Isolation and Identification of Brucella using PCR in Indigenous Dogs in the County of Neyshabur and the Suburbs

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Abstract

Brucellosis is a chronic zoonotic disease caused by *Brucella* species that continues to pose major public health and veterinary challenges, particularly in endemic regions. While ruminants are traditionally recognized as the primary reservoirs, the role of domestic dogs, especially those in close contact with livestockremains underexplored. This study investigated the presence of *Brucella melitensis* in indigenous dogs in Neyshabur and its surrounding rural areas using a combination of serological, bacteriological, and molecular diagnostic methods. A total of 100 blood samples were collected from herding and stray dogs. Initial screening by the Rose Bengal Plate Test (RBPT) identified 31 seropositive samples. These samples were subjected to PCR using both genus-specific (B4/B5) and species-specific (*B. melitensis*) primers. Only three samples (9.7% of RBPT-positive cases), all from herding dogs, were PCR-positive for *Brucella melitensis*, and none were positive among stray dogs. The isolated organisms were further characterized and confirmed as *B. melitensis* Biovar 1 through biotyping. No significant association was found between infection and sex, while a statistically significant relationship was observed between infection status and dog type ($p = 0.013$), indicating a higher risk among herding dogs. The results of this study suggest that dogs involved in herding activities may serve as epidemiological bridges between infected livestock and other animals or humans. The findings underscore the limitations of serological testing alone due to false positives and support the inclusion of PCR as a critical tool for definitive diagnosis. Enhanced molecular surveillance of brucellosis in companion and working dogs is recommended for effective disease control and for minimizing zoonotic risk in endemic regions like northeastern Iran.

Keywords: *Brucella*, Iran, molecular surveillance, PCR, Rose Bengal Plate Test (RBPT)

1. Introduction

Brucellosis is a globally distributed zoonotic disease that continues to impose significant economic and public health burdens in many developing countries, including Iran. The disease is caused by facultative intracellular bacteria of the genus *Brucella*, with *Brucella melitensis* considered the most virulent species affecting humans and small ruminants such as sheep and goats (1, 2). Infected animals typically shed the organism through reproductive discharges, including placental tissues, aborted fetuses, and vaginal secretions, contaminating the environment and facilitating transmission through direct contact or ingestion (3, 4). While cattle, sheep, and goats are recognized as the primary reservoirs of brucellosis, dogsparticularly those used in herding or guarding livestockcan also become infected. These animals are frequently exposed to contaminated tissues during parturition or abortion events in infected herds. As a result, they may act as asymptomatic carriers of *B. melitensis*, posing a zoonotic risk to humans, especially those living or working in close contact with farm animals (5, 6). Evidence from recent Iranian studies confirms the presence of *B. melitensis* biovars in dogs, emphasizing their potential role in the complex epidemiology of brucellosis (5).

In Iran, brucellosis remains endemic in many regions, including the northeast. The province of Khorasan Razavi, with its extensive livestock farming and transboundary animal movements, is considered a high-risk zone (5, 6). Despite the national vaccination program targeting livestock, sporadic outbreaks continue to occur, and the role of secondary reservoirs such as dogs is often overlooked in surveillance efforts. Traditional serological tests such as the Rose Bengal Plate Test (RBPT) are commonly used for initial screening due to their simplicity and affordability. However, these tests can yield false positives, especially in animals exposed to cross-reacting antigens or those with chronic infections (7, 8). In contrast, molecular diagnostic methods, particularly polymerase chain reaction (PCR), offer higher specificity and sensitivity and have been increasingly used in epidemiological studies of brucellosis (9, 10).

Given the potential role of indigenous dogs in the maintenance and transmission of *Brucella melitensis*, this study aimed to assess the prevalence of infection in herd and stray dogs in the rural and urban areas of Neyshabur. Additionally, we sought to compare the diagnostic performance of serological and molecular methods in identifying infected animals.

2. Material and methods

2.1 Sample Collection

This cross-sectional study was conducted on a total of 100 indigenous dogs from Neyshabur and surrounding rural areas of Razavi Khorasan Province, Iran. The sampled dogs were classified into two groups: herd dogs (n=50), which were in regular contact with livestock, and stray dogs (n=50), which roamed freely in peri-urban areas. Dogs were selected randomly, without consideration of age, sex, or clinical history, in order to provide a representative population sample.

