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Enzootic epidemiology of *Brucella* in livestock in central Gansu Province after the National Brucellosis Prevention and Control Plan

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Abstract

Brucellosis remains one of the most common zoonoses spread worldwide, inducing enormous economic losses to the livestock industry and posing serious health threats to humans. Brucellosis re-emerged in China in the mid-1990s and reached a historically high level in 2015. The National Brucellosis Prevention and Control Plan (NBPCP) was initiated from 2016 to 2020. However, the present epidemiological status in livestock has not been elucidated, and whether *Brucella* variation occurred remains unclear. This study performed an extensive serological investigation in ruminant livestock from 2019 to 2021 in central Gansu Province, China. In total, 11,296 samples from 337 farms were collected to detect the specific antibodies of *Brucella*. The yearly average serological prevalence of *Brucella* at the flock level and individual level declined from 11.32% to 8.26% and 1.17% to 0.57%, respectively. The apparent individual-level seroprevalence of small and large ruminants was 0.89% and 0.52%, respectively. The brucellosis distribution has shifted from pastoral areas to agro-pastoral areas. Flock size and gender may be major risks of *Brucella* infection. Then, the *B. melitensis* TZ strain was isolated from female Tibetan sheep blood cell lysates. Phonotypical characterization demonstrated that it belongs to *B. melitensis.* biovar 3, and multilocus sequencing typing results indicated that it belongs to ST8. The whole genome and subsequent phylogenetic analysis demonstrated that the *B. melitensis* TZ strain is genetically more closely related to the *B. melitensis* QH61 strain. The *B. melitensis* TZ strain has similar growth characteristics to the *B. melitensis* 16 M strain. Overall, our study suggests that after strengthening control and prevention measures based on the NBPCP, there is a very low prevalence or absence of *B. melitensis* in the central Gansu Province of China, and the genotype of an epidemic strain of *Brucella* in Northwest China is relatively stable.

Keywords *Brucella*, NBPCP, Epidemiology, Ruminant, Isolation, Genotype

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Introduction

Brucellosis remains a serious worldwide bacterial zoonotic disease with enormous economic losses and a global public health problem and is still epidemic in Africa, Central Asia, the Middle East, and Latin America (Celli 2019; Bagheri Nejad et al. 2020; Dean et al. 2012; Heavey 2019). *Brucella* spp. is the main cause of Brucellosis, a gram-negative *α-proteobacteria* intracellular pathogen (Ahmed et al. 2016). *Brucella* spp. causes a serious threat to human and animal health (Percin 2013). It is classified as a class B2 agent and a class B pathogen by the World Health Organization (WHO) and the *Law of the People's Republic of China on Prevention and Treatment of Infectious Diseases*, respectively. Sheep, goats, cattle, and pigs are the dominant animal reservoirs of human brucellosis (Franco et al. 2007). *B. melitensis*, *B. abortus* and *B. suis* are major pathogens of animal and human brucellosis worldwide. *Brucella* can be transmitted to humans through consuming contaminated animal products or direct contact infection (Pappas et al. 2005). Typically, *B. melitensis* remains the most dangerous to humans and is considered the leading causative pathogen of human brucellosis (Díaz 2013).

