al.* (2019), Khan *et al.* (2021a), Awais *et al.* (2024), and Hussain *et al.* (2025), and reflects the density-dependent nature of brucellosis transmission that makes herd size the most actionable structural target for disease control.

Age showed a clear dose-response gradient in Model 1, with risk rising progressively from younger to older animals. In the Model 2, only the oldest age group retained significance, likely because reproductive covariates in that model captured a substantial portion of the age-associated risk that operates through the gravid uterus and gestational immunosuppression. The association of older age with brucellosis seropositivity is consistently reported across endemic settings and represents one of the most robust epidemiological patterns in the literature (Al-Majali *et al.*, 2009; Mai *et al.*, 2012; Li *et al.*, 2024)

Male sex was the strongest individual-level predictor of seropositivity in Model 1. This is atypical given the preferential localization of *Brucella* in the gravid uterus and erythritol-rich placental tissues of females (Anderson & Smith, 1965). Although higher male seropositivity is uncommon, it is not without precedent, as this difference between sexes is consistent with reports by Chimana *et al.* (2010), who recorded more seropositive cases in bulls compared to females. However, our findings are contrary to other reports that showed significantly higher prevalence in females than males (Junaidu *et al*., 2008, 2011) or no difference between sexes (Bayemi *et al*., 2009).

Similarly, farming system was not independently significant in either model, confirming that its apparent univariable association is largely mediated by herd size, species composition, and management intensity rather than representing an independent risk pathway, consistent with observations by Saeed *et al.* (2020) and Arif *et al.* (2019) in comparable Pakistani settings.

In the female-only model, pregnancy was independently associated with substantially higher odds of seropositivity, particularly consistent with the preferential replication of *Brucella* in the gravid uterus compounded by the physiological immunosuppression of advanced gestation that facilitates bacterial persistence (Khurana *et al*., 2021). This finding is consistent with the observation by Haileselassie *et al.* (2011) but contrary to reports by Ibrahim *et al.* (2010).

Abortion history did not reach significance after multivariable adjustment, which is consistent with a previous study performed by Shabbir *et al.* (2013). However, our findings are contrary to other reports that showed significantly higher prevalence in females with an abortion history (Deresa *et al.*, 2020; Yanti *et al.*, 2021; Tekle *et al.*, 2023). This contrast may result from the under-reporting of abortion events by the farmer (Bronner *et al.*, 2014).

Repeat breeding history was also a significant independent predictor, consistent with various previous studies that had also reported a significant association of repeat breeding history with prevalence of brucellosis in bovines (Khan *et al.*, 2020; Saeed *et al.*, 2020; Khan *et al.*, 2021b). Contrarily, Patel *et al.* (2014) reported a non-significant association of repeat breeding history in bovines.

Conclusions: Bovine brucellosis is endemic in Faisalabad Division with an iELISA-confirmed seroprevalence of 14.7%, posing a significant zoonotic risk to public health and livestock productivity. Larger herd size, older age, male sex, pregnancy status, and repeat breeding history

were identified as key risk factors, providing a locally validated evidence base for targeted intervention. The substantial between-herd variation identified underscores the importance of herd-level interventions over individual animal treatment. Furthermore, the district-stratified epidemiological data generated establish the first regional baseline for brucellosis in Faisalabad Division, with direct implications for disease surveillance and control programs across Punjab and Pakistan. Using two diagnostic tests in parallel is recommended to maximize diagnostic accuracy and minimize false-positive and false-negative classifications in endemic settings.

Limitations: The absence of a comprehensive sampling frame for livestock holdings in the study area posed a methodological challenge, as cattle and buffalo are distributed across both organized farms and unregistered smallholdings, introducing the potential for selection bias. To address this, the study was conducted in collaboration with the Livestock and Dairy Development Department, Government of Punjab, ensuring that animals from both organized and small-scale farms across the study area were represented in the sample.

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Consent to Publish: Written informed consent was obtained from farmers/owners of the animals to use the data generated from this study for research and publication purposes without revealing their identity.

Generative AI statement: The authors declare that no Gen AI/DeepSeek was used in the writing/creation of this manuscript.

REFERENCES

Agriculture Development Plan, 2023. The Urban Unit, Government of Punjab, Lahore, Pakistan. Retrieved from https://urbanunit.gov.pk/Download/publications/Files/17/2023/Faisalabad%20Regional%20Development%20Plan%20-%20Agriculture%20Development_compressed%20(1).pdf#page=21.58

Akhtar R, Iqbal MZ, Ullah A, *et al.*, 2019. Comparative association of slc11a1 (Nramp1) gene with susceptibility and resistance to brucellosis in various cattle and buffaloes breeds of Pakistan. Pakistan Veterinary Journal 39(4):612-614.

