

Molecular Epidemiology of Human Brucellosis: Identification through *bvrR*/*bvrS* Gene Analysis

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Abstract—*Brucellosis* in humans is one of the most important zoonotic diseases worldwide, causing serious public health and economic burdens. This study determined the prevalence of human brucellosis in different occupational groups in Erbil Governorate, Kurdistan Region, Iraq, and evaluated serological, bacteriological, and molecular approaches for detecting *Brucella* spp. In total, 300 blood samples were collected from six occupational groups (herder families, farmer families, clinical patients, restaurant chefs and staff, butchers, and bakers) between April and November 2024. Samples were tested using the Rose Bengal test (RBT), enzyme-linked immunosorbent assay (ELISA), blood culture, and polymerase chain reaction (PCR) targeting the *bvrR* and *bvrS* virulence genes. Data were analyzed using the Statistical Package for the Social Sciences, with $p < 0.05$ considered statistically significant. Overall positivity rates were highest for RBT (10.3%), followed by ELISA (7.0%), while blood culture and PCR each detected 16 positive cases (5.3%). Herder families showed the highest positivity rates; farmer families were positive only for RBT. ELISA positivity was significantly associated with occupation, while RBT and PCR showed borderline significance. RBT and ELISA positivity were significantly higher among rural than urban residents, with no significant association with age or gender. Phylogenetic analysis of the *bvrR* and *bvrS* sequences showed close clustering with reference *Brucella* strains in GenBank, with sequence identities of 99.75–100% for *bvrR* and 99.05–100% for *bvrS*. Sequences were deposited in GenBank under accession numbers PZ165384-PZ165399 and PZ204958-PZ204973. Our findings highlight the burden of brucellosis in animal-exposed populations and the utility of serology and molecular tools for disease surveillance and diagnosis.

Index Terms—*Brucellosis*, Enzyme-linked immunosorbent assay, Occupational exposure, Polymerase chain reaction, Rose Bengal test.

I. INTRODUCTION

Brucellosis remains an important zoonotic infection throughout the world and still poses a serious threat to public health, especially in developing countries. It is caused by Gram-negative, facultative intracellular bacteria of the genus *Brucella* that can infect humans and a wide variety of domestic and wild animals. The disease is still prevalent in the Middle East, the Mediterranean basin, Africa, Asia, and Latin America. It has a major negative effect on both health and the economic system. (Dadar, et al., 2024; Khoshnood, et al., 2022; Nejad, et al., 2020; Suresh, et al., 2022). Human infection occurs mainly through direct contact with infected animals or the ingestion of unpasteurized milk and dairy products. Consequently, individuals with occupational exposure to livestock, such as herders, farmers, butchers, slaughterhouse workers, and veterinarians, are at increased risk of infection (Khairullah, et al., 2024; Qureshi, et al., 2023). Human brucellosis is characterized by non-specific clinical manifestations, including fever, night sweats, fatigue, headache, and musculoskeletal pain, making clinical diagnosis difficult without laboratory confirmation (Ferrero, et al., 2020; Mambote, 2024; Zhang, et al., 2022). If left untreated, the disease may become chronic and involve multiple organ systems. Therefore, accurate laboratory diagnosis is essential for appropriate patient management and disease control. The diagnosis of human brucellosis is based on bacteriological, serological, and molecular techniques. Although bacterial culture remains the reference method, its diagnostic value is limited by the slow growth of *Brucella*, reduced sensitivity in chronic infections, and biosafety requirements (Di Bonaventura, et al., 2021; Yagupsky, et al., 2019). Serological tests, including the Rose Bengal Test (RBT) and enzyme-



linked immunosorbent assay (ELISA), are widely used because they are rapid, simple, and suitable for screening large populations (Barkay, et al., 2024; Lukumbagire, et al., 2021; Vicente, et al., 2026). Furthermore, polymerase chain reaction (PCR) has been reported as a rapid and highly sensitive method for detection of *Brucella* DNA, surpassing conventional diagnostic methods. The PCR detection uses the *bvrR* and *bvrS* genes as molecular targets, which encode a two-component regulatory system crucial for the pathogenicity, intracellular survival, and virulence of *Brucella* species (Altamirano-Silva, et al., 2023; Loubet, et al., 2024; Rivas-Solano, et al., 2023). These highly conserved genes are valuable molecular markers for the detection and molecular characterization of *Brucella* isolates and are widely used in epidemiological investigations. Despite the endemic nature of brucellosis in Iraq, limited information is available regarding its prevalence among different occupational groups in Erbil Governorate. Furthermore, few studies have combined serological, bacteriological, molecular, and phylogenetic analyses to investigate human brucellosis in the same population. Therefore, the present study aimed to determine the prevalence of human brucellosis among six occupational groups in Erbil Governorate, Kurdistan Region, Iraq, using the RBT, ELISA, blood culture, and PCR targeting the *bvrR* and *bvrS* virulence genes. In addition, phylogenetic analysis of the obtained sequences was performed to evaluate their genetic relationships with reference *Brucella* strains available in GenBank.