Bovine brucellosis has been successfully eradicated in Japan, several European countries, Australia, New Zealand, and Canada (Samadi et al. 2010; Abernethy et al. 2011). However, animal brucellosis still poses a serious risk to human health in some regions, especially some developing countries based on the ruminant livestock industry (OIE 2018). Furthermore, the epidemic in some less-developed countries is still serious due to the complex epidemiological situation of *B. melitensis* in ruminant livestock (Lacasta et al. 2015). Brucellosis in humans was first noted as Malta fever in 1905 (Boone 1905) and was prevalent during the 1950s–1970's in China. It was under control until the early 1990s due to the introduction of diagnostic criteria, clinical symptoms, laboratory test criteria, and treatment protocols for humans (Jiang et al. 2020). According to the China Center for Disease Control (CDC), human brucellosis re-emerged and increased greatly from the mid-1990s, and the distribution extended from the northern to the southern provinces (Chen et al. 2013; Zhong et al. 2013). It was reported that human brucellosis has been distributed in 31 provinces of China, of which 95.2% (159,667) were from northern provinces (Tao et al. 2021). The reported human infection cases increased 7.8% annually from 2007 to 2017 (Guan et al. 2018). The number of livestock increased significantly to meet the increasing demand for consumption of meat and milk from the 1990s. However, quarantine in frequent livestock product trade lags (Tan et al. 2015; Chen et al. 2013). Under such a situation, animal brucellosis re-emerged in most northern regions of China and expanded to southern provinces (Wang et al. 2018; Cao et al. 2018b, c, a). Brucellosis is endemic, even epidemic, particularly in Gansu, Xinjiang, Ningxia, Qinghai, and Inner Mongolia Autonomous Region (Zhou et al. 2018).

Northwest provinces of China are the historical epidemic zone of brucellosis. Small and large ruminant livestock are primarily raised by pastoral farmers and are a great source of milk and meat for local farmers. In 2013, human brucellosis in central Gansu Province numbered 24, while the data increased to 272 in 2017 (Man et al. 2018). In addition, yak individual-level and flock-level seroprevalence in Tibet was 2.8% and 18.2% in 2015, respectively (Zeng et al. 2017). The average positive rate of *Brucella*-specific antibodies in dairy cows reached 1.07% in 15 provinces of China in 2011 (Ning et al. 2013). The yearly average anti-brucellosis seropositive rates of domestic animals in Qingyang Gansu increased to 4.75% in 2015 (Cao et al. 2018b, c, a). As infected animals are the main sources of human brucellosis, most cases are closely related to their careers (veterinarians, farmers, and slaughterhouse workers) (Godfroid et al. 2005). Therefore, effective control measures for animal brucellosis are crucial for human brucellosis control. To effectively control and prevent the epidemic of human and animal brucellosis, "The National Brucellosis Prevention and Control Plan (NBPCP) (2016–2020) was commenced by The Ministry of Agriculture and National Health Commission of China from 2016 to 2020, with effective control measures in animals and humans at the national level. Following the implementation of the NBPCP program, the human brucellosis number declined from 47,139 (3.4/100,000) to 37,947 (2.7/100,000) between 2016 and 2018 (Tao et al. 2021). However, the epidemiological status of animal brucellosis has not been assessed immediately before and after the end of NBPCP, and whether Brucella variation took place should be a concern.

This study aimed to carry out the first epidemiological evaluation of Brucella infection in ruminant livestock before and after the end of NBPCP and to realize the present epidemiological data and possible risk factors for animal brucellosis for further eradication of brucellosis. Therefore, random sampling was used to perform a comprehensive serological survey of ruminant livestock brucellosis from 2019 to 2021. In addition, bacterial isolation from blood cells was first performed on asto animals, MLST analysis, and whole-genome sequencing were performed to elucidate the molecular characteristics of endemic *Brucella* spp., and physiological feature detection was also implemented to assess the kinetics of the isolated strain.

Results

Flock-level seroprevalence declined post-NBPCP

To analyze the epidemic situation at the flock level, 337 farms were tested for *Brucella* antibodies. Thirty-two farms contained at least one or more seropositive animals. Therefore, the flock-level seroprevalence was 9.50% (95% CI: 8.43%–17.13%). From 2019 to 2021, the flock-level seroprevalence was 11.32% (95% CI: 4.61%-20.28%), 9.09% (95% CI: 4.00%-22.44%), and 8.26% (95% CI: 3.41%-21.92%), respectively. It showed a decreasing trend from 2019 to 2021 (Fig. 1A). The flock-level seroprevalence of County G and County M was similar but different from the changing trend of County T and County L.

