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Species Diversity of *Brucella* spp. Infecting Humans in Tongliao City, China

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Abstract

Objective: Human brucellosis poses a substantial public health threat in Tongliao; however, the species diversity of *Brucella* strains infecting humans remains unclear. **Methods:** A cross-sectional study was conducted among consecutive outpatients at a brucellosis hospital (August-September 2024). Demographic, clinical, and symptom data were collected via questionnaire. Serum samples were tested with SAT, and *Brucella* species/biovars were identified by nested-PCR. Sequencing data were used for phylogenetic analysis (neighbor-joining tree) to assess strain clustering. **Results:** The cohort comprised 42.17% (97/230) initial cases, 53.48% (123/230) follow-up cases, and 4.35% (10/230) health examiners. Clinical presentations included fever only (10.43%), fever with limb soreness (18.26%), limb soreness only (43.91%), and asymptomatic infection (26.96%). Serologically, 23.04% (53/230) had serum agglutination test (SAT) titers ≥ 1:100, 22.17% (51/230) had titers of 1:25 - 1:50, and 33.91% (78/230) were negative. Nearly half (49.57%, 114/230) had taken medication prior to consultation. *Brucella* was detected in 43.48% (100/230) of cases by nested-PCR. Species distribution among positive samples was *B. abortus* (70.00%), *B. melitensis* (11.74%), and *B. suis* (3.00%), with multiple biovars identified for each species. Phylogenetic analysis of 100 sequences resolved them into 9 distinct clades (Groups 1 - 9), revealing clear biovar-clade associations: *B. abortus* biovar 1 clustered in Groups 1 and 8, biovar 2 in Group 2; *B. melitensis* biovars

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1, 2, and 3 were respectively assigned to Groups 5, 4, and 3/9; *B. suis* biovars 1 and 2 were placed in Groups 6 and 7. **Conclusion:** This study first reveals the high species and genetic diversity of *Brucella* infecting humans in Tongliao, showing a dominance by *B. abortus* with co-circulation of *B. melitensis* and *B. suis*. The high diversity indicates complex epidemic origins and control challenges, calling for integrated strategies and enhanced molecular surveillance.

Keywords

Brucella, Serum Agglutination Testing, Clinical Symptoms, Molecular Epidemiology

1. Introduction

Brucellosis is a highly prevalent zoonotic disease. The new cases ranged from 5 to 12.5 million every year in the world [1]. This systemic zoonotic disease has a series of symptoms such as fever, limb joint soreness, etc. [2]. Brucellosis seriously affected people's physical health, especially slaughterhouse workers, farmers, shepherds, veterinarians, laboratory technicians, and traders [3]. PCR testing revealed Brucella infection rates of 13.83% in beef cattle, 9.09% in cows, and 7.68% in sheep, with 14 Brucella strains isolated from positive specimens, comprising 11 *B. melitensis*, 2 *B. abortus*, and 1 *B. suis* [4]. As of 2024, Tongliao City had a cattle inventory of 384.019 million heads and a sheep inventory of 642.301 million heads [5]. However, there is currently no available information regarding the *Brucella* antibody positivity rate in the cattle and sheep populations in Tongliao City. The positive rate of *Brucella* serum agglutination test (SAT) was 0.26% (37/13,999) and 1.09% (304/27,947) in sheep and cattle respectively in 2008 according to statistics from national monitoring points in China [6]. Studies showed that the seropositive rate in humans and animals was positively correlated with the incidence rate [7]. And *Brucella* species and biovars have obvious host specificity [8] [9]. However, the distribution of *Brucella* species and biovars in human brucellosis was 109:1 based on data from 21 brucellosis monitoring sites in China from 2005 to 2016 [6]. The above data and research results indicated that the distribution of *Brucella* species and biovars, which were isolated from humans in China, was inconsistent with the infection situation in different animal hosts. In order to understand the distribution pattern of *Brucella* species and biovars in the population of brucellosis, we conducted a cross-sectional study to analyze the distribution of *Brucella* species and biovars in human brucellosis.