II. MATERIALS AND METHODS

A. Study Design and Sample Collection

The study was conducted in Erbil Governorate, Iraq, between April and November 2024. In total, 300 blood samples were obtained from suspected brucellosis cases, including 125 samples from herder families, 50 samples from farmer families, 50 samples from clinical patients, and 25 samples from butchers, bakers, and restaurant chefs/staff. A venous blood sample (5–10 mL) was collected, transported at 4°C, and processed within 4–6 h. Samples were inoculated on selective *Brucella* agar plates and cultured at 37°C with 5–10% carbon dioxide for 14–21 days and monitored. Suspected *Brucella* colonies were subcultured to purity and presumptively identified by colony morphology and biochemical assays (catalase, oxidase, urease, H₂S generation, thionine and basic fuchsin). The study protocol was reviewed and approved by the Faculty of Science and Health, Department of Medical Microbiology, Koya University, with Ethical Approval Code No. DMMB-4-24, May 5, 2024. The study was conducted confidentially, and participation was voluntary. All procedures were in accordance with the ethical standards of the Declaration of Helsinki. Written informed consent was obtained from all participants before sample collection.

B. RBT

Torax Biosciences (United Kingdom) detected *Brucella* antibodies by the following procedure: 0.03 mL of serum

was mixed with an equal volume of antigen on a white tile or enamel plate, producing a zone about 2 cm in diameter. The mixture was gently mixed for 4 min at room temperature, and any visible clumping indicates a positive result (Kaki, et al., 2025; Verma, 2013).

C. ELISA

The indirect ELISA kit (Demeditec Diagnostics, Germany) was utilized for the estimation of several antibodies raised against human brucellosis specifically. Sera, controls, reagents, and coated microplates were handled as per the manufacturer's instructions. The absorbance was read at an optical density (OD) of 450 nm using the ELISA Microplate Reader manufactured by Chromate (USA). Eventually, the efficiency of the test was evaluated, and the critical value (CUT OFF) was obtained at 0.299. If the OD value for samples was greater than or equal to CUT OFF, then they were considered positive. However, it is also intriguing to take seropositive ODs and assign them into four categories based on the severity of infection: Light (<0.4), moderate (0.41–0.6), severe (0.61–0.8), and grave (0.800 or greater).

D. Molecular Analysis

DNA extraction

All samples of the colony bacteria were extracted using a DNA extraction kit (Amplisens, Korea) in accordance with the manufacturer's instructions. An ultraviolet (UV) transilluminator (Bio-Rad, USA) was used to visualize the extracted DNA on a 2% agarose gel stained with a safety dye (Green-DNA Dye, Biotech, USA) after quantification using a NanoDrop spectrophotometer (Biometrics, USA) at a wavelength of 260/280 nm. The isolated genomic DNA was stored at –20°C for further molecular analysis.

PCR

Conventional PCR employing species-specific primers that target the *bvrS* and *bvrR* genes detected *Brucella* spp. The primers 5'-CGCTTTATCGTTGATCTTCC-3' (forward) and 5'-AATCACCATTGCGGACAAT-3' (reverse) amplified *Brucella* spp., producing a 474 bp amplicon (In this study), while the primers 5'-CCTAATTTGTACTGCAAAG-3' (forward) and 5'-TTCAATCTTACCCTTAACCG-3' (reverse) amplified *Brucella* spp., producing a 963 bp amplicon (Sola-Landa, et al., 1998). The amplification was initiated by a denaturation step at 95°C for 3 min, followed by 1 cycle of denaturation at 95°C for 30 s, 39 cycles of annealing at 57.5°C for 30 s, and 39 cycles of extension at 72°C for 1 min. Finally, one cycle of 5 min of extension at 72°C was performed. The PCR reaction was performed in 20 µL containing 10 µL of HotStarTaq DNA polymerase master mix buffer (Addbio, South Korea), 1 µL of each primer, 5 µL of DNA template, and 3 µL of nuclease-free water. The PCR products were separated by electrophoresis on 2% agarose gel stained with ethidium bromide. The process was performed during 40 min at 120 V and 100 amps, and the results were visualized by a UV trans-illuminator (Lee, et al., 2012; Lorenz, 2012).

DNA sequencing

For nucleotide sequencing, 18 µL of PCR product from each sample was transferred into 1.5 mL microcentrifuge tubes. After that, the samples were sent to the ExoGenic center in Erbil for sequencing. DNA was sequenced using the SeqStudio™ Genetic Analyzer with Sanger sequencing methodology. The nucleotide sequences were submitted to the GenBank database and provided the following accession numbers (PZ204958, PZ204959, PZ204960, PZ204961, PZ204962, PZ204963, PZ204964, PZ204965, PZ204966, PZ204967, PZ204968, PZ204969, PZ204970, PZ204971, PZ204972, and PZ204973) for *bvrS* *Brucella* spp. and (PZ165384, PZ165385, PZ165386, PZ165387, PZ165388, PZ165389, PZ165390, PZ165391, PZ165392, PZ165393, PZ165394, PZ165395, PZ165396, PZ165397, PZ165398, and PZ165399) for *bvrR* *Brucella* spp.

