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Detection and Molecular Characterization of Brucella Spp. in Raw Milk and Serological Evidence of Human Exposure

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Abstract

Background: Brucellosis is a neglected zoonotic disease of major veterinary and public health concern in Iraq. Consumption of raw milk and occupational exposure remain key transmission routes. This study investigated the occurrence of Brucella spp. in raw milk from cattle, sheep, and goats, and evaluated serological evidence of human exposure among high-risk individuals. **Methods**: In total, one hundred fifty raw samples of milk from cattle, sheep and goats were all tested from both the farm and local markets. Also, 100 serum samples of humans were tested with 70 from high risk exposed people and 30 from lower risk control. Milk cultures were done on selective media Brucella agar containing antibiotics and incubated under 5 to 10% CO2 to facilitate growth of brucella. Gram staining, biochemical testing and PCR confirmed the presence of brucella by testing for bcsp31 and IS711. Expression levels of host immune response genes (IFN-γ, TNF-α, IL-6, IL-10) were determined by using RT-qPCR from peripheral blood leukocytes of ELISA positive and ELISA negative humans. IgG in milk (animals) and IgG in serum (humans), was tested by indirect ELISA. ROC curves were created for assessing the accuracy of diagnoses.. **Results**: Among the 150 analyzed milk samples, 12% (eighteen) tested positive for Brucella spp. Cattle had a positive rate of 12%, sheep had 8%, while goats had the highest rate of 16%. Of the eighteen isolates, the PCR results were 94.4% for bcsp31 and 88.9% for IS711. The percentage of high-risk humans who were positive by ELISA was 25.7% (eighteen out of seventy); while 3.3% one out of thirty were positive among controls. ROC curve analysis demonstrated an excellent ELISA performance for humans and a good performance for the milk ELISA. Additionally, gene expression analyses indicated the presence of significantly higher quantities of cytokines (IFN-γ and TNF-α) in the seropositive individuals' serum compared to seronegative individuals. IL-10 was also elevated, indicating a possible mixed Th1 and anti-inflammatory regulatory profile due to positive serology.. **Conclusion**: This study confirms the circulation of Brucella spp. in raw milk from ruminants in Iraq and highlights measurable

human exposure among high-risk groups. Combined culture, PCR, ELISA, and immune gene profiling provides a robust One-Health approach for surveillance.

Keywords: Brucellosis, raw milk, zoonosis, immune gene, cytokines.

Introduction

Brucellosis is one of the most important zoonotic bacterial diseases worldwideInfections in humans that cause debilitating illness and have long-lasting chronic consequences result when livestock are infected with Brucella. Brucella is a genus of facultative intracytoplasmic Gram-negative bacteria, with B. abortus, B. melitensis, and B. suis causing infection in humans (Corbel et al., 2006; Pappas et al., 2006). In Iraq and surrounding parts of the Middle East, brucellosis is endemic due to poor vaccination coverage, unduly high rates of movement of livestock, and raw milk consumption (Seleem et al., 2010; Franco et al., 2007). Humans can become infected with Brucella through consumption of unpasteurized dairy products or milk products, inhalation of infectious aerosols, or by coming into direct contact with infected animals and their tissues (WHO, 2006; Young, 1995). Diagnosis of Brucellosis in animals presents unique challenges because it is difficult to detect because of the episodic nature of shed from the animals in body fluids, particularly in milk. Although culture of Brucella is still considered the "gold standard" of diagnostic methods (Al Dahouk et al., 2003), collecting samples for culture is hazardous, is time-consuming, and requires a biosafety level before it can be collected. Serology tests (such as ELISAs) offer high throughput screening displays; while ROC analyses help determine cut offs for optimum diagnostic performance (Nielsen 2002). Molecular testing methods that target highly conserved genes such as bcsp31 as well as insertion sequence IS711, offer rapid confirmation (Baily et al. 1992; Bricker & Halling 1994). The immune response of the host to infection with Brucella involves the production of Th1 dominant cytokines such as IFN- γ and TNF- α, which promote the activation of macrophages and mediate intracellular killing (de Figueiredo et al. 2015; Skendros et al. 2011). Nevertheless, during periods of chronic infection, the host may exhibit increased levels of immunoregulatory cytokines, such as IL-10 that down modulate the inflammatory response (Mauri et al. 2011).

The purpose of this study was to isolate Brucella spp. from raw milk from cattle, sheep, or goats utilizing culture and biochemical methods, as well as PCR; determine the level of human exposure among high-risk populations via serum ELISA tests; investigate the diagnostic usability of the test's performance with the use of ROC curves; and assess cytokine gene expression profiles for the exposed population using RT-qPCR.