2. Materials and Methods

2.1. Sample Source and Epidemiology Investigation

A cross-sectional study was conducted from all outpatients who visited the Mon-

golian Medical Hospital in Tongliao city from August to September 2024. This hospital mainly treats patients with brucellosis. The brucellosis is diagnosed by clinical physicians according to the human brucellosis diagnosis standard (WS 269-2019). Th fresh blood samples were collected from all outpatients. The individual case investigation included patient name, gender, age, date of onset, contact history, medical history, medicant, etc. The clinical symptoms and signs included fever and limb soreness, *etc*. Laboratory tests include serum agglutination test (SAT). Physicians determined who needed to undergo serum tube agglutination test based on their condition.

2.2. Clinical Blood Samples

3 mL venous blood from each outpatient was collected. Of 3 mL venous blood, 1mL was placed into a 3 mL sterile tube containing the anticoagulant (ethylene diamine tetraacetie acid, EDTA). Genomic DNA was extracted from 200 μL anticoagulated blood by using Blood/Cell/Tissue genomic DNA extraction kit (DNeasy Blood&Issue Kit QIAGEN, Germany). Remainder 2 mL blood was placed into a sterile tube without anticoagulants. And allowed it to stand at room temperature for 24 hours. The serum was separated and subjected to a tube agglutination test using *Brucella* antigen.

The DNA extraction from both positive and negative *Brucella* specimens was performed simultaneously during the process of extracting blood samples.

2.3. Nested Polymerase Chain Reaction for *IS711* Gene

Based on the previously established method (Tian, 2021) [10], a nested-PCR brief description as follows: The first round PCR primers were F1:5'-GCGCATGCGAGATGGACGAA-3' and R1:5'-AGCAGACGAAGCCTTACAGATGAG-3'. The second round PCR primers: F2:5'-GAATGCGGTCAATGTTTTCTCGC-3' and R2:5'-ATATCTTCCGGGGCGAGTTGGTA-3'; the reaction system for the first round PCR included: 2 × Ex Taq Mastermix 12.5 μL, primer F1 and primer R1 each 0.4 μL (10 μ mol/L), DNA template 4 μL from blood, and double distilled water supplemented to 25 μL. PCR amplification reaction conditions: pre-denaturation at 95˚C for 4 min; denaturation at 95˚C for 30 sec, annealing at 55˚C for 30 sec, extension at 72˚C for 30 sec, 30 cycles; extend at 72˚C for 5 min. The reaction system and conditions for the second round were basically the same as those of the first round PCR. Except the primers F2 and R2, and the DNA template was 2 μL from the first round PCR products. The PCR products from the second round PCR will be sequenced using primers F2 and R2. The PCR amplification for positive and negative *Brucella* control samples was performed simultaneously during the process of PCR amplification for the DNA from blood samples.

2.4. Data Process and Bioinformation Analysis

Excel 2017 software was used for data organization and analysis. MEGA 5.1 software was used to construct an adjacent evolutionary tree to analyze the phylogenetic re-

lationships by the unweighted pair group method arithmetic averages (UPGMA).

3. Results

3.1. Basic Characteristics of Study Participants

A total of 230 outpatients were enrolled in this study, including 156 males and 74 females, with a male-to-female ratio of 2.1:1. The median age was 47 years (range: 8 - 80 years), and the average age was 45.5 years. Among the participants, 97 (42.2%) were initial cases, 123 (53.5%) were follow-up cases, and 10 (4.3%) were health examiners.

Clinical presentations included fever only (24 cases, 10.4%), fever with limb soreness (42 cases, 18.3%), limb soreness only (101 cases, 43.9%), and asymptomatic infection (62 cases, 26.9%). One case presented with testicular pain. Regarding serum agglutination test (SAT) results, 53 patients (23.0%) had antibody titers ≥ 1:100, 51 (22.2%) had titers between 1:25 and 1:50, 78 (33.9%) were seronegative, and 48 (20.9%) were not tested. In terms of medication history before consultation, 116 patients (50.4%) had not taken any medication, while 114 (49.6%) reported prior use of medication. The onset duration was less than 1 month in 108 patients (46.9%), 1 - 6 months in 59 (25.7%), 6 months to 1 year in 24 (10.4%), and over 1 year in 29 (12.6%) (Table 1).

Table 1. Detection results on time of onset, clinical symptoms, drug in 230 outpatients.