Phylogenetic analysis

Phylogenetic analyses of *Brucella* spp. commonly employ virulence- and immunogenicity-associated loci (for example, *bvrR* and *bvrS*) to infer evolutionary relationships and place strain diversity against reference genomes. These markers can be analyzed as individual-locus trees or concatenated in multilocus sequence analysis (MLSA) depending on the study objectives and the extent of shared sequence regions (Rivas-Solano, et al., 2023). The National Center for Biotechnology Information database and the BLASTn algorithm identified homologous sequences of two specific genes (*bvrS* and *bvrR*) for *Brucella* spp. Sequences generated here were queried with BLASTn against NCBI GenBank, aligned with Clustal X v2.1 and refined in BioEdit v7.2, and subjected to maximum-likelihood phylogenetic reconstruction in MEGA v12 (Tamura-Nei, partial deletion, 1,000 bootstraps); trees were visualized and annotated in iTOL to examine the genetic similarity and differences between the isolates over time.

E. Statistical Analysis

Data were analyzed using GraphPad Prism (version 10) and IBM Statistical Package for the Social Sciences Statistics (version 26.0). Descriptive statistics were used to break down the data for age, gender, and place of residence and are presented as means ± standard deviations for continuous variables and frequencies with percentages for categorical variables. The prevalence of brucellosis among the six occupational groups was compared using the Pearson Chi-square (χ^2) test based on the RBT, ELISA, and PCR. Agreement between the three diagnostic techniques was assessed using Cohen's kappa (κ) statistic. Two-sided $p < 0.05$ was considered statistically significant.

III. RESULTS

A. Molecular Analysis

Target genes *bvrR* and *bvrS* were verified by comparing the size of amplicons with the expected fragment lengths to confirm successful PCR amplification on agarose gel electrophoresis. After DNA extraction and PCR amplification, the products were separated on 1–2% agarose

gels, stained with ethidium bromide, and visualized to reveal discrete bands characteristic of each primer's target nucleotide sequences, while differentiating between non-specific products or "primer dimers." This step was used to confirm the presence or absence of the expected band patterns for each gene target (*bvrR* and *bvrS*) to validate source-directed amplification before downstream applications such as sequencing or phylogenetic analysis (Rivas-Solano, et al., 2022). PCR amplification results confirming successful detection of both *bvrS* and *bvrR* genes are presented below. Fig. 1 shows the agarose gel electrophoresis of the *bvrS* gene, while Fig. 2 displays the corresponding *bvrR* gene amplification products.

B. Phylogenetic Tree Analysis

Phylogenetic analyses of *Brucella* spp. commonly employ virulence- and immunogenicity-associated loci, including *bvrR* and *bvrS*, to infer evolutionary relationships and evaluate strain diversity against reference genomes. These markers can be analyzed individually or as part of MLSA, depending on the study objectives and the availability of shared sequence regions (Rivas-Solano, et al., 2023). The *bvrR/bvrS* two-component regulatory system plays a critical role in bacterial cell invasion and intracellular survival. A total of 16 *Brucella* isolates were included in the molecular analysis. Sequencing of the *bvrR* and *bvrS* genes generated 32 nucleotide sequences, comprising 16 *bvrR* sequences and 16 *bvrS* sequences. The nucleotide sequences generated in this study were deposited in the NCBI GenBank database. The *bvrS* gene sequences were assigned accession numbers PZ204958 - PZ204973, while the *bvrR* gene sequences were assigned accession numbers PZ165384 - PZ165399. These sequences were queried using BLASTn against the NCBI GenBank database, aligned using Clustal X version 2.1, and refined with BioEdit version 7.2. Phylogenetic trees were reconstructed using the maximum-likelihood method implemented in MEGA version 12 under the Tamura-Nei

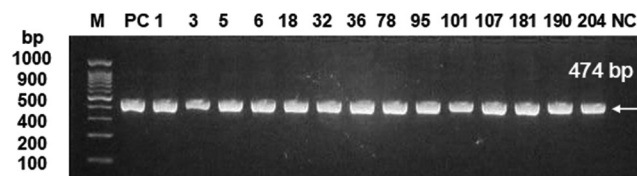


Fig. 1. Agarose gel electrophoresis of the polymerase chain reaction product of the *bvrS* gene of *Brucella* spp. in colony samples. M=DNA ladder (100bp scale), 1–14: Samples, NC: Negative control.

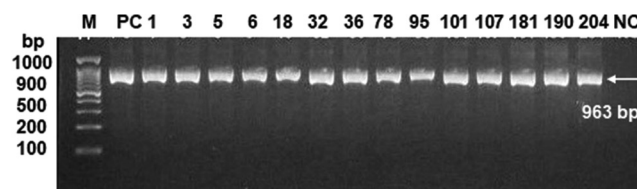


Fig. 2. Agarose gel electrophoresis of the polymerase chain reaction product of the *bvrR* gene of *Brucella* spp. in colony samples. M=DNA ladder (100bp scale), 1–14: Samples, NC: Negative control.

substitution model with partial deletion and 1,000 bootstrap replicates. The resulting phylogenetic trees were visualized and annotated using iTOL.