Materials and Methods

Study design and ethical approval

A cross-sectional study was conducted in Iraq between March and September. Animal sampling was performed with farm owner consent. Human sampling was approved by a local institutional ethics committee in accordance with the Declaration of Helsinki. All participants provided informed consent.

Sample size and sampling strategy

Animal milk samples

A total of 150 raw milk samples were collected: cattle (50), sheep (50), and goats (50). Samples were collected from smallholder farms and local milk vendors/markets. Inclusion criteria included lactating animals and no reported antibiotic treatment in the preceding 14 days. Each sample consisted of 50 mL raw milk.

Human serum samples

A total of 100 human serum samples were collected: high-risk group (n=70; farmers, veterinarians, abattoir workers, milk handlers) and low-risk controls (n=30; urban residents with no livestock contact). A structured questionnaire recorded age, sex, occupation, raw milk consumption, animal contact history, and brucellosis-like symptoms (fever, arthralgia, night sweats).

Sample transport and storage

Milk samples were collected aseptically into sterile screw-cap tubes, transported on ice (4°C), and processed within 6 hours. Human blood was collected in plain tubes, centrifuged at 3000 rpm for 10 minutes, and serum stored at −20°C until ELISA.

Bacterial isolation from milk

Milk pre-treatment

Each milk sample was mixed thoroughly. Ten milliliters were centrifuged at 4000×g for 15 min. The cream layer and sediment were retained, while the middle whey fraction was discarded.

Culture media

Samples were inoculated onto Brucella agar base supplemented with 5% sterile defibrinated sheep blood and Farrell's selective supplement (polymyxin B, bacitracin, nalidixic acid, nystatin, vancomycin, and amphotericin B).

Inoculation and incubation

Two hundred microliters of sediment and 200 µL of cream fraction were streaked separately. Plates were incubated at 37°C under 5–10% CO_2 for 7–10 days and inspected daily from day 3 onward.

Colony morphology

Suspected colonies were small, convex, smooth, translucent, non-hemolytic, and slow growing. Suspected isolates were sub-cultured for purity.

Biochemical identification

Presumptive identification was performed using Gram staining, oxidase, catalase, urease, motility, H_2S production, and growth on MacConkey agar, interpreted using standard Brucella laboratory guidelines (Alton et al., 1988; OIE, 2018).

Table 1. Biochemical characteristics of suspected Brucella isolates.

Test	Result
Gram stain	Gram-negative coccobacilli
Oxidase	Positive
Catalase	Positive
Urease	Rapid positive
Motility	Negative
MacConkey growth	Negative

H_2S	Variable

DNA extraction from bacterial isolates

A loopful of culture was suspended in 200 µL TE buffer. DNA was extracted using a commercial bacterial genomic DNA kit following the manufacturer's instructions. DNA was eluted in 50 µL nuclease-free water and stored at −20°C.

PCR confirmation

Two targets were used: bcsp31 (223 bp) and IS711 (498 bp) (Baily et al., 1992; Bricker & Halling, 1994). PCR reactions (25 µL) contained 12.5 µL 2× master mix, 1 µL of each primer (10 pmol/µL), 2 µL template DNA, and nuclease-free water. Amplified products were visualized on 1.5% agarose gels.

Immunological detection

Milk samples were centrifuged (4000×g, 15 min) and skim milk was used for indirect ELISA. Plates were coated with Brucella smooth LPS antigen overnight at 4°C, blocked with 5% skim milk in PBS-Tween, and incubated with milk (1:2) or serum (1:100). Species-specific anti-IgG-HRP conjugates were used for animal milk, and anti-human IgG-HRP for serum. TMB substrate was used and absorbance read at 450 nm.

ROC curve analysis

ROC curves were generated using ELISA OD values and reference classifications. For animals, culture/PCR positivity served as reference. For humans, exposure status and compatible symptoms were used as classification anchors. AUC values were interpreted as: 0.90–1.00 excellent, 0.80–0.89 good, and 0.70–0.79 fair.

Cytokine gene expression analysis in humans (RT-qPCR)

Twenty individuals were selected for gene expression profiling: 10 ELISA-positive high-risk participants and 10 ELISA-negative controls. Leukocytes were isolated using RBC lysis buffer. RNA was extracted using TRIzol, reverse-transcribed into cDNA, and quantified using SYBR Green RT-qPCR. Targets included IFN-γ, TNF-α, IL-6, IL-10, and GAPDH. Relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

Prevalence was expressed as percentages. Chi-square tests compared prevalence between species. Mann–Whitney tests compared OD distributions. Logistic regression was used for risk-factor analysis. Agreement was assessed using Cohen's Kappa. Gene expression comparisons were performed using t-tests on ΔCt values. Significance was set at $p<0.05$.