Of the 337 flocks sampled in this study, 32.05% (108/337) were from County L, 16.32% (55/337) were from County M, 22.26% (75/337) were from County G, and 29.38% (99/337) were from County T. Although the flock-level seroprevalence (12.00%) of County G was the highest, there was no significant difference compared with the three other counties ($P > 0.05$) (Fig. 1B). This phenomenon may be related to the distribution of farms and frequent market transactions with neighboring provinces.

The flock-level seroprevalence of sheep, goat, cattle and white yak farms was 8.14%, 19.23%, 9.38% and 14.29%, respectively. Although the flock-level seroprevalence of goat farms was the highest, there was no significant difference among the other three species' farms ($P > 0.05$) (Fig. 1C). Of all farms sampled, the number of farms with flock sizes less than 100 and more than 100 were 49.85% (168/337) and 50.15% (169/337), respectively. The flock-level seroprevalences were 2.98% (95% CI: 0.87%-10.86%) and 15.98% (95% CI: 13.35%-40.18%), respectively. The flock-level seroprevalence of less than 100 group farms was significantly different from that of the group of more than 100 farms according to the *t* test ($P < 0.01$) (Fig. 1D). Moreover, small and large ruminant farms showed no

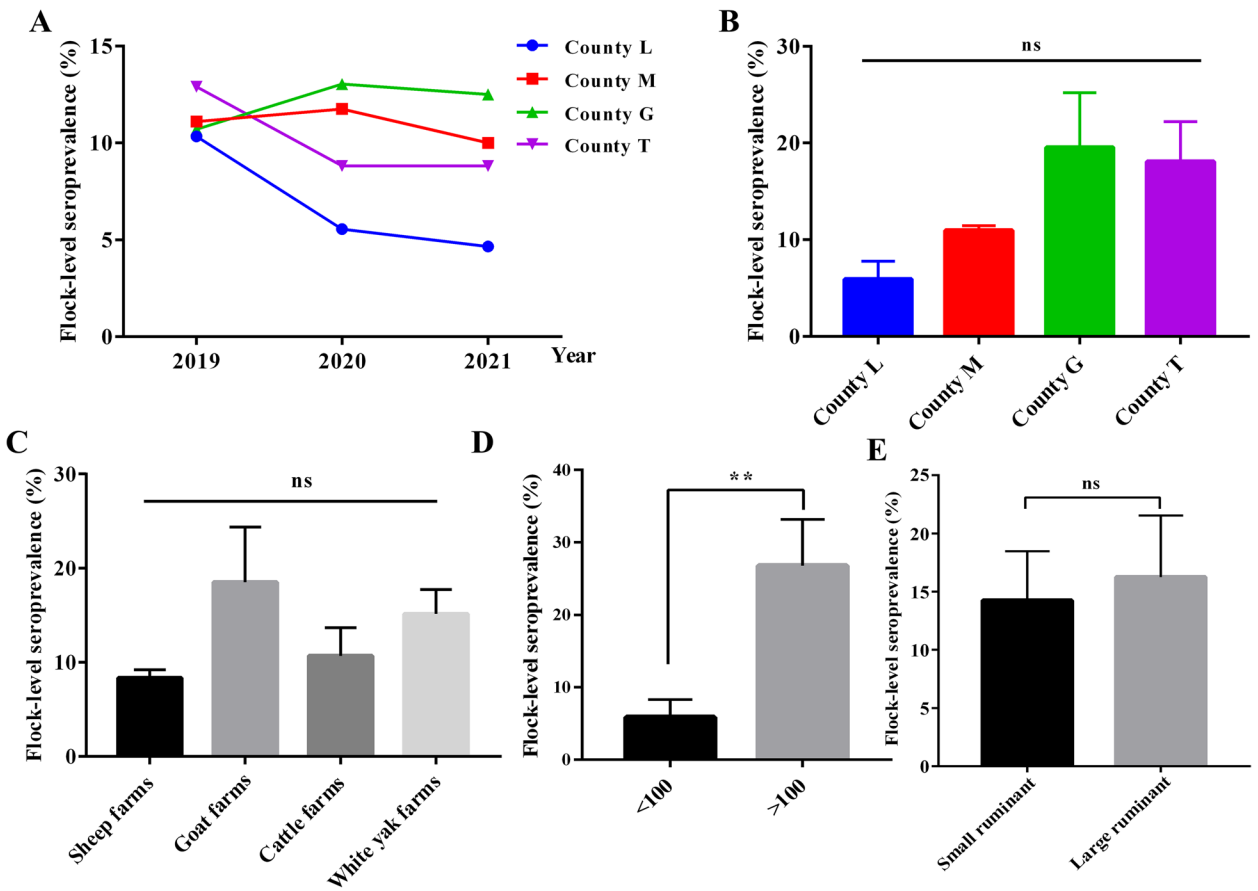

Fig. 1 The flock-level seroprevalence of animal brucellosis from 2019 to 2021. **A** The trend of flock-level seroprevalence in 4 counties from 2019 to 2021. The Y-axis represents the average flock-level positive rate of the county with seroprevalence % ± SE (p), and the X-axis represents years. **B** Differential analysis of seroprevalence in different counties; **C** Differential analysis of seroprevalence in different species of farms; **D** Differential analysis of seroprevalence in different flock sizes of farms; **E** Differential analysis of seroprevalence in small and large ruminant farms. ns: no significant difference at $P > 0.05$. *Significant difference at $P < 0.05$. ** Extremely significant difference at $0.01 < P < 0.05$

significant differences ($P > 0.05$) (Fig. 1E). In addition, the geographical distribution of seropositive farms was mainly concentrated in agro-pastoral transition and rural counties, which demonstrated that the trend has transferred from pastoral to agro-pastoral regions. These data indicate that the flock-level seroprevalence declined post-NBPCP.