The phylogenetic analysis comparing our sequenced isolates with reference *Brucella* strains from GenBank is illustrated in Fig. 3 (*bvrS* gene sequences) and Fig. 4 (*bvrR* gene sequences). These figures demonstrate the evolutionary relationships and genetic clustering patterns observed.

C. Clinical Data Analysis

In our study, we enrolled 300 participants ranging from 4 to 85 years old, with a mean age of 33.40 ± 16.455 years. The highest number of people were from herder families ($n = 125$; 41.7%), as shown in Table II. The mean age in this group was 32.81 ± 17.632 years. This group was followed by farmers and clinical personnel, with each group contributing 50 participants (16.7%). Their ages were, respectively, 39.10 ± 13.917 and 37.58 ± 19.309 years. Families of chief restaurant workers, butchers, and cooks provided 25 participants each (8.3%) with mean ages between 26.68 and 29.40 years. Among the 300 patients, 156 (52%) were male and 144 (48%) were female; individual groupings even included only men - chiefs, butchers, and bakers formed the majority of these people. 137 (45.7%) of the participants lived in cities, while 163 (54.3%) resided in rural areas.

The rate of *Brucella* detection using RBT, ELISA, and PCR among occupational groups all differed greatly, as shown in Table I. Herder families had the highest positive rates: RBT came in at 16.8% (21/125), ELISA was 12.8% (16/125), and PCR was 9.6% (12/125). Clinical patients had uniform rates (8.0%, 6.0%, and 6.0%), while farming families saw minimal detection (RBT 6.0%, ELISA/PCR 0%). Chi-square tests were used to confirm significant associations between occupation and test results (ELISA: $\chi^2 = 10.27$, $p = 0.02$; RBT: $\chi^2 = 10.19$, $p = 0.07$; PCR: $\chi^2 = 12.87$, $p = 0.06$). As a whole, RBT had the highest positivity of all methods used (10.3%). This was followed by ELISA (7.0%) and then PCR (5.3%). Serological methods proved most effective for the 300 highest-risk participants.

Demographic and cited occupational risk factors for *Brucella* infection revealed distinct patterns. As shown in Table I, the age distribution revealed the highest positivity among the younger age groups (0–20 and 21–40 years old, respectively: RBT 7.2%, 12.1%, and 13.0%); and no cases occurred in people over 60 ($p > 0.05$). Herder families had the highest occupational risk (RBT 16.8%, ELISA 12.8%, PCR 9.6%), with ELISA showing a statistically significant association ($p = 0.02$). There were few gender differences (males: RBT 10.3%, ELISA 5.8%, PCR 3.2%; females: RBT 10.4%, ELISA 8.3%, PCR 7.6%; $p > 0.05$). Rural residence

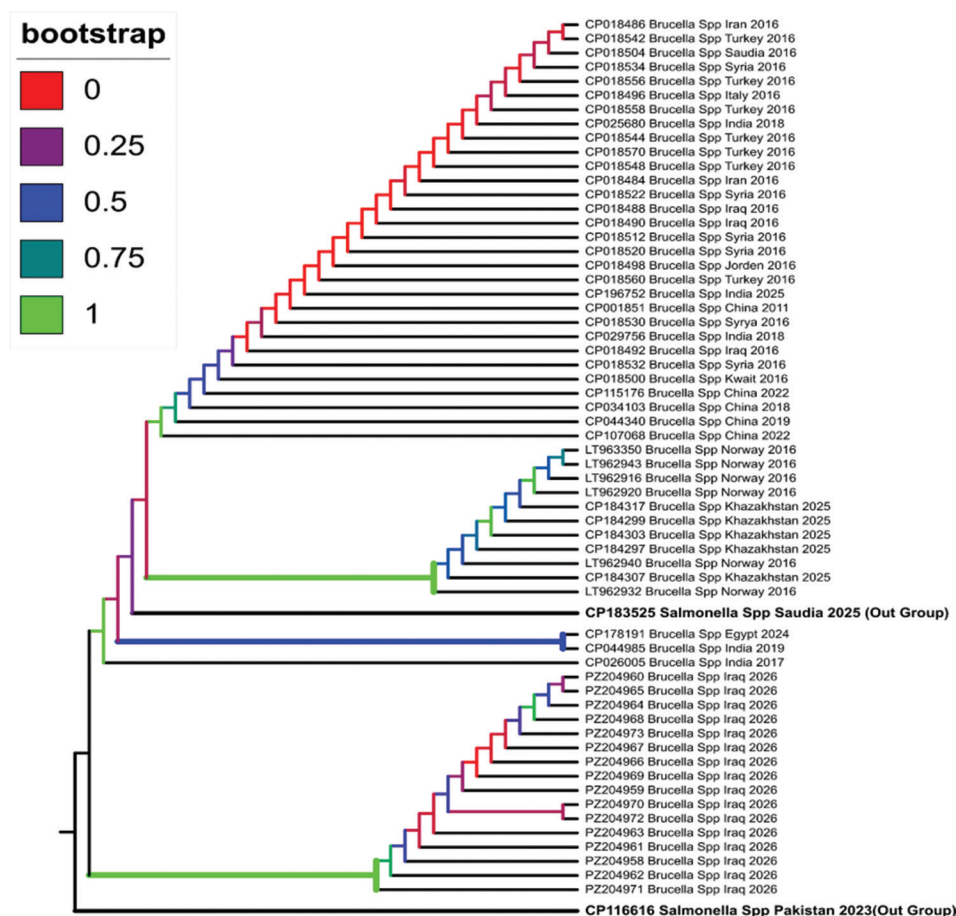


Fig. 3. Illustrates the evolutionary relationships between the *bvrS* sequences of the *Brucella* isolates identified in this investigation and a few isolates from GenBank using the neighbor-joining method.