Disease characteristics	Symptom characteristics					Drug use			Onset time				Total
	Fever	Fever + Limb soreness	Limb soreness	Testicular pain	Asympto-matic	user	Unuser	health examin-ers	<1 month	1 - 6 month	6 months - 1 year	>1 year	
Detected cases	24	42	101	1	62	116	114	10	108	59	24	29	230
Positive case	10	18	41	1	30	45	55	3	42	29	16	10	100
Positivity rate	41.67%	42.86%	40.59%	100%	48.39%	38.79%	47.37%	30.00%	38.89%	49.15%	66.67%	34.48%	43.48%

3.2. Detection and Speciation of *Brucella* by *IS711* Gene

Among 230 outpatients, 100 (43.5%) tested positive for the *Brucella IS711* gene. Of these positive samples, 70 (70.0%) were identified as *B. abortus*, comprising 66 (94.3%) biovar 1 and 4 (5.7%) biovar 2. A total of 27 (27.0%) positive sequences were identified as *B. melitensis*, distributed as biovar 1 (n = 2, 7.4%), biovar 2 (n = 3, 11.1%), and biovar 3 (n = 22, 81.5%). The remaining 3 (3.0%) were *B. suis*, including one biovar 1 and two biovar 2 (Table 2).

Table 2. Distribution of 100 *Brucella IS711*-positive samples.

Brucella species		*B. abortus*		*B. melitensis*			*B. suis*	
Biovars		bv. 1	bv. 2	bv. 1	bv. 2	bv. 3	bv. 1	bv. 2
No.		66	4	2	3	22	1	2
Total	100	70		27			3	

3.3. Case Analysis

Among initial cases (n = 97), the positivity rate was 43.3% (42/97), while among follow-up cases (n = 123), it was 44.7% (55/123). Among asymptomatic health examiners (n = 10), the positivity rate was 30.0% (3/10). Regarding species distribution, initial and follow-up cases demonstrated high consistency: *B. abortus* accounted for 69.0% (29/42) and 70.9% (39/55), respectively; *B. melitensis* accounted for 26.2% (11/42) and 27.3% (15/55), respectively; and *B. suis* constituted a minor proportion (4.8% in initial cases and 1.8% in follow-up cases). The biovar composition remained similarly stable: *B. abortus* was predominantly biovar 1 (96.6% in initial cases and 92.3% in follow-up cases), while *B. melitensis* was predominantly biovar 3 (63.6% in initial cases and 93.3% in follow-up cases). Among the positive samples from health examiners, two of *B. abortus* biovar 1 and one strain of *B. melitensis* biovar 3 were identified. Overall, no significant differences in *IS711* detection rates were observed across different case types, and the species/biovar distribution patterns remained consistent, suggesting that this molecular assay demonstrates consistent diagnostic performance across patients with varying clinical statuses and indicating the persistent circulation of dominant strains in this region.

3.4. Symptom Analysis

Of 230 outpatients, 1) 10.43% (24/230) was fever only. 41.67% (10/24) was positive for *IS711* gene including 60.00% (6/10) was *B. abortus*, 30.00% (3/10) was *B. melitensis*, and 10.00% (1/10) was *B. suis*. 2) 18.26% (42/230) was fever and limb soreness. 42.86% (18/42) was positive for *IS711* gene including 72.22% (13/18) was *B. abortus*, 22.22% (4/18) was *B. melitensis*, and 5.56% (1/18) was *B. suis*. 3) 43.91% (101/230) was limb soreness only. 40.59% (41/101) was positive for *IS711* gene including 68.29% (28/41) was *B. abortus* and 31.71% (13/41) was *B. melitensis*. 4) 26.96% (62/230) was asymptomatic cases. 48.39% (30/62) was positive for *IS711* gene including 63.33% (19/30) was *B. abortus biovars* 1, 10.00% (3/30) was *B. abortus biovars* 2, 20.00% (6/30) was *B. melitensis biovars* 3, 3.33% (1/30) was *B. melitensis biovars* 1, 3.33% (1/30) was 1 *B. suis biovars* 2. 5) There was one case had testicular pain, which was *B. abortus biovars* 1 (Table 1).