Individual-level seroprevalence decreased post-NBPCP

Based on the survey results of epidemiological investigation and observation of animal brucellosis in assigned farms in 22 counties, the positive rates of anti-brucellosis of sheep and cattle at the individual level were 3.3% and 3.1%, respectively. The flock-level prevalence rates were 34% and 29% in 2015, respectively (Ministry of Agriculture and Rural Affairs of the People's Republic of China 2016). As the historical brucellosis area, the positive rate of anti-brucellosis of sheep in county L at the individual level was 11.34% in 2014 (Wei et al. 2019). The average flock-level prevalence rates of the four counties were 38.1% and 47.62%, and the average individual-level apparent seroprevalence of sheep breeding farms was 6.61% and 4.79% in 2016 and 2017, respectively (Zhao et al. 2019).

To assess the epidemiological status immediately before and after the end of NBPCP in 2020, 11 296 serum samples were collected from sheep, goat, cattle, and white yak in four counties of central Gansu Province to monitor *Brucella* antibodies, and 182 samples tested positive. As a result, the apparent individual-level seroprevalence was 0.85% (95% CI: 0.24%-0.51%), and the estimated true seroprevalence was 0 (95% CI: 0) when considering the test specificity and sensitivity.

From 2019 to 2021, the apparent seroprevalence was 1.17% (95% CI: 0.17%-0.66%), 0.94% (95% CI: 0.15%-0.69%), and 0.57% (95% CI: 0.08%-0.51%), respectively. The annual variation tendency decreased from 2019 to 2021 (Fig. 2A). The yearly apparent seroprevalence was between 0.47% and 1.62% in the four counties. The results indicate that the serological prevalence rate of animal brucellosis has decreased significantly since the NBPCP was implemented.

As a total of 11,296 serum samples from this study, 30.16% (3407/11,296) were from County L, 28.97% (3272/11,296) were from County M, 16.76% (1893/11,296) were from County G, and 24.11% (2724/11,296) were from County T. The apparent seroprevalence was 0.41% (95% CI: 0.03%-0.30%), 0.95% (95% CI: 0.09%-1.04%), 1.43% (95% CI: 0.17%-0.88%) and 0.88% (95% CI: 0.14%-0.63%), respectively. The apparent seroprevalence of County M showed no statistically significant difference from that of County L ($P > 0.05$) (Fig. 2B).