Al-Majali AM, Talafha AQ, Ababneh MM, *et al.*, 2009. Seroprevalence and risk factors for bovine brucellosis in Jordan. Journal of Veterinary Science 10(1):61-65.

Alamian S, Amiry K, Bahreinipour A, *et al.*, 2023. Evaluation of serological diagnostic tests for bovine brucellosis in dairy cattle herds in an endemic area: a multicenter study. Tropical Animal Health and Production 55(2):104.

Anderson J and Smith H, 1965. The metabolism of erythritol by *Brucella abortus*. Microbiology 38(1):109-124.

Andrade RS, Oliveira MM, Bueno Filho JSS, *et al.*, 2024. Accuracy of serological tests for bovine brucellosis: a systematic review and meta-analysis. Preventive Veterinary Medicine 222:106079.

Anonymous, 2022. Brucellosis (infection with *Brucella abortus*, *B. melitensis* and *B. suis*). In WOAH Terrestrial Manual 2022. World Organisation for Animal Health. Retrieved from https://www.woah.org/fileadmin/Home/eng/Health_standards/tahm/3.01.04_BRUCELLOSIS.pdf

Aranguiz AA and Spoelstra M, 2025. Trends and outlook of dairy production in Pakistan. Wageningen Livestock Research, Public Report 1567. Retrieved from https://edepot.wur.nl/691204

Arif S, Thomson PC, Hernandez-Jover M, *et al.*, 2019. Bovine brucellosis in Pakistan; an analysis of engagement with risk factors in smallholder farmer settings. Veterinary Medicine and Science 5(3):390-401.

Asgedom H, Damena D and Duguma R, 2016. Seroprevalence of bovine brucellosis and associated risk factors in and around Alage district, Ethiopia. SpringerPlus 5(1):851.

Audigé L, 2005. Veterinary Epidemiologic Research: I. Dohoo, W. Martin, H. Stryhn, Atlantic Veterinary College, Charlottetown, PE, Canada, 2003. Preventive Veterinary Medicine 68:289-292.

Awais MM, Khadim G, Akhtar M, *et al.*, 2024. A study on the epidemiology of brucellosis in bovine population of peri-urban and rural areas of district Multan, southern Punjab, Pakistan. BMC Veterinary Research 20(1):39.

Bayemi P, Webb E, Nsongka M, *et al.*, 2009. Prevalence of *Brucella abortus* antibodies in serum of Holstein cattle in Cameroon. Tropical Animal Health and Production 41(2):141-144.

Boukary AR, Saegerman C, Abatih E, *et al.*, 2013. Seroprevalence and potential risk factors for *Brucella* spp. infection in traditional cattle, sheep and goats reared in urban, periurban and rural areas of Niger. PLoS ONE 8(12):e83175.

Bronner A, Hénaux V, Fortané N, *et al.*, 2014. Why do farmers and veterinarians not report all bovine abortions, as requested by the clinical brucellosis surveillance system in France? BMC Veterinary Research 10(1):93.

Cadmus S, Adesokan H and Stack J, 2008. The use of the milk ring test and Rose Bengal test in brucellosis control and eradication in Nigeria. Journal of the South African Veterinary Association 79(3):113-115.

Chimana HM, Muma JB, Samui KL, *et al.*, 2010. A comparative study of the seroprevalence of brucellosis in commercial and small-scale mixed dairy-beef cattle enterprises of Lusaka province and Chibombo district, Zambia. Tropical Animal Health and Production 42(7):1541-1545.

Corbel MJ, 2020. Microbiology of the genus *Brucella*. In Brucellosis. CRC Press: Boca Raton, FL, USA, pp:53–72.

Deka RP, Magnusson U, Grace D, *et al.*, 2018. Bovine brucellosis: prevalence, risk factors, economic cost and control options with particular reference to India- a review. Infection Ecology & Epidemiology 8(1):1556548.

Deresa B, Tulu D, Deressa FB, 2020. Epidemiological investigation of cattle abortion and its association with brucellosis in Jimma zone, Ethiopia. Veterinary Medicine: Research and Reports 2020(11):87-98.