3.5. Serum Antibody Levels Analysis

Of 230 outpatients, 1) 23.04% (53/230) with serum antibody levels was above 1:100, and 43.40% (23/53) was positive for *IS711* gene. Among them, 52.17% (12/23) was *B. abortus biovars* 1, 13.04% (3/23) was *B. abortus biovars* 2, 4.35% (1/23) was *B. melitensis biovars* 1, 26.09% (6/23) was *B. melitensis biovars* 3, 4.35% (1/23) was *B. suis biovars* 1. 2) 22.17% (51/230) with serum antibodies was at 1:25-1:50. And 39.22% (20/51) was positive for *IS711* gene including 65.00% (13/20) was *B. abortus biovars* 1, 5.00% (1/20) was *B. abortus biovars* 2, 5.00% (1/20) was *B. melitensis biovars* 1, 20.00% (4/20) was *B. melitensis biovars* 3,

5.00% (1/20) was *B. suis biovars* 2. 3) 33.91% (78/230) was negative serum antibodies, and 38.46% (30/78) was positive for *IS711* gene. 70.00% (21/30) was *B. abortus biovars* 1, 3.33% (1/30) was *B. melitensis biovars* 1, 6.66% (2/30) was *B. melitensis biovars* 2, 16.67% (5/30) was *B. melitensis biovars* 3, 3.33% (1/30) was *B. suis biovars* 2. 4) 20.87% (48/230) was not tested for SAT, and 56.25% (27/48) was positive for *IS711* gene. Among them, 74.07% (20/27) was *B. abortus biovars* 1, 25.93% (7/27) was *B. melitensis biovars* 3 (Table 3).

Table 3. Detection results on *IS711* gene in different serum antibody titers.

Serum antibody titer	1:25 - 1:50	1:100 and above	Negative	Not detected SAT	Total
Detected cases	51	53	78	48	230
Positive cases	20	23	31	27	100
Positivity rate	39.22%	43.40%	39.74%	56.25%	43.48%

3.6. Medicant Analysis

Of 230 outpatients, 50.43% (116/230) was not use medicant before seeking medical treatment, 38.79% (45/116) was positive for the *IS711* gene including 68.89% (31/45) was *B. abortus*, 26.67% (12/45) was *B. melitensis*, and 4.44% (2/45) was *B. suis*. 49.57% (114/230) was drug users before seeking medical treatment, 48.25% (55/114) was positive for the *IS711* gene including 70.91% (39/55) was *B. abortus*, 27.27% (15/55) was *B. melitensis*, and 1.82% (1/55) was *B. suis*.

3.7. Analysis of Onset Time

Of 230 outpatients, 1) 46.96% (108/230) was less than 1 month for onset time. Among them, 38.89% (42/108) was positive for *IS711* gene including 71.43% (30/42) was *B. abortus*, 23.81% (10/42) was *B. melitensis*, and 4.76% (2/42) was *B. suis*; 2) 49.15% (29/59) was positive for *IS711* gene in 59 cases with 1 month to 6 months for onset time including 68.97% (20/29) was *B. abortus*, 27.59% (8/29) was *B. melitensis*, and 3.45% (1/29) was *B. suis*; 3) 10.43% (24/230) was 6 months to 1 year for onset time. Among them, 66.67% (16/24) was positive for *IS711* gene including 56.25% (9/16) was *B. abortus* and 43.75% (7/16) was *B. melitensis*; 4) 12.61% (29/230) was over 1 year. Among them, 34.48% (10/29) was positive for *IS711* gene including 90.00% (9/10) was *B. abortus* and 10.00% (1/10) was *B. melitensis*; 5) 4.35% (10/230) was health examiner. Among them, 30.00% (3/10) was positive for *IS711* gene including 2 *B. abortus* and 1 *B. melitensis*.

3.8. Regional Distribution of Cases

In the Left Banner area of Tongliao city, 52.17% (12/23) of outpatients were *IS711*-positive, comprising 8 *B. abortus* biovar 1 and 4 *B. melitensis* biovar 3. In Zhalai Banner (Tongliao), 66.67% (4/6) were positive, with equal distribution of *B. abortus* and *B. melitensis*. Kailu region (Tongliao) showed 30.77% (4/13) positivity, all identified as *B. abortus*. Notably, all 4 cases from Shenyang (Liaoning province)

were *B. melitensis* biovar 3, while 3 cases from Heilongjiang province were exclusively *B. abortus* biovar 1. These findings suggest regional variations in both detection rates and species distribution, with mixed *B. abortus* and *B. melitensis* circulation in some Tongliao areas versus single-species dominance in other regions, potentially reflecting distinct epidemiological patterns or exposure sources across geographical areas.