Blood samples were collected aseptically from the cephalic or jugular vein of each dog. For molecular and bacteriological analysis, 2 mL of whole blood was drawn into EDTA-containing

tubes. For serological testing, 3 mL of blood was collected into plain tubes and allowed to clot. Serum was separated by centrifugation at 3,000 rpm for 10 minutes. All samples were stored at −21°C and transported under cold-chain conditions to the laboratory for further analysis.

2.2 Serological Test

Serological screening was initially performed using the Rose Bengal Plate Test (RBPT) following standard procedures. Briefly, a drop of serum was mixed with a drop of *Brucella* A and M antigen on a clean glass plate and gently agitated. The presence of visible agglutination within 4 minutes was considered a positive result; absence of clumping was interpreted as negative (11). To confirm RBPT-positive or equivocal results, the Wright standard tube agglutination test was carried out. A control tube was included in each set for comparative interpretation. Agglutination results were evaluated based on the clarity of the supernatant: a clearer supernatant compared to the control indicated a positive reaction, while a turbid or opaque supernatant was considered negative. In addition, the 2-Mercaptoethanol (2-ME) test was performed on all positive and suspected samples to detect IgG antibodies and differentiate between acute and chronic infections. Results from all serological tests were recorded and compiled for final comparative analysis (7, 11-14).

2.3 Culture of Blood Samples

Blood samples were cultured in selective *Brucella* broth medium (Oxoid, Basingstoke, UK), supplemented with a combination of growth enhancers and selective antibiotics, including polymyxin B (2500 IU), bacitracin (12,500 IU), cycloheximide (5000 mg), nalidixic acid (250 mg), nystatin (50,000 IU), and vancomycin (1000 mg). The cultures were incubated at 37°C in an atmosphere containing 10% carbon dioxide for five days. Following incubation, samples were subcultured onto selective Brucella agar and incubated under the same conditions for an additional seven days.

Colonial morphology was examined, and suspected colonies were subjected to preliminary biochemical identification using catalase, oxidase, urease, nitrate reduction, hydrogen sulfide (H_2S) production, and carbohydrate fermentation (glucose and lactose) tests (15). Rose Bengal antigen derived from *Brucella abortus* was also used for agglutination testing, as it reacts with most Brucella species except *Brucella canis* (16). Classical biotyping of isolates was performed based on the criteria established by Alton et al. (12), including assessment of CO_2 dependency, H_2S production, agglutination with monospecific A and M antisera, growth in media containing thionin and fuchsin dyes, agglutination with acriflavine, and susceptibility to lysis by the brucellosis-specific phage Tb (supplied by the Razi Institute, Iran). Biotyping results were interpreted according to established taxonomic standards in the literature.

2.4 DNA Extraction

The extraction of genomic DNA from blood samples was performed with the MBST DNA extraction kit (made in Iran), following the instructions of the supplier. The quality and yield of extracted DNA was determined using the Nanodrop spectrophotometric method and confirmed by agarose gel electrophoresis. DNA samples were stored at −20°C for later molecular analysis.

2.5 PCR Test for Identifying the Genus and Species of *Brucella*

The PCR was performed utilizing a master mix that was ready-to-use from Viragen (Amplicon, Denmark). The reaction mixture comprised 25 µL with 12.5 µL of master mix, 1 µL of each of the primers (10 pmol/µL), 3 µL of DNA template, and 7.5 µL of nuclease-free distilled water (6, 17). The primers and the conditions for the PCR cycling are provided in Table 1. Distilled water served as the negative control, while reference strain of *Brucella melitensis* (M16) obtained from microbial collection of the Faculty of Veterinary Medicine, University of Tehran served as positive controls.

Amplification was conducted utilizing a 512-TC thermocycler (Techne, UK). PCR products were separated on a 2% agarose gel at 90 V in 1X TBE buffer for 70 minutes utilizing a 100 bp DNA ladder (Synaclone, Cat. No. 901644PR [7031SL]) for estimation of fragment size. The gels were stained with ethidium bromide (C7721MR) at 1ug/mL, and the bands were observed under UV illumination (Bioword, Germany).