Fero E, Juma A, Koni A, *et al.*, 2020. The seroprevalence of brucellosis and molecular characterization of *Brucella* species circulating in the beef cattle herds in Albania. PloS One 15(3):e0229741.

Freire ML, Machado de Assis TS, Silva SN, *et al.*, 2024. Diagnosis of human brucellosis: systematic review and meta-analysis. PLoS Neglected Tropical Diseases 18(3):e0012030.

Getachew T, Getachew G, Sintayehu G, *et al.*, 2016. Bayesian estimation of sensitivity and specificity of rose bengal, complement fixation,

and indirect ELISA tests for the diagnosis of bovine brucellosis in Ethiopia. Veterinary Medicine International 2016(1):8032753.

González-Espinoza G, Arce-Gorvel V, Mémet S, *et al*., 2021. "*Brucella*: reservoirs and niches in animals and humans." Pathogens 10(2): 186.

Haileselassie M, Kalayou S, Kyule M, *et al*., 2011. Effect of *Brucella* infection on reproduction conditions of female breeding cattle and its public health significance in Western Tigray, northern Ethiopia. Veterinary Medicine International 2011(1):354943.

Holt HR, Bedi JS, Kaur P, *et al*., 2021. Epidemiology of brucellosis in cattle and dairy farmers of rural Ludhiana, Punjab. PLoS Neglected Tropical Diseases 15(3):e0009102.

Hussain A, Hussain S, Chaudhry M, *et al*., 2025. Prevalence and herd-level risk factors associated with *Brucella* infection in smallholders keeping large ruminants. Research in Veterinary Science 183:105506.

Ibrahim N, Belihu K, Lobago F, *et al*., 2010. Sero-prevalence of bovine brucellosis and its risk factors in Jimma zone of Oromia Region, south-western Ethiopia. Tropical Animal Health and Production 42(1):35-40.

Iqbal M, Fatmi Z, Khan MA, 2020. Brucellosis in Pakistan: a neglected zoonotic disease. Journal of Pakistan Medical Association 70(9):1625-1626.

Jamil T, Khan AU, Saqib M, *et al*., 2021. Animal and human brucellosis in Pakistan. Frontiers in Public Health 9:660508.

Jamil T, Melzer F, Saqib M, *et al*., 2020. Serological and molecular detection of bovine brucellosis at institutional livestock farms in Punjab, Pakistan. International Journal of Environmental Research and Public Health 17(4):1412.

Junaidu AU, Oboegbulem SI and Salihu MD, 2008. Seroprevalence of brucellosis in Prison farm in Sokoto, Nigeria. Asian Journal of Epidemiology 1(1):24-28.

Junaidu AU, Oboegbulem SI and Salihu MD, 2011. Serological survey of *Brucella* antibodies in breeding herds. Journal of Microbiology and Biotechnology Research 1(1):60-65.

Kairu-Wanyoike S, Nyamwaya D, Wainaina M, *et al*., 2019. Positive association between *Brucella* spp. seroprevalences in livestock and humans from a cross-sectional study in Garissa and Tana River Counties, Kenya. PLoS Neglected Tropical Diseases 13(10):e0007506.

Khan AU, Melzer F, Hendam A, *et al*., 2020. Seroprevalence and molecular identification of *Brucella* spp. in bovines in Pakistan—investigating association with risk factors using machine learning. Frontiers in Veterinary Science 7:594498.

Khan I, Ali S, Hussain R, *et al*., 2021a. Serosurvey and potential risk factors of brucellosis in dairy cattle in peri-urban production system in Punjab, Pakistan. Pakistan Veterinary Journal 41:459-462.

Khan J, Sadiq A, Basharat N, *et al*., 2025. Seroprevalence and associated risk factors of brucellosis in human and animals blood. Journal of Applied Animal Research 53(1):2483796.

Khan MR, Rehman A, Khalid S, *et al*., 2021b. Seroprevalence and associated risk factors of bovine brucellosis in district Gujranwala, Punjab, Pakistan. Animals 11(6):1744.

Khurana SK, Sehrawat A, Tiwari R, *et al*., 2021. Bovine brucellosis–a comprehensive review. Veterinary Quarterly 41(1):61-88.

Laine CG, Johnson VE, Scott HM, *et al*., 2023. Global estimate of human brucellosis incidence. Emerging Infectious Diseases 29(9):1789.

Landis JR and Koch GG, 1977. The measurement of observer agreement for categorical data. Biometrics:159-174.