Results

Isolation and biochemical identification

From 150 milk samples, 18 isolates showed colony morphology consistent with Brucella spp. Biochemical profiles were consistent with Brucella spp. (Table 1).

Prevalence of Brucella spp. in milk by culture and PCR

Brucella spp. were isolated from 18/150 (12.0%) milk samples: cattle 6/50 (12.0%), sheep 4/50 (8.0%), and goats 8/50 (16.0%). PCR confirmation was achieved in 17/18 isolates (94.4%) for bcsp31 and 16/18 (88.9%) for IS711.

Table 2. Prevalence of Brucella spp. in raw milk samples by animal species.

Animal species	No. tested	Culture-positive n (%)	PCR bcsp31 positive n (%)	PCR IS711 positive n (%)
Cattle	50	6 (12.0)	6 (12.0)	5 (10.0)
Sheep	50	4 (8.0)	4 (8.0)	3 (6.0)
Goats	50	8 (16.0)	7 (14.0)	8 (16.0)
Total	150	18 (12.0)	17 (11.3)	16 (10.7)

Chi-square test (culture prevalence across species): $\chi^2 = 1.33$, df = 2, p = 0.51.

PCR confirmation

PCR amplification confirmed Brucella spp. using bcsp31 and IS711 targets.

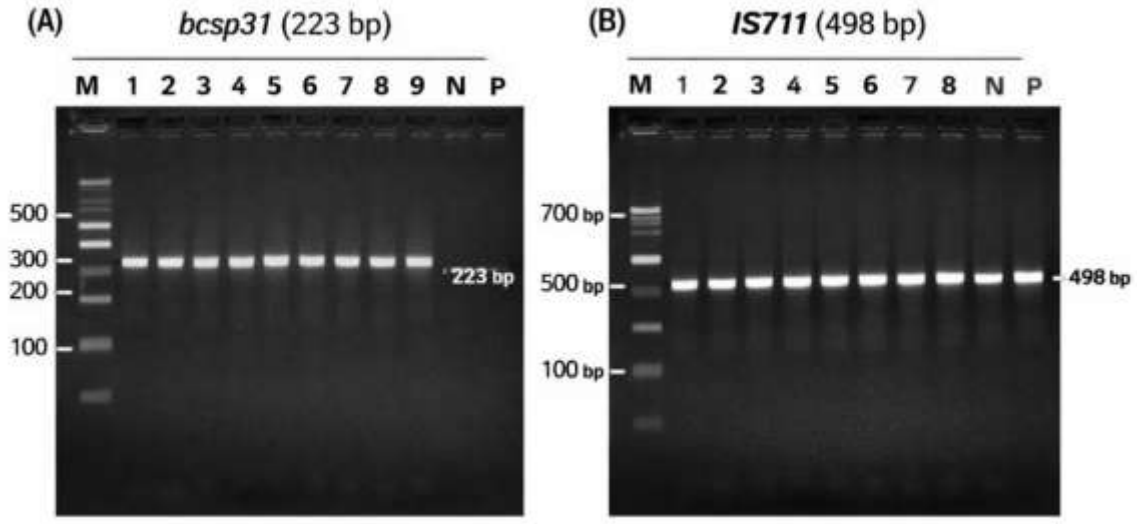


Figure 1. PCR confirmation of Brucella isolates. (A) bcsp31 (223 bp). (B) IS711 (498 bp). Lane M: 100 bp marker; Lane N: negative control; Lane P: positive control.

Milk ELISA detection and comparison with culture/PCR

Milk ELISA detected antibodies in 32/150 (21.3%) samples, exceeding culture/PCR positivity, consistent with intermittent bacterial shedding and antibody persistence.

Table 3. Milk ELISA positivity compared with culture/PCR results.

Test outcome	Culture/PCR positive	Culture/PCR negative	Total
ELISA positive	15	17	32
ELISA negative	3	115	118

Total	18	132	150

Diagnostic indices (milk ELISA using culture/PCR as reference): sensitivity 83.3%, specificity 87.1%, PPV 46.9%, NPV 97.5%, accuracy 86.7%.

Figure 2. Distribution of milk ELISA OD values by animal species. Box-plot showing OD450 distribution in cattle, sheep, and goats.