3.9. Cluster Analysis of *IS711* Sequences

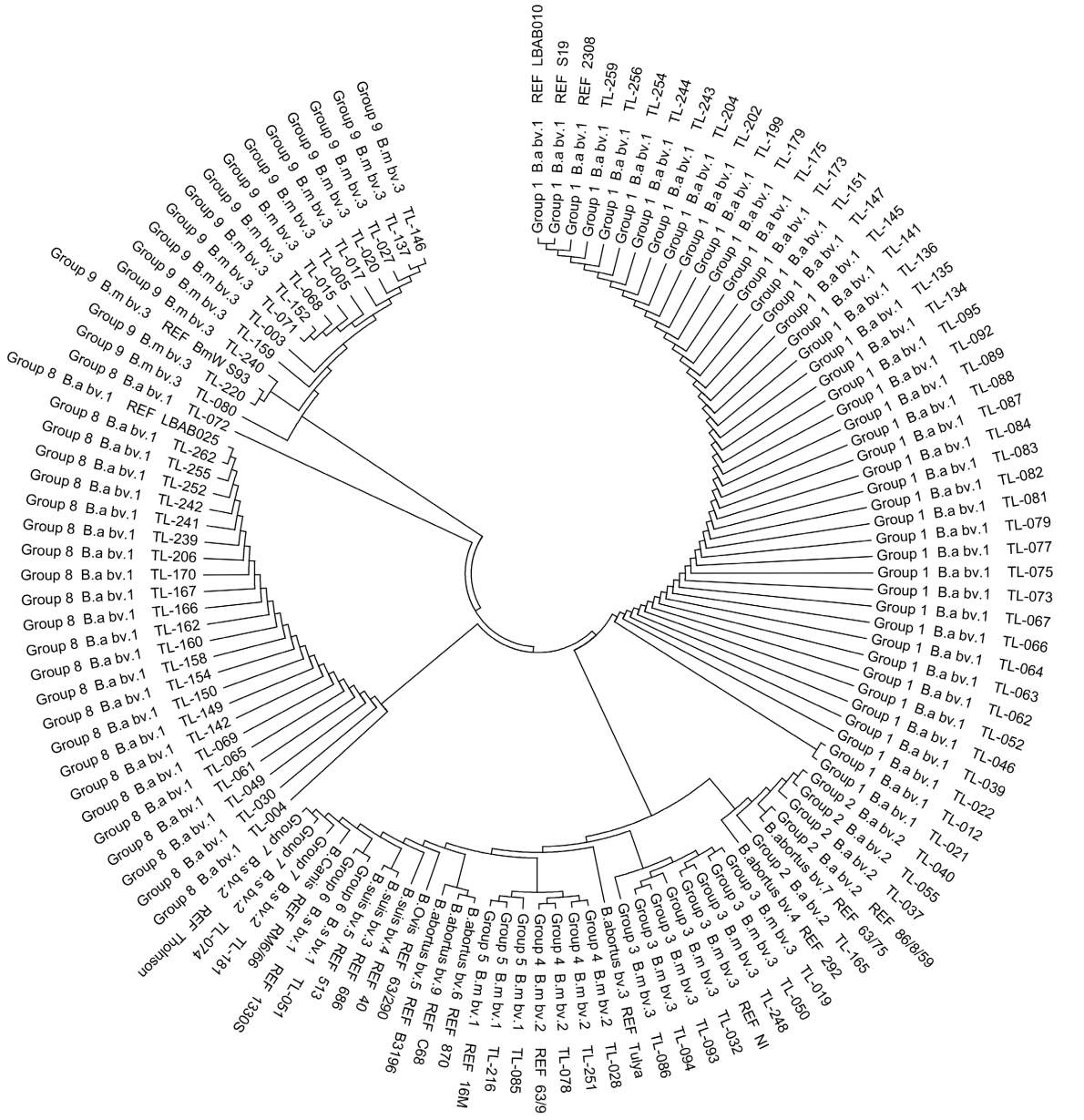

Figure 1. Cluster analysis of 100 sequences of the *Brucella IS711* gene. Note: Cluster analysis revealed that these sequences formed nine distinct groups, with each group corresponding to specific species and biovars. This clustering pattern demonstrates a strong correlation between *IS711* sequence variation and the classical species/biovar classification.