Table 1. Primer Sequences Used for PCR Amplification of *Brucella* Genus and *Brucella melitensis*

primer	Target	Nucleotide sequence	Bp	reference
B4 B5	*Brucella* genus	F: TGGCTCGGTTGCCAATATCAA R: CGCGCTTGCCTTTCAGGTCTG	214bp	33
Br.m	*Brucella melitensis*	F: AAATCGCGTCCTTGCTGGTCTGA R: TGCCGATCACTTAAGGGCCTTCAT	731bp	34

2.6. Statistical Analysis

Data were analyzed using SPSS software version 26.0 (IBM Corp., Armonk, NY, USA). The prevalence of Brucella infection was calculated as the percentage of seropositive and PCR-positive cases among the total number of animals tested. Descriptive statistics were used to summarize categorical variables (e.g., sex, dog type). The Chi-square test was used to assess the association between variables such as sex, lifestyle (herd vs. stray), and infection status. A p-value of less than 0.05 was considered statistically significant.

3. Result

3.1 Serological Findings

The RBPT identified 31 seropositive dogs, representing an overall apparent seroprevalence of 31.0%. The highest proportion of seropositive animals was detected in herd females (50.0%), followed by stray females (40.0%), stray males (28.6%), and herd males (25.0%) (Table 2). These results suggest widespread exposure to *Brucella spp*. across both stray and herding dog populations.

3.2 Bacteriological Isolation and Biotyping

Blood cultures from RBPT-positive samples were incubated on Brucella-selective agar. Colonies appeared after one week and were characterized by smooth, convex, grey morphology and absence

of hemolysis. Growth was not observed on MacConkey agar. Biochemical tests showed positive catalase, oxidase, urease, and nitrate reduction activity, with negative hydrogen sulfide (H_2S) production.

All isolates grew in the presence of basic fuchsine and thionin and were CO_2-independent. No lysis occurred with the Brucella-specific Tb phage. Classical biotyping identified all isolates as *Brucella melitensis* Biovar 1, based on agglutination with M-specific antiserum and standard phenotypic profiles (21).

3.3 Molecular Detection by PCR

PCR analysis was conducted on all 31 seropositive blood samples. Using genus-specific primers (B4/B5), 3 samples (9.7%) yielded a distinct 223 bp product, confirming the presence of Brucella DNA. All three positive samples originated from herd dogs, indicating likely occupational exposure through close contact with infected livestock (Figure 2).

Subsequent testing of the genus-positive samples with *B. melitensis*-specific primers (Br.m) produced a clear 731 bp amplicon in all cases, confirming *Brucella melitensis* species-level infection (Figure 3). No amplification was detected in stray dogs or RBPT-negative controls.

3.4 Statistical Associations

Statistical analysis revealed no significant association between PCR-confirmed infection and the sex of the dogs ($\chi^2 = 0.09$, df = 1, $p = 0.76$). In contrast, a significant association was found between infection and dog type: herd dogs were significantly more likely to test positive by PCR than stray dogs ($\chi^2 = 6.21$, df = 1, $p = 0.013$). Fisher's exact test was applied for contingency tables with expected cell counts <5.

Table 2. Seroprevalence and PCR Positivity of *Brucella* spp. Among Indigenous Dogs in Neyshabur, Iran

Group	n	RBPT Positive n (%)	PCR Positive n (%)
Herd Males	40	10 (25.0%)	2 (5.0%)
Herd Females	10	5 (50.0%)	1 (10.0%)
Stray Males	35	10 (28.6%)	0 (0.0%)
Stray Females	15	6 (40.0%)	0 (0.0%)
Total	100	31 (31.0%)	3 (3.0%)