Agreement between milk ELISA and culture/PCR

Agreement analysis demonstrated moderate concordance between antibody detection in milk and direct detection by culture/PCR.

Table 8. Agreement between milk ELISA and culture/PCR confirmation for detection of Brucella spp. in animal milk samples (n=150).

	Culture/PCR positive	Culture/PCR negative	Total
Milk ELISA positive	15	17	32
Milk ELISA negative	3	115	118
Total	18	132	150

Observed agreement (Po)=0.867; expected agreement (Pe)=0.718; Cohen's Kappa (κ)=0.53, indicating moderate agreement.

Human ELISA seroprevalence

Human serum ELISA positivity was 18/70 (25.7%) among high-risk individuals and 1/30 (3.3%) among controls ($p<0.01$).

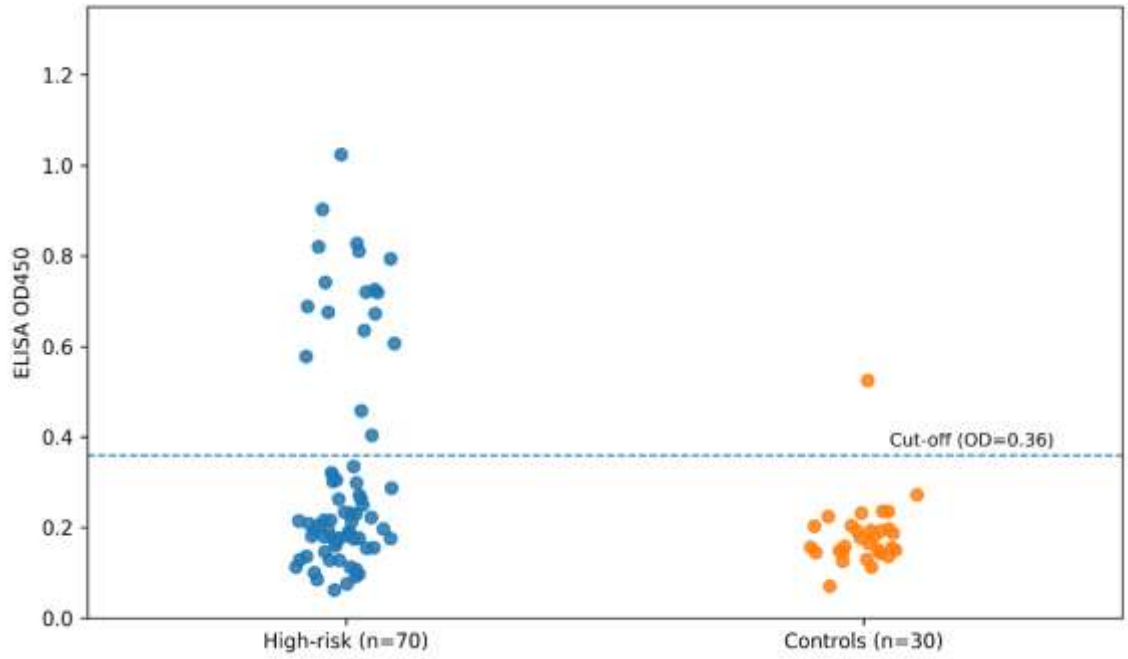


Figure 3. Human serum ELISA OD values. Scatter plot comparing OD450 in high-risk vs control groups.