Li W, Ouyang H, Zhao Z, *et al*., 2024. Trends and age-period-cohort effect on incidence of brucellosis from 2006 to 2020 in China. Acta Tropica 260:107475.

Loubet P, Magnan C, Salipante F, *et al*., 2024. Diagnosis of brucellosis: combining tests to improve performance. PLoS Neglected Tropical Diseases 18(9):e0012442.

Mai HM, Irons PC, Kabir J, *et al*., 2012. A large seroprevalence survey of brucellosis in cattle herds under diverse production systems in northern Nigeria. BMC Veterinary Research 8(1):144.

Manheim D and Lewis G, 2022. High-risk human-caused pathogen exposure events from 1975-2016. F1000Research 10:752.

Moreno E, Blasco JM and Moriyón I, 2022. Facing the human and animal brucellosis conundrums: the forgotten lessons. Microorganisms 10(5):942.

Nasir A, Perveen Z and Ikram-ul-Haq M, 2005. Comparative study of standard and modified serum agglutination tests for the diagnosis of brucellosis in animals. Pakistan Veterinary Journal 25(1):33-34.

Ntivuguruzwa JB, Babaman Kolo F, Mwikarago EI, *et al*., 2022. Seroprevalence of brucellosis and molecular characterization of *Brucella* spp. from slaughtered cattle in Rwanda. PloS One 17(11):e0261595.

Otte MJ and Gumm ID, 1997. Intra-cluster correlation coefficients of 20 infections calculated from the results of cluster-sample surveys. Preventive Veterinary Medicine 31(1):147-150.

Pakistan Economic Survey, 2025–2026. Economic Advisor's Wing, Finance Division, Government of Pakistan, Islamabad, Pakistan. Retrieved from https://www.finance.gov.pk/survey/chapter_26/2_Agriculture.pdf

Patel M, Patel P, Prajapati M, *et al*., 2014. Prevalence and risk factor's analysis of bovine brucellosis in peri-urban areas under intensive system of production in Gujarat, India. Veterinary World 7(7):509-516.

Paweska J, Potts A, Harris H, *et al*., 2002. Validation of an indirect enzyme-linked immunosorbent assay for the detection of antibody against *Brucella abortus* in cattle sera using an automated ELISA workstation. Onderstepoort Journal of Veterinary Research 69(1):61-77.

Pourhoseingholi MA, Vahedi M and Rahimzadeh M, 2013. Sample size calculation in medical studies. Gastroenterology and Hepatology from Bed to Bench 6(1):14-17.

Saeed U, Ali S, Latif T, *et al*., 2020. Prevalence and spatial distribution of animal brucellosis in central Punjab, Pakistan. International Journal of Environmental Research and Public Health 17(18):6903.

Shabbir MZ, Khalid RK, Freitas DM, *et al*., 2013. Serological evidence of selected abortifacients in a dairy herd with history of abortion. Pakistan Veterinary Journal 33(1):19-22.

Shirwany ASAK, Awais MM, Masood S, *et al*., 2026. A snapshot of brucellosis as an occupational health hazard in animal handlers of an agroecological zone of southern Punjab, Pakistan. Journal of Agromedicine 31(1):30-43.

Tekle M, Legesse M and Mamo G, 2023. Seropositivity, comparison between the efficiency of serological tests and risk factors of *Brucella* infection in small ruminants with history of abortion in the Afar Region of North-Eastern Ethiopia. Veterinary Medicine: Research and Reports:245-252.

Ullah I, Naz S, Khattak US, *et al*., 2024. Prevalence and associated risk factors of *Brucella abortus* and *Brucella* melitensis in humans and cattle populations: a comprehensive study. Comparative Immunology, Microbiology and Infectious Diseases 115:102276.

Ullah Q, Jamil H, Lodhi LA, *et al*., 2019. Brucellosis is significantly associated with reproductive disorders in dairy cattle of Punjab, Pakistan. Pakistan Journal of Zoology 51:1995-1997.

Ullah S, Jamil T, Asif M, *et al*., 2022. Brucellosis remains a neglected disease in District Muzaffargarh of Pakistani Punjab: a call for multidisciplinary collaboration. German Journal of Veterinary Research 2:35-38.

Yanti Y, Sumiarto B, Kusumastuti TA, *et al*., 2021. Seroprevalence and risk factors of brucellosis and the brucellosis model at the individual level of dairy cattle in the West Bandung District, Indonesia. Veterinary World 14(1):1-10.