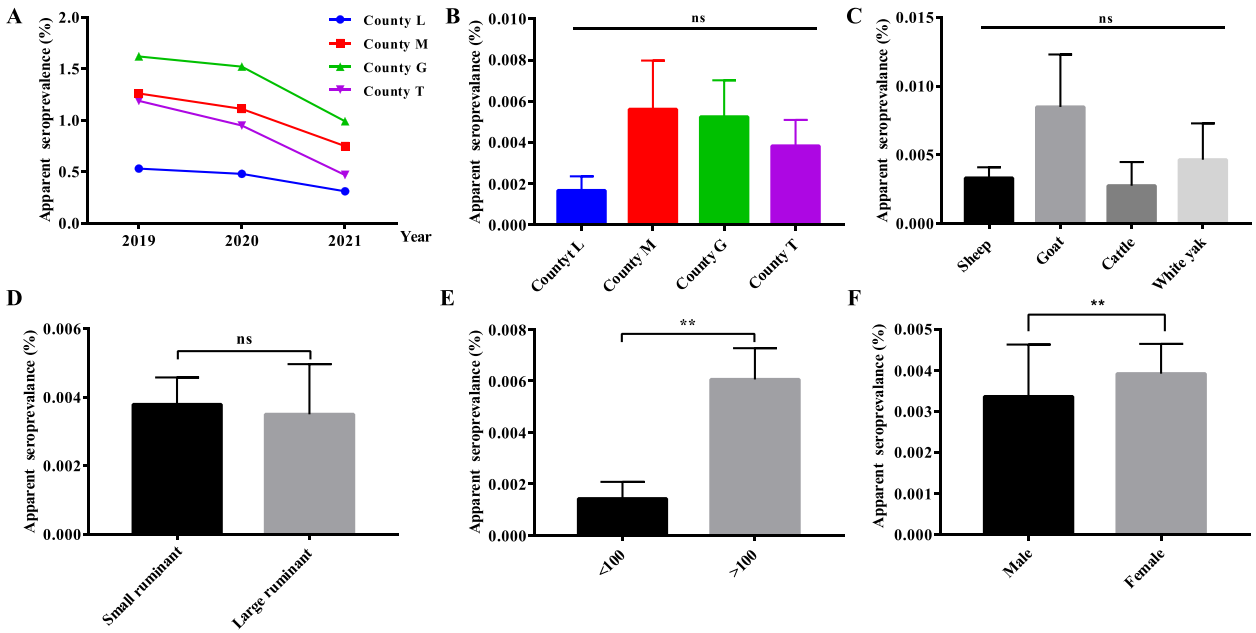

Fig. 2 The individual-level apparent seroprevalence of animal brucellosis from 2019 to 2021. **A** The trends of apparent seroprevalence in four counties from 2019 to 2021; the Y-axis represents the average individual-level positive rate of the county with seroprevalence % ± SE (p), and the X-axis represents years. **B** Differential analysis of apparent seroprevalence in different counties; **C** Differential analysis of apparent seroprevalence in different species; **D** Differential analysis of apparent seroprevalence in small and large ruminants; **E** Differential analysis of apparent seroprevalence in different flock sizes; **F** Differential analysis of apparent seroprevalence in different sexes. ns: no significant difference at $P > 0.05$. *Significant difference at $P < 0.05$. ** Extremely significant difference at $0.01 < P < 0.05$

Serum samples from sheep, goats, cattle, and white yaks were 85.60% (9 669/11 296), 4.19% (473/11 296), 5.88% (664/11 296), and 4.34% (490/11 296), respectively. The apparent seroprevalence was 0.86% (95% CI: 0.18%-0.48%), 1.48% (95% CI: 0.06%-1.64%), 0.45% (95% CI: 0–0.63%), and 0.61% (95% CI: 0–1.02%), respectively. Goats showed the highest apparent seroprevalence and no statistically significant difference compared with cattle, which showed the lowest seroprevalence ($P > 0.05$) (Fig. 2C). The apparent seroprevalence rates of different species may be directly related to their breeding patterns. For instance, goats are mainly grazed or mixed with sheep, whereas cattle are usually concentrated on farms. In addition, the apparent seroprevalence of small and large ruminant groups was 0.89% (95% CI: 0.22%-0.53%) and 0.52% (95% CI: 0.06%-0.64%), respectively. Brucellosis in small ruminant groups was more serious with the largest number and the most extensive distribution compared with the large ruminant groups but showed no significant difference ($P > 0.05$) (Fig. 2D).