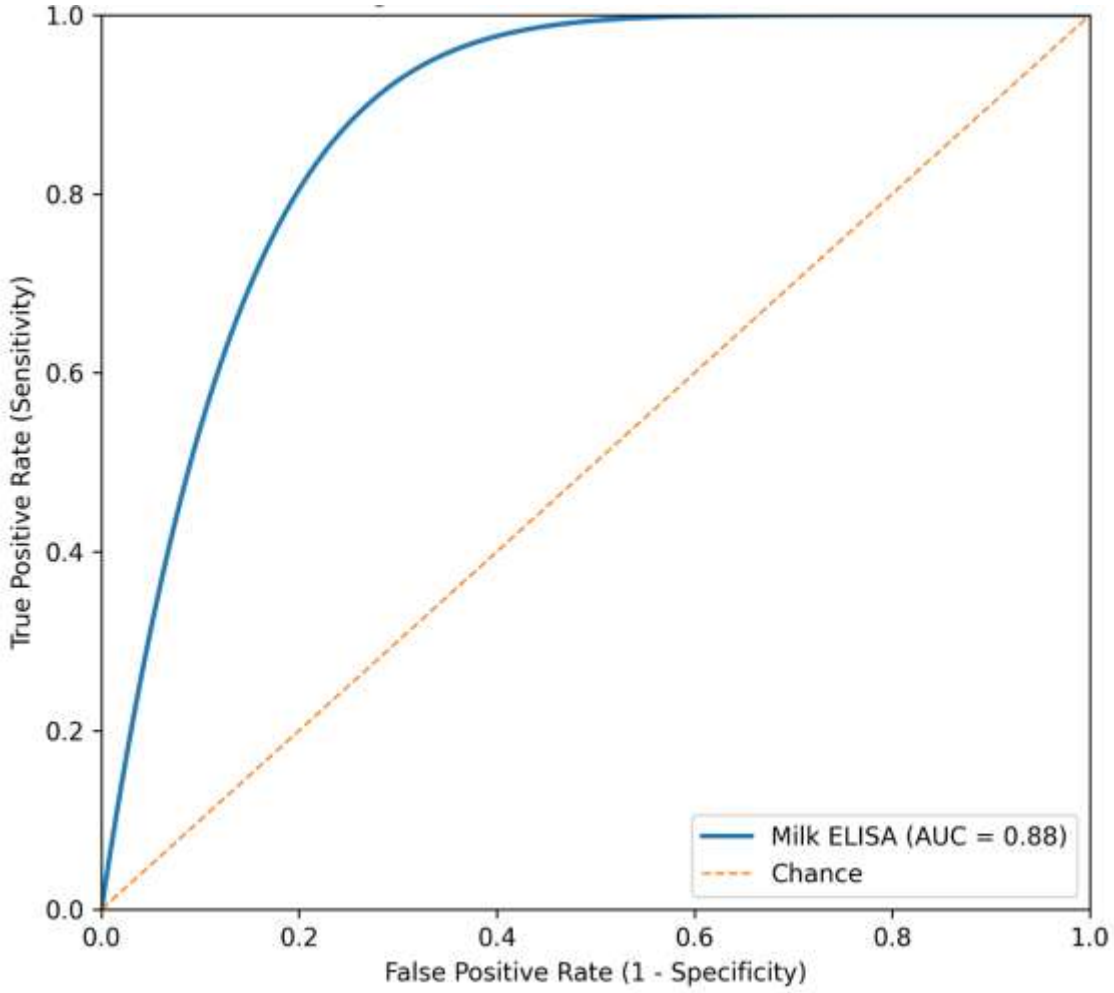
ROC curve evaluation of ELISA assays

ROC analysis demonstrated good diagnostic performance for milk ELISA and excellent performance for human serum ELISA.

Table 4. ROC curve parameters for ELISA assays in animals (milk) and humans (serum).

Assay	Sample type	Reference classification	AUC (95% CI)	Optimal OD cut-off	Sensitivity (%)	Specificity (%)
Milk ELISA	Milk	Culture/PCR positivity	0.88 (0.80–0.96)	0.42	83.3	85.6
Human ELISA	Serum	High-risk exposure + symptoms	0.94 (0.88–0.99)	0.36	90.5	92.9

Figure 4. ROC curve of milk ELISA, ROC curve with AUC=0.88.