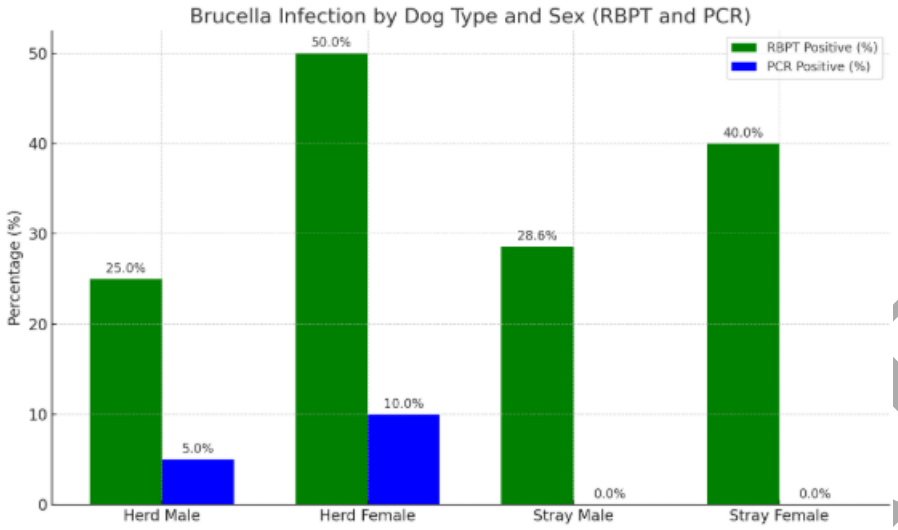

Figure 1 Seropositivity of *Brucella spp.* across dog categories based on RBPT results

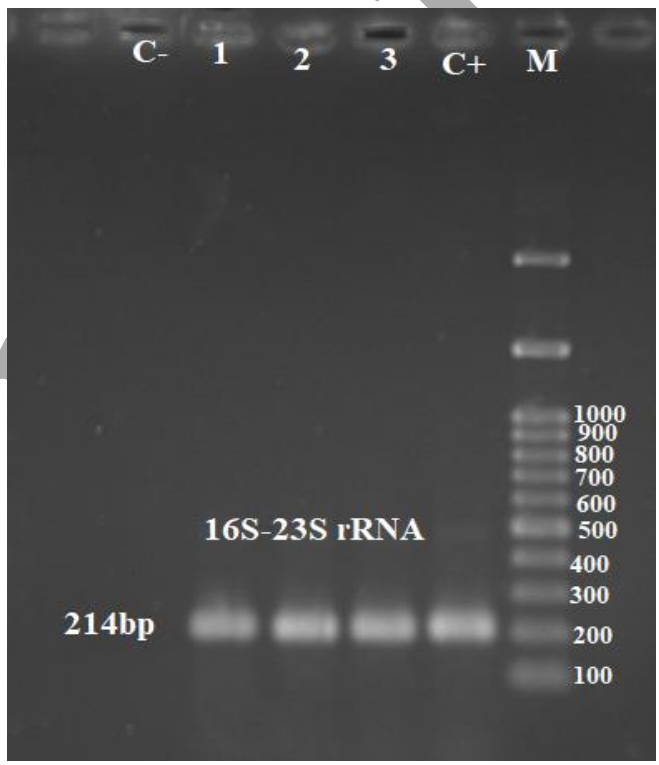

Figure 2: Gel electrophoresis image showing 223 bp PCR products amplified with Brucella genus

Figure 3: Gel electrophoresis image showing 731 bp PCR products from *Brucella melitensis*-specific primers (Br.m).

4. Discussion

Brucellosis was a chronic and widespread zoonotic disease caused by bacteria of the genus Brucella, presenting a persistent global threat to both human and animal health. Endemic in many regions, including parts of the Middle East, it remained challenging to detect, control, and eradicate due to complex transmission dynamics involving ecological, occupational, and animal–human interface factors (1, 3, 18). Identifying local reservoirs and understanding species-specific transmission pathways were therefore critical components of brucellosis surveillance and control strategies.

In this study, we identified *Brucella melitensis* Biovar 1 in indigenous dog populations from Neyshabur and its surroundings, with PCR confirmation in 9.7% of RBPT-seropositive cases. Notably, molecular positivity was confined to herding dogs, underscoring the role of close, repeated contact with livestock, particularly infected sheep and goats as a risk factor for cross-species transmission. This finding was consistent with prior evidence that *Brucella spp*. Could infect non-preferred hosts through prolonged exposure (5, 19).

Despite relatively high RBPT seropositivity in stray females (40%) and males (28.6%), none of these animals tested positive via PCR. This disparity reflected the limited specificity of serological assays, particularly in stray populations that might have encountered environmental antigens or unrelated Gram-negative bacteria. By contrast, PCR-confirmed prevalence among herd females

(10%) and males (5%) supported the conclusion that occupational exposure, rather than incidental contact, played a key role in the transmission of *B. melitensis* to dogs.