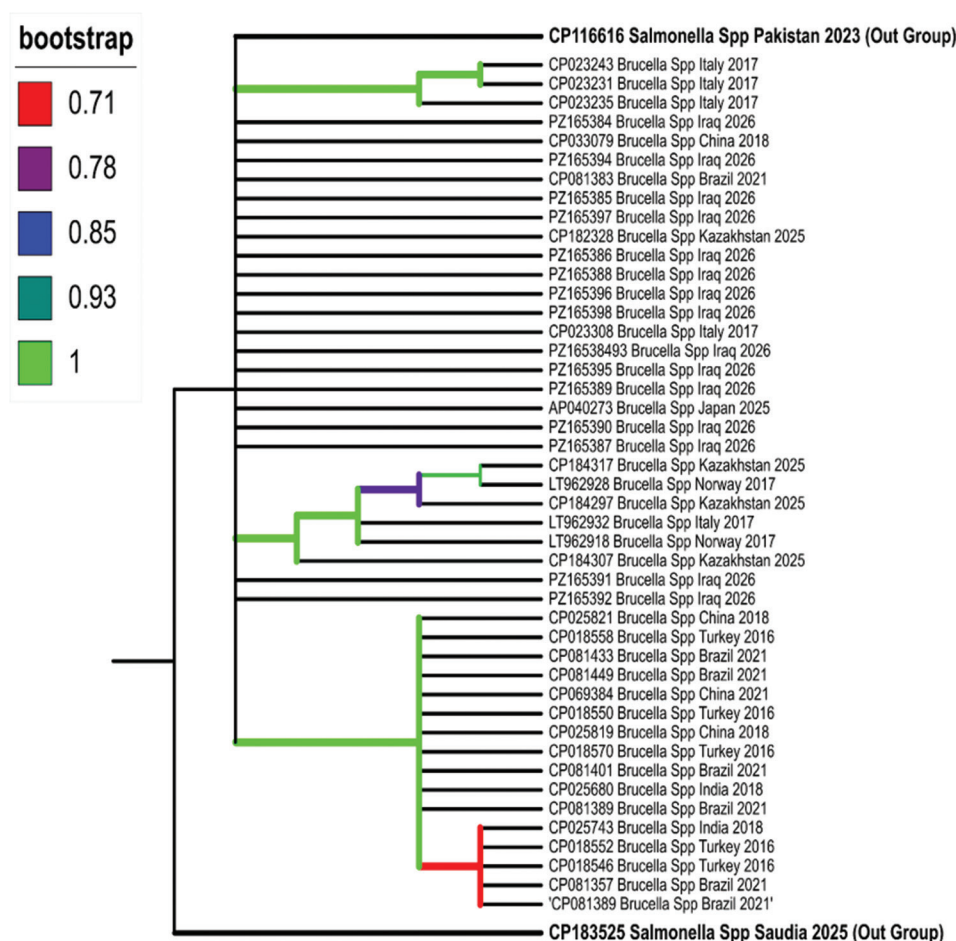


Fig. 4. Illustrates the evolutionary relationships between the *bvrR* sequences of the *Brucella* isolates identified in this investigation and a few isolates from GenBank using the neighbor-joining method.

TABLE I
SOCIODEMOGRAPHIC CHARACTERISTICS AND SEROPREVALENCE/MOLECULAR DETECTION OF BRUCELLOSIS BY OCCUPATIONAL GROUP

Category	N	Mean age±standard deviation	Male n (%)	Female n (%)	Urban n (%)	Rural n (%)	RBT Positive n (%)	ELISA Positive n (%)	PCR Positive n (%)
Herder families	125	32.81±17.63	55 (35.3)	70 (48.6)	0 (0.0)	125 (76.7)	21 (16.8)	16 (12.8)	12 (9.6)
Farmer families	50	39.10±13.92	13 (8.3)	37 (25.7)	12 (8.8)	38 (23.3)	3 (6.0)	0 (0.0)	0 (0.0)
Clinical patients	50	37.58±19.31	13 (8.3)	37 (25.7)	50 (36.5)	0 (0.0)	4 (8.0)	3 (6.0)	3 (6.0)
Restaurant workers	25	26.68±12.46	25 (16.0)	0 (0.0)	25 (18.2)	0 (0.0)	1 (4.0)	1 (4.0)	0 (0.0)
Butchers	25	29.40±9.47	25 (16.0)	0 (0.0)	25 (18.2)	0 (0.0)	1 (4.0)	0 (0.0)	0 (0.0)
Bakers	25	27.28±12.53	25 (16.0)	0 (0.0)	25 (18.2)	0 (0.0)	1 (4.0)	1 (4.0)	1 (4.0)
χ^2 (df=5)							10.19	10.27	12.87
p value							0.07	0.02	0.06
Total	300	33.40±16.46	156	144	137	163	31	21	16