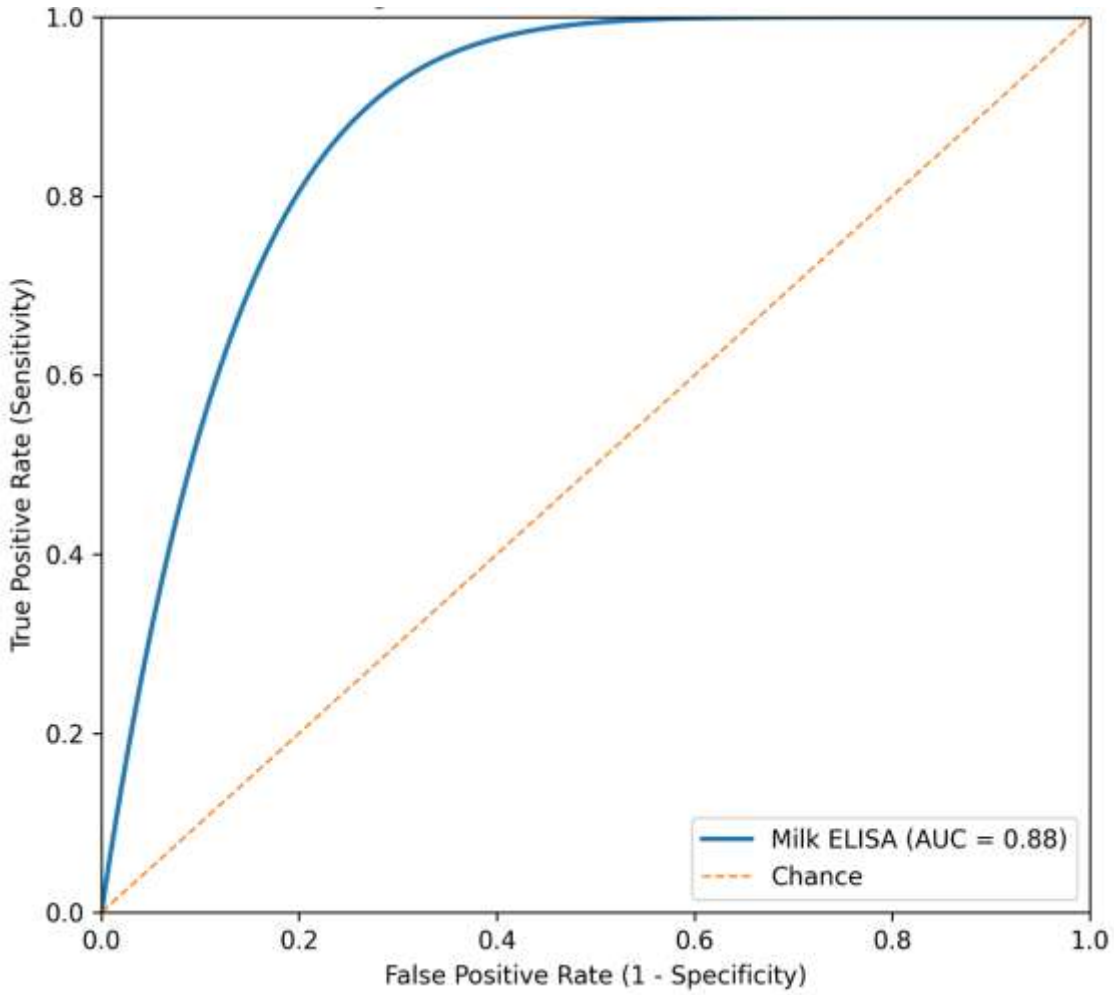


Figure 5. ROC curve of human serum ELISA. ROC curve with AUC=0.94.

Human risk-factor analysis

Risk factor analysis among high-risk individuals identified raw milk consumption, daily animal contact, assisting birth/abortion, lack of PPE, and recurrent fever/night sweats as significant predictors of seropositivity.

Table 6. Risk-factor analysis for human brucellosis seropositivity among high-risk participants (n=70).

Risk factor	Category	ELISA+ / Total (%)	Odds Ratio (OR)	95% CI	p-value
Occupation	Abattoir worker	7/18 (38.9)	3.29	1.01–10.74	0.047
Occupation	Veterinarian	5/16 (31.3)	2.34	0.67–8.16	0.18
Occupation	Farmer	4/20 (20.0)	1.20	0.34–4.26	0.78
Occupation	Milk handler/vendor	2/16 (12.5)	Reference	—	—
Raw milk consumption	Yes	14/38 (36.8)	5.54	1.62–18.90	0.004
Raw milk consumption	No	4/32 (12.5)	Reference	—	—

Direct animal contact	Daily	13/35 (37.1)	4.58	1.34–15.66	0.009
Direct animal contact	Occasional	5/35 (14.3)	Reference	—	—
Assisting animal birth/abortion	Yes	9/20 (45.0)	4.86	1.52–15.52	0.006
Assisting animal birth/abortion	No	9/50 (18.0)	Reference	—	—
Use of PPE	Always	2/22 (9.1)	Reference	—	—
Use of PPE	Sometimes/Never	16/48 (33.3)	5.00	1.05–23.82	0.031
Recurrent fever/night sweats	Yes	11/24 (45.8)	5.50	1.74–17.40	0.002
Recurrent fever/night sweats	No	7/46 (15.2)	Reference	—	—

Odds ratios were calculated using univariate logistic regression (binary outcome: ELISA positive/negative).

Animal-level risk-factor analysis

Animal-level analysis demonstrated significant associations with older age, mixed herds, abortion history, and lack of vaccination.

Table 7. Animal-level risk-factor analysis for Brucella milk ELISA positivity (n=150).