The findings of this study were aligned with those of Alamian and Dadar (2020), who reported *B. melitensis* in 38.1% of seropositive herding dogs from Tehran, Qom, and Alborz provinces using PCR, with 6 dogs also culture-positive (5). The higher detection rate in their study compared to our 9.7% PCR-positive rate might have reflected regional variation in livestock infection rates, diagnostic sensitivity, or differences in herd management practices. They also confirmed the presence of both Biovar 1 and 2, which indicated a broader spectrum of circulating strains.

International studies corroborated our epidemiological observations. In Brazil, Keid et al. (2015) found a *Brucella canis* seroprevalence of 20.9% in 753 dogs, with no significant association between sex and infection status (20), a pattern that was mirrored in our results ($p = 0.76$). Conversely, a statistically significant association between *Brucella* infection and dog type ($p = 0.013$) highlighted herd dogs as a high-risk group due to continuous exposure to infected ruminants.

Age-related risk was not directly assessed in our study, but Mosallanejad et al. found a higher prevalence of *B. canis* antibodies in older dogs in Ahvaz, suggesting that duration and intensity of exposure, as seen in working dogs, might have been more critical than age alone (21). Similarly, Behzadi et al. (2011) reported 10.62% *B. canis* seropositivity in imported dogs in Shiraz (22), whereas our findings pointed to local transmission of *B. melitensis* in native, non-imported dogs, emphasizing differing transmission cycles for each *Brucella* spp.

The diagnostic superiority of PCR was also supported by Aras et al. (2011), who demonstrated its efficacy in post-mortem detection of *B. canis* in lymphatic tissue (23). Our study confirmed that PCR was a reliable, non-invasive, and field-applicable method for detecting *Brucella* in canine blood samples, especially when combined with species-specific primers and sequence confirmation.

Serological assays such as RBPT, Wright, and ME2 remained useful for preliminary screening but were subject to cross-reactivity and low specificity, especially in endemic areas. Known cross-reactants include *Yersinia enterocolitica*, *Salmonella* spp., *Campylobacter fetus*, and *Vibrio cholerae*, potentially inflating false positive rates (7, 9, 10, 15, 19, 24). False negatives also occurred during early or chronic stages of infection, or in immunocompromised hosts (18, 19). In our study, only 3 of 31 RBPT-positive dogs (9.68%) were PCR-confirmed, underscored the limited predictive value of serology alone and reaffirmed the necessity of molecular diagnostics in brucellosis surveillance.

This study highlighted the role of herding dogs in Neyshabur as potential reservoirs of *Brucella melitensis* due to their close contact with infected livestock. While serological tests like RBPT aided in initial screening, their limitations such as cross-reactivity and false positives emphasized the need for more accurate diagnostics. PCR offered distinct advantages by enabling rapid, sensitive, and specific detection, even in asymptomatic or atypical hosts. The confirmed presence of *B. melitensis* in native herding dogs signaled an ongoing risk to both animal and human populations. Effective control required integrating molecular diagnostics into surveillance,

vaccinating livestock, and educating animal owners. Lessons from countries with successful control programs showed that combining these measures could significantly reduce transmission and support long-term eradication efforts, especially in endemic regions like Iran.

5. Conflict of interests

The authors declare no conflict of interests.

6. Authors Contributions

Study supervision: F.Z.GH

Study concept and design: F.Z.GH, I.A.T

Acquisition of data: B.E, F.Z.GH, I.A.T

Drafting of the manuscript: H.A.A, I.A.T

All authors reviewed the manuscript.

7. Ethics committee Approval

We declare that all ethical standards related to animal health and welfare have been respected in present study.

8. Data availability

The data that supporting the findings of this study are available upon request from the corresponding author.

9. Acknowledgment

We would to thank all of our collogues at Department of Clinical Sciences, Babol Campus, Islamic Azad University, Babol, Iran and Faculty of Veterinary Medicine, University of Tehran, Iran for their kind cooperation.

10. Funding/ Support

The authors confirm that they did not receive any financial assistance for the research, authorship, and/or publication of this article.

Artificial intelligence (AI) tools (ChatGPT, version GPT-5) were used only for grammar checking and translation assistance. No part of the analysis, interpretation, or original writing of this article was generated by AI.

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