ELISA: Enzyme-linked immunosorbent assay, PCR: Polymerase chain reaction, RBT: Rose Bengal test

emerged as the most significant risk factor, with substantially higher rates (RBT 13.5%, ELISA 9.8%, PCR 7.4%) versus urban dwellers (RBT 6.6%, ELISA 3.8%, PCR 2.9%), while only RBT and ELISA showed statistical significance ($p = 0.03$ and 0.025 , respectively). As shown in Table II, the rural herder occupation is the primary brucellosis risk factor.

The kappa values (0.611–0.799) for comparison of the three methods of *Brucella* detection suggest a moderate overall agreement of the results. In Table III, the RBT presented a high similarity with ELISA (79.0%, $\kappa = 0.790$, $p < 0.0001$)

and a fair degree of agreement with PCR (61.1%, $\kappa = 0.611$, $p < 0.0001$). ELISA and PCR showed good consensus (79.9%, $\kappa = 0.799$, $p < 0.0001$). All comparisons presented significant ($p < 0.001$) results, which implies that there is no random agreement. Kappa values (0.611–0.799) suggest general integrity, but the Rose Bengal-PCR concordance ($\kappa = 0.611$) is lower than anticipated. This could be because of systematic variation or because the two tests are used in different ways or for different purposes. These results suggest

TABLE II
SOCIODEMOGRAPHIC AND OCCUPATIONAL CHARACTERISTICS OF STUDY PARTICIPANTS

Characteristic	Category	Herder family (%)	Farmer family (%)	Clinical patient (%)	Chef restaurant (%)	Butcher (%)	Baker (%)	Total (%)
Age (years)	0–20	34 (49.3)	6 (8.7)	8 (11.6)	10 (14.5)	4 (5.8)	7 (10.1)	69 (100.0)
	21–40	51 (36.4)	20 (14.3)	23 (16.4)	12 (8.6)	18 (12.9)	16 (11.4)	140 (100.0)
	41–60	29 (42.0)	22 (31.9)	12 (17.4)	2 (2.9)	3 (4.3)	1 (1.4)	69 (100.0)
	61>	11 (50.0)	2 (9.1)	7 (31.8)	1 (4.5)	0 (0.0)	1 (4.5)	22 (100.0)
	Total	125 (41.7)	50 (16.7)	50 (16.7)	25 (8.3)	25 (8.3)	25 (8.3)	300 (100.0)
Gender	Male	55 (35.3)	13 (8.3)	13 (8.3)	25 (16.0)	25 (16.0)	25 (16.0)	156 (100.0)
	Female	70 (48.6)	37 (25.7)	37 (25.7)	0 (0.0)	0 (0.0)	0 (0.0)	144 (100.0)
	Total	125 (41.7)	50 (16.7)	50 (16.7)	25 (8.3)	25 (8.3)	25 (8.3)	300 (100.0)
Residence	Urban	0 (0.0)	12 (8.8)	50 (36.5)	25 (18.2)	25 (18.2)	25 (18.2)	137 (100.0)
	Rural	125 (76.7)	38 (23.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	163 (100.0)
	Total	125 (41.7)	50 (16.7)	50 (16.7)	25 (8.3)	25 (8.3)	25 (8.3)	300 (100.0)

TABLE III
COMPARATIVE AGREEMENT OF ROSE BENGAL, ELISA, AND PCR TESTS FOR BRUCELLOSIS

Test comparison	Agreement (%)	Kappa value	p-value
Rose Bengal versus ELISA	79.0	0.790	<0.0001
Rose Bengal versus PCR	61.1	0.611	<0.0001
ELISA versus PCR	79.9	0.799	<0.0001

ELISA: Enzyme-linked immunosorbent assay, PCR: Polymerase chain reaction

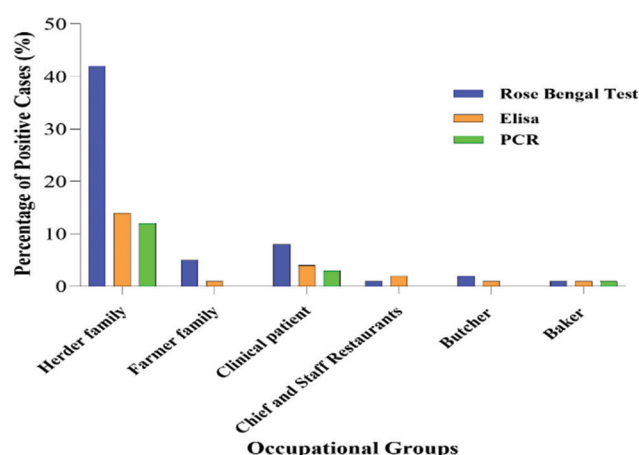


Fig. 5. Distribution of *Brucella* detection by diagnostic method (Rose Bengal test, enzyme-linked immunosorbent assay, and polymerase chain reaction) across occupational groups in Erbil, Kurdistan, Iraq.

that the combination of several methods for diagnosis is advisable, which is supported by the fact that serological methods (Rose Bengal and ELISA) were more easily matched among themselves than with any single PCR result. As shown in Fig. 5, the distribution of *Brucella* and method to detection varied significantly across occupational groups.