Cluster analysis of *IS711* sequences from 100 *Brucella* sequences were divided into 9 groups (Group 1 to Group 9) (**Figure 1**). Group 1 consists of 42 *B. abortus* biovars 1. Group 2 consists of 4 *B. abortus* biovars 2. Group 3 consists of 7 *B. melitensis* biovars 3. Group 4 consists of 3 *B. melitensis* biovars 2. Group 5 consists of 2 *B. melitensis* biovars 1. Group 6 consists of 1 *B. suis* biovars 1. Group 7 consists of 2 *B. suis* biovars 2. Group 8 consists of 24 *B. abortus* biovars 1. Group 9 consists of 15 *B. melitensis* biovars 3. This clustering pattern demonstrates a strong correlation between *IS711* sequence variation and the classical species/biovar classification, with each biovar forming distinct phylogenetic clusters, while the separation of *B. abortus* biovar 1 into two groups (Group 1 and 8) and *B. melitensis* biovar 3 into two groups (Group 3 and 9) suggests potential genetic diversity within these predominant biovars in the study region.

4. Discussions

Brucella spp. classification is based on factors such as host specificity, pathogenicity, and phenotypic traits [11]. To date, there are 12 recognized species and 19 biovars within the *Brucella* genus. The most frequently encountered species causing brucellosis in humans and animals are *Brucella melitensis* and *Brucella abortus*, followed by *Brucella suis*. Other species can occasionally lead to human infections [12]. The clinical manifestations of brucellosis often resemble those of various infectious and non-infectious diseases, which underscores the importance of laboratory testing as the most reliable diagnostic method [13].

Detection of *Brucella* typically involves microbial culture and serological testing. While bacterial isolation is considered the "gold standard" for diagnosis, it often results in false-negative outcomes influenced by the culture medium, the bacterial load in the specimen, and the specific species of *Brucella* being tested [14]. Serological methods, such as the standard serum agglutination test (SAT), remain valuable diagnostic tools despite their limitations, including potential cross-reactions, false positives, and an inability to accurately reflect the severity of the disease [15].

In China, the isolation and cultivation of *Brucella* from blood samples are primarily conducted using fully automated blood culture systems. However, these methods can be labor-intensive and time-consuming. Meeting the required CO_2 levels for optimal growth presents significant challenges, particularly for *B. abortus* strains, which require an environment with 5% - 10% CO_2 for initial cultivation [13] [16]. This difficulty may explain the predominance of *B. melitensis* strains reported in the literature, which contrasts with the species commonly found in livestock such as cattle and sheep.

In our study, the observed ratio of *B. abortus* to *B. melitensis* was 2.59:1 (70/27), indicating a higher prevalence of *B. abortus* in human brucellosis cases. This finding aligns with the distribution patterns of *Brucella* species documented in European countries and South Korea [8]. Specifically, *B. abortus* biovar 1 constituted 71.80% of *B. abortus* in these regions, while in Tongliao city, *B. abortus* biovar 1

accounted for 94.29% (66/70) of the identified *B. abortus*, further supporting the consistency with European data.

The positive rates for initial and follow-up cases were recorded at 43.30% and 44.72%, respectively. Notably, the positive rate among drug users was 48.33% (29/60). Additionally, the positive rates were 66.67% (16/24) for cases with an onset time of 6 months to 1 year and 33.33% (10/30) for those with an onset time exceeding 1 year. This suggests that follow-up cases are particularly important for treatment strategies, as they exhibited a higher positive rate compared to initial diagnosis cases, especially for those within the 6-month to 1-year timeframe.