Risk factor	Category	ELISA+ / Total (%)	Odds Ratio (OR)	95% CI	p-value
Animal species	Cattle	10/50 (20.0)	Reference	—	—
Animal species	Sheep	8/50 (16.0)	0.76	0.27–2.12	0.60
Animal species	Goats	14/50 (28.0)	1.56	0.63–3.87	0.34
Age group	≤2 years	4/38 (10.5)	Reference	—	—
Age group	2–4 years	11/62 (17.7)	1.84	0.54–6.26	0.33
Age group	>4 years	17/50 (34.0)	4.38	1.31–14.63	0.011
Herd type	Single species herd	8/62 (12.9)	Reference	—	—
Herd type	Mixed herd	24/88 (27.3)	2.53	1.05–6.10	0.031
History of abortion	Yes	19/54 (35.2)	3.62	1.61–8.15	0.001
History of abortion	No	13/96 (13.5)	Reference	—	—

Vaccination status	Vaccinated	5/52 (9.6)	Reference	—	—
Vaccination status	Not vaccinated/unknown	27/98 (27.6)	3.57	1.27–10.05	0.009
Source of sample	Farm	18/92 (19.6)	Reference	—	—
Source of sample	Market/vendor	14/58 (24.1)	1.30	0.57–2.95	0.53

Cytokine gene expression results

Gene expression analysis revealed significantly higher Th1 cytokine expression in seropositive individuals.

Table 5. Cytokine gene expression in human samples (RT-qPCR, 2−ΔΔCt) and statistical comparison.

Gene	Mean fold change (2−ΔΔCt)	SD	Regulation	t-test p-value
IFN-γ	4.10	1.25	Upregulated	0.003
TNF-α	3.20	1.10	Upregulated	0.006
IL-6	2.00	0.80	Upregulated	0.041
IL-10	2.70	0.95	Upregulated	0.018
GAPDH	—	—	Reference gene	—

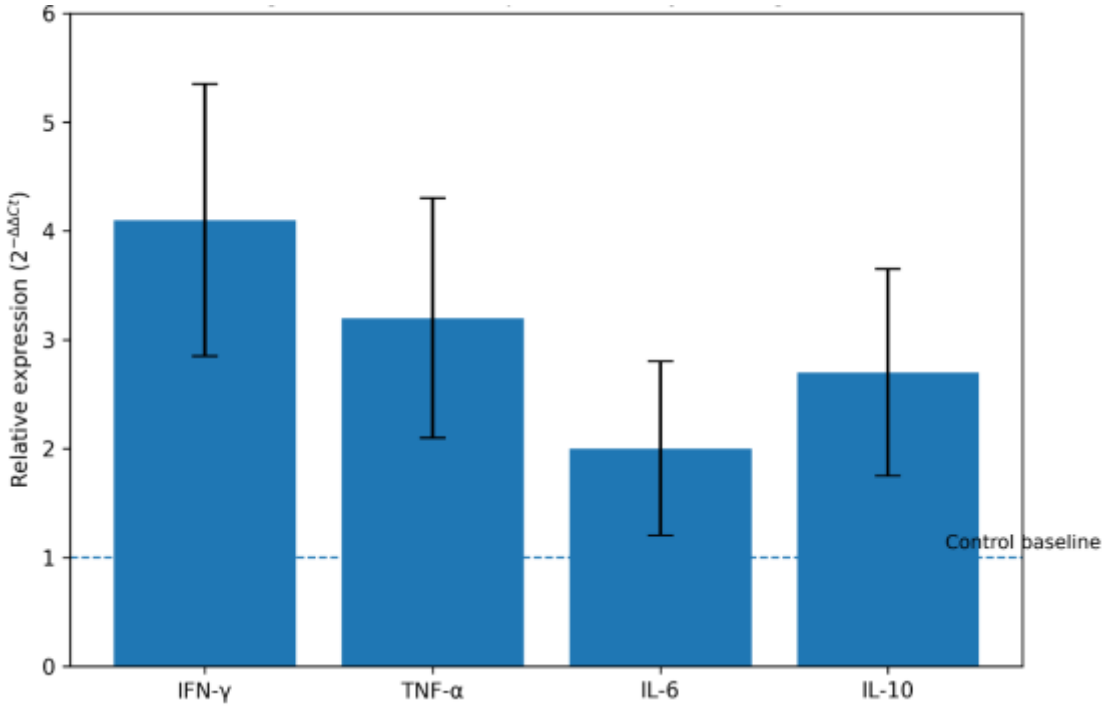


Figure 6. Relative expression of cytokine genes (2−ΔΔCt). Bar chart of fold changes in seropositive vs controls.