Most participants were young adults aged 21–40 years (46.7%), and men constituted 52% of the sample (Table II). In terms of occupation, most participants were from herder families (41.7%), followed by clinical patients (16.7%). A few restaurant workers, butchers, and bakers made small percentages. In terms of place of residence, rural residents mainly include herders and farmers, while urban residents include clinical patients and bakers. These urban-rural and occupational patterns reflect differences in exposure

pathways: rural livestock-related work and healthcare settings are particularly conducive to zoonotic transmission.

IV. DISCUSSION

The prevalence of human brucellosis in Iraq is still a serious public health problem. The results of this study are in agreement with those found in the Kurdistan Region. The overall positivity rates of RBT, ELISA, and PCR were 10.3%, 7.0%, and 5.3%, respectively (Table I). These results are in line with the results of (Mustafa, Khaleel and Lami, 2024), who found 10.7% of RBT-positive patients among 2085 suspected patients in Erbil. This strong agreement between two different surveys conducted in the same governorate externally validates the present findings and shows that brucellosis continues to circulate in the area at a similar level. The same Erbil-based study also found significantly higher infection rates among people who lived in rural areas and had direct contact with animals. This pattern was reflected in our data: Herder families, who all lived in rural areas, had the highest positivity rates for all three tests (16.8% RBT, 12.8% ELISA, and 9.6% PCR; Table I), and living in a rural area was significantly linked to seropositivity (RBT 13.5% vs. 6.6% in urban participants, $p = 0.03$; ELISA 9.8% vs. 3.8%, $p = 0.025$; Table I). When combined, these results support the theory that human brucellosis in northern Iraq mostly affects rural households raising small ruminants (Almashhadany, et al., 2026; Ilyas, et al., 2024; Khairullah, et al., 2024).

The occupational distribution of positivity revealed important differences even within the broad category of “rural” work. Farmer families, despite residing in rural areas, showed markedly lower positivity than herder families (6.0% RBT and 0% for both ELISA and PCR; Table I). This finding indicates that residence alone does not account for exposure risk; rather, the intensity and nature of animal contact appear to be the decisive factor. Herders are typically involved in daily, close-range activities such as assisting parturition, handling aborted fetal material and placentas, and consuming raw milk and dairy products, all of which are recognized high-risk practices for *Brucella* transmission (Khairullah, et al., 2024; Pereira, et al., 2020; Qureshi, et al., 2024), whereas farming activities in this setting were largely crop-based with comparatively limited direct animal contact. Governorate-level surveillance

data, which typically classify all rural inhabitants as a single risk category, would not capture this distinction, and the stratified approach used here therefore adds meaningful detail to the regional epidemiological picture (Mustafa, Khaleel and Lami, 2024). Clinical patients, an entirely urban subgroup, showed intermediate and internally consistent positivity (8.0% RBT, 6.0% ELISA, 6.0% PCR), lower than hospital-based series restricted to clinically suspected cases (Khalid, 2023) but higher than would be expected in an unselected healthy urban population, reflecting the fact that this subgroup was drawn from individuals already presenting with compatible symptoms. Restaurant workers, butchers, and bakers, who had comparatively limited or indirect contact with live animals, showed the lowest positivity overall (Table I), further supporting an exposure-dependent gradient of risk across occupations.

Consistent with the biology of the infection, RBT yielded the highest positivity, followed by ELISA and then PCR (10.3%, 7.0%, and 5.3%, respectively; Table I). RBT is a highly sensitive slide-agglutination screening test but is prone to false positives arising from cross-reactivity with other Gram-negative organisms and from residual antibody titers in previously exposed or treated individuals (Kaki, et al., 2025; Verma, 2013). ELISA, which quantifies specific antibody classes, is more specific and better able to distinguish active from past infection, which likely explains its somewhat lower positivity relative to RBT. PCR and blood culture, in contrast, depend on the presence of viable organisms or circulating bacterial DNA in the sample and are therefore less likely to be positive once bacteremia has resolved, even though specific antibodies persist (Javadi, et al., 2026; Di Bonaventura, et al., 2021; Yagupsky, et al., 2019). This hierarchy of positivity rates, serology exceeding molecular and culture yields, has been repeatedly documented in other Middle Eastern series (Dadar, et al., 2024; Etemady, et al., 2019) and supports the recommendation that serological screening be combined with a confirmatory molecular or bacteriological test rather than used in isolation.