Research by Navarro E and Vrioni G suggests that around half of the cases may still test positive for DNA detection after completing the treatment regimen [17] [18]. The persistence of DNA can last from several months to six months, with some cases extending to 1 - 3 years or longer [19]. Our findings are consistent with these observations. A single PCR-positive result, particularly at low viral loads or high Ct values, does not suffice to determine disease activity or infectivity [18]. To evaluate the presence of viable bacteria, both specimen isolation and culture, as well as DNA detection methods, were employed. A reduction in DNA load alongside symptom improvement indicates a scenario of "residual DNA/low-level DNA" during treatment. Conversely, a rebound in DNA load along with symptom recurrence or the emergence of new focal manifestations should raise concerns for relapse or reinfection [20].

The positive sequences from outpatients in the Left Banner and Kailu regions were predominantly *B. abortus*. In contrast, the sequences from patients in Zhalai Banner were evenly split between *B. abortus* and *B. melitensis*. Cases from Shenyang City, Liaoning province, primarily involved *B. melitensis* biovar 3, while those from Heilongjiang province were mainly *B. abortus* biovar 1. Therefore, targeted measures will be implemented to prevent and control brucellosis in cattle and sheep based on the epidemiological context of human brucellosis across different regions.

Fever and limb soreness were identified as the primary symptoms in human brucellosis, affecting 72.61% (167/230) of patients. The positive detection rate was 48.39% (30/62) among asymptomatic outpatients. Additionally, the positive rates were 38.46% (30/78) for serum antibody-negative individuals and 56.25% (27/48) for those without SAT testing. The strains were mainly *B. abortus*, suggesting that symptoms attributed to *B. abortus* strains tend to be milder and may not be taken seriously by clinicians. This could also contribute to the lower detection rates for *B. abortus* strains.

Phylogenetic analysis indicated that *B. abortus* biovar 1 could be divided into two distinct groups. Sequences from Group 1 were closely related to REF 2308 and REF LBAB010, the latter of which was isolated from Para state in northwestern Pakistan [21]. Based on the *IS711* gene, *B. abortus* biovar 1 in Group 8 was closely related to LBAB025, which was isolated from Rio Grande do Sul state in southwestern Brazil [21]. All *B. abortus* biovar 1 demonstrated associations with Brazilian strains, corroborating previous studies that indicated genetic homology be-

tween Chinese and Brazilian strains [8]. Similarly, *B. melitensis* biovar 3 strains were categorized into two groups (Group 3 and Group 9). Sequences from Group 3 were closely related to the REF-NI strain identified as *B. melitensis* biovar 3 in Inner Mongolia, China [22]. Sequences from Group 9 were closely related to the REF BmW S93 strain, isolated from blood specimens in Ulanqab City, Inner Mongolia Autonomous Region [23]. The geographic distance of approximately 1033 kilometers between Ulanqab City and Tongliao City suggests genetic homology among *B. melitensis* in Inner Mongolia. The sequence similarity of strains based on the single *IS711* gene should be interpreted with caution, while whole-genome SNP analysis offers insights into genetic relatedness across the entire genome.

In summary, this study elucidates the distribution patterns of *Brucella* infections within the population of Tongliao City, revealing a predominance of *B. abortus* strains, followed by *B. melitensis* and *B. suis*. The predominant species and biovars in this region are *B. abortus* biovar 1 and *B. melitensis* biovar 3. Clinical presentations indicated similar detection rates for fever and limb soreness. In terms of SAT testing, the positive detection rates varied based on serum antibody levels, with the highest rates observed in individuals with antibody levels exceeding 1:100, followed by those with levels of 1:25 - 1:50, and finally, negative serum antibodies. The onset time also influenced detection rates, with the highest rates seen in cases occurring 1 month to 6 months prior, followed by less than 1 month, over 1 year, and finally 6 months to 1 year. Cluster analysis revealed that the 100 positive samples could be categorized into 9 distinct groups corresponding to various *Brucella* species and biovars. Furthermore, *IS711* sequences of *B. abortus* biovar 1 exhibited associations with Brazilian strains, while *B. melitensis* strains in Inner Mongolia demonstrated genetic homology.

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Ethical Statements

This study had approval from Ethics Committee of Tongliao Center for Disease Control and Prevention.

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Conflicts of Interest

All authors declare no conflict of interest.

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