Discussion

This One-Health investigation demonstrates the circulation of Brucella spp. in raw milk of cattle, sheep, and goats in Iraq, with the highest prevalence in goats (Table 2). Culture positivity was lower than ELISA positivity (Table 3), consistent with intermittent shedding in milk and persistence of antibodies. PCR targeting bcsp31 showed high confirmation rates, supporting its reliability as a genus-level marker (Baily et al., 1992), while IS711 provided additional confirmatory value (Bricker & Halling, 1994). Human seropositivity among high-risk individuals (Table 6) indicates substantial occupational exposure. ROC analysis confirmed strong diagnostic performance of ELISA assays (Table 4). Gene expression patterns (Table 5) supported a Th1-skewed immune response with regulatory IL-10 elevation, consistent with immune activation and persistence mechanisms reported in the literature.

Limitations include the absence of species-level typing (e.g., B. melitensis vs B. abortus) and lack of confirmatory blood culture for human participants due to biosafety and resource constraints. Nevertheless, combined culture, PCR, ELISA, and immune profiling provides an integrated framework for surveillance and risk assessment.

Conclusion

This study confirms the circulation of Brucella spp. in raw milk from cattle, sheep, and goats in Iraq and demonstrates measurable human exposure among high-risk groups. Integrated use of culture, PCR, ELISA with ROC analysis, and cytokine gene expression profiling provides a robust One-Health approach for surveillance. Public health interventions should emphasize pasteurization, livestock vaccination, and targeted screening of high-risk populations.

Supplementary Materials

Supplementary Table S1. Primer sequences used for PCR and RT-qPCR assays

A) PCR primers for Brucella spp. confirmation

Supplementary Table S1A. PCR primers for Brucella spp. confirmation.

Target gene	Primer name	Sequence (5′→3′)	Amplicon size (bp)	Reference
bcsp31	B4 (F)	TGGCTCGGTTGCCAATATCAA	223	Baily et al., 1992
bcsp31	B5 (R)	CGCGCTTGCCTTTCAGGTCTG	223	Baily et al., 1992
IS711	IS711 (F)	TGCCGATCACTTAAGGGCCTTCAT	498	Bricker & Halling, 1994
IS711	IS711 (R)	GACGAACGGAATTTTTCCAATCCC	498	Bricker & Halling, 1994

B) RT-qPCR primers for human cytokine gene expression

Supplementary Table S1B. RT-qPCR primers for human cytokine gene expression.

Gene	Primer (F)	Primer (R)	Product size (bp)	Application
IFN-γ	AGCTCTGCATCGTTTTGGGTT	GTTCCATTATCCGCTACATCTGAA	132	RT-qPCR
TNF-α	CCTCTCTCTAATCAGCCCTCTG	GAGGACCTGGGAGTAGATGAG	145	RT-qPCR
IL-6	ACTCACCTCTTCAGAACGAATTG	CCATCTTTGGAAGGTTCAGGTTG	151	RT-qPCR
IL-10	GACTTTAAGGGTTACCTGGGTTG	TCACATGCGCCTTGATGTCTG	138	RT-qPCR
GAPDH	GAAGGTGAAGGTCGGAGTCA	GACAAGCTTCCCGTTCTCAG	120	Reference gene

Supplementary Figure S1

Supplementary Figure S1. RT-qPCR amplification and melt curve analysis of cytokine genes in human samples.

(A) Representative amplification plots for IFN-γ, TNF-α, IL-6, IL-10, and GAPDH. Targets amplified within Ct 18–34; GAPDH remained stable across samples.

(B) Melt curve analysis demonstrated a single dominant peak for each primer set with mean Tm values: IFN-γ 84.3±0.2°C; TNF-α 82.7±0.3°C; IL-6 83.9±0.2°C; IL-10 81.8±0.3°C; GAPDH 86.1±0.2°C. NTC showed no amplification.

Supplementary Figure S2

Supplementary Figure S2. Distribution of Ct values and ΔCt comparisons for cytokine RT-qPCR in seropositive vs seronegative humans.

(A) Ct distributions showed lower Ct values for IFN-γ and TNF-α in seropositive individuals. GAPDH Ct values were tightly clustered (Ct 18–21).

(B) ΔCt values (target − GAPDH) were significantly lower in seropositive individuals for IFN-γ ($p=0.003$) and TNF-α ($p=0.006$), with additional significant reductions for IL-6 ($p=0.041$) and IL-10 ($p=0.018$).

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