The degree of agreement between the three diagnostic methods, assessed by Cohen's kappa, was consistent with this biological rationale (Table III). RBT and ELISA, both antibody-based assays, showed the closest agreement (79.0%, $\kappa = 0.790$), and ELISA and PCR also agreed well (79.9%, $\kappa = 0.799$), whereas RBT and PCR showed only moderate agreement (61.1%, $\kappa = 0.611$). Because RBT and ELISA measure the same immunological response, they would be expected to classify the same individuals similarly; PCR, which detects a fundamentally different analyte, is expected to diverge from serology whenever antibodies persist in the absence of detectable DNA, or, conversely, when DNA is transiently present before a full antibody response has developed. All three pairwise comparisons were statistically significant ($p < 0.0001$), indicating that the observed agreement was well above what would be expected by chance and supporting the use of PCR as a valuable complementary confirmatory tool rather than a replacement for serological screening.

With respect to demographic risk factors, no statistically significant association was observed between infection and

either age or gender (Table II). Positivity was broadly similar across the 0–20, 21–40, and 41–60-year age groups, and no cases occurred among participants older than 60 years, suggesting that occupational and behavioral exposure, rather than age per se, is the principal determinant of infection risk in this population. Likewise, the near-equal overall positivity between males (RBT 10.3%, ELISA 5.8%, PCR 3.2%) and females (RBT 10.4%, ELISA 8.3%, PCR 7.6%) is consistent with regional reports in which women are heavily involved in milking, processing of dairy products, and animal husbandry, activities that narrow the traditional gender gap in exposure typically described for brucellosis. In contrast, rural residence and herding occupation emerged as the two strongest and most consistent risk factors identified in this study, corroborating the conclusions of national and regional reviews that prioritize rural, livestock-keeping communities for targeted surveillance and control programs (Dadar, et al., 2024; Mustafa, Khaleel and Lami, 2024).

Molecular screening targeting the *bvrR* and *bvrS* genes, which encode the two-component regulatory system essential for *Brucella* intracellular survival and virulence (Altamirano-Silva, et al., 2023; Rivas-Solano, et al., 2023), confirmed the presence of *Brucella* spp. in all 16 culture-positive isolates and yielded 32 nucleotide sequences deposited in GenBank. BLAST analysis showed very high sequence identity with reference strains (99.75–100% for *bvrR* and 99.05–100% for *bvrS*), and phylogenetic reconstruction placed the local isolates in close clusters with reference *Brucella* sequences, confirming their genus-level identity and close genetic relatedness to strains circulating elsewhere. However, because *bvrR* and *bvrS* are highly conserved, core regulatory genes, they provide limited discriminatory power for species- or biovar-level differentiation among closely related *Brucella* species (Rivas-Solano, et al., 2023), and the present analysis could therefore not resolve species identity beyond the genus level. This is an intrinsic limitation of the chosen markers rather than of the amplification or sequencing procedures themselves, and it highlights the value of complementing *bvrR/bvrS*-based screening with additional species-specific markers, such as *bcs31* or the *omp* genes, or with whole-genome sequencing, when precise species identification is epidemiologically required.

The present study has several strengths, including the simultaneous application of four independent diagnostic approaches (RBT, ELISA, blood culture, and PCR), the inclusion of six distinct occupational groups spanning both rural and urban settings, and the integration of serological, bacteriological, and molecular data with phylogenetic analysis in a single population, an approach that has rarely been applied together in previous studies from this region. Certain limitations should nonetheless be acknowledged. The cross-sectional design does not allow causal inference regarding the risk factors identified. Several occupational subgroups, particularly restaurant workers, butchers, and bakers, contained relatively small sample sizes, which may have limited the statistical power to detect associations within these groups. Molecular analysis was restricted to blood samples, which may underestimate the true prevalence of chronic or localized brucellosis, since *Brucella* DNA is not

always detectable in peripheral blood in such cases. Finally, the *bvrR/bvrS* target, while suitable for genus-level detection and general phylogenetic placement, was not designed to differentiate between closely related *Brucella* species, a limitation that should be addressed in future studies using additional or whole-genome markers.

V. CONCLUSION

This is the first epidemiological study on human brucellosis in the region that used a combined diagnostic approach including serological testing (RBT and ELISA), bacterial culture, and molecular approach by PCR. Molecular screening for the *bvrR* and *bvrS* genes confirmed *Brucella* spp. The highest infection rates were observed among herder families. Sequence and phylogenetic analyses showed that the identified isolates grouped closely with reference *Brucella* sequences in GenBank, suggesting strong genetic similarity. However, consistent with previous knowledge as to the low discriminatory ability of the *bvrR* and *bvrS* genes to differentiate closely related *Brucella* species, the approaches used provided only partial resolution at the species level and could not provide conclusive species identification. The findings provide important baseline information on the epidemiology and molecular features of human brucellosis in the region, highlighting the need for a combination of serological, culture-based, and molecular methods in disease surveillance. Further studies should include more specific genetic markers such as *bcs31* and *omp* genes, or whole-genome sequencing, to accurately determine the circulating species of *Brucella* and improve epidemiological understanding. The findings should be viewed in light of the cross-sectional study design, the small sample sizes in some occupational categories, and the resource-intensive nature of the diagnostic methods used.

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