For different flock size groups, 18.87% (2 132/11 296) were from a herd scale of less than 100, and 81.13% (9164/11,296) were from a flock size above 100. The apparent seroprevalences were 0.23% (95% CI: 0.01%-0.27%) and 0.99% (95% CI: 0.37%-0.85%). Therefore, there was a significant difference between different flock size herds according to the *t* test ($P < 0.01$) (Fig. 2E). Male livestock comprised 16.33% of the samples in this study (1845/11,296), and female livestock comprised 83.67% (9451/11,296). The apparent seroprevalences were 0.70% (95% CI: 0.09%-0.58%) and 0.88% (95% CI: 0.02%-0.53%). The seroprevalence of the female group was higher than that of the male group and was extremely statistically significant ($P < 0.01$) (Fig. 2F). Flock size and sex may be the major risk factors influencing the prevalence of animal brucellosis. These results are consistent with the epidemiological characteristics of *Brucella*. These data suggest that individual-level seroprevalence decreased post-NBPCP.

The isolated bacterial strain belongs to *B. melitensis* biovar 3

To study the possible epidemic *Brucella* strain in the study area, 27 seropositive blood samples with EDTA anticoagulant were selected as the source for *Brucella* isolation. As a result, four suspected bacterial clones isolated from 2-year-old female Tibetan sheep blood cell lysates were observed on *Brucella* selective plates as early as 72 h and were obtained after 120 h of culturing at 37°C without CO_2. The results indicated that the seropositive blood cell lysates could be a source for *Brucella* isolation from animals. The bacterial colony showed a honey-like appearance with a smooth surface, shiny and translucent, small and convex. The microscopic results showed that the isolates were gram-negative coccobacilli, mostly in a beaded arrangement singly or in pairs. The isolate is catalase, oxidase and urease positive, but hydrogen sulfide production is negative. The classical biotyping assay of the four bacterial clones was agglutinated with the monospecific anti-A serum and anti-M serum, not with the classical biotyping assay of these bacterial clones with the monospecific anti-A and anti-M serum, not the monospecific anti-R serum. The isolated strain grew normally on plates containing thionin or basic fuchsin dyes, while the bacteriophage Tb test was negative and the Bk_2 test was positive (Table 1). These data suggest that the isolate belongs to *B. melitensis* biovar 3.

MLST confirmed that the isolated bacteria belonged to ST8

Since four bacterial clones were isolated from the same blood sample, classical biovar typing and genus-specific results confirmed that these clones belong to the same *Brucella* strain. Therefore, specific PCR and MLST assays were performed on this strain to analyze the genotype. Specific PCR of the *Brucella* genus targeting the BCSP31 and OMP25 genes amplified 224 and 628 bp, respectively, and the results confirmed the isolated strain as *Brucella* (Fig. 3A, B). The enhanced AMOS-PCR further verified the isolated strain as *B. melitensis* with 731 bp PCR amplicons (Fig. 3C). The MLST analysis results indicated

Table 1 Classical biotyping assay for *Brucella* isolates *B.melitensis* TZ

Brucella spp. (Strain)	CO_2 requirement	H_2S production	Urease activity	Thionin blue[a]			Basic fuchsin[b]		Monospecific antisera			*Brucella* phage group	
				a	b	c	b	c	A	M	R	Tb	Bk_2
B.melitensis bv. 1 (M5-90)	-	-	+	+	+	+	+	+	-	+	-	-	+
B.abortus bv.1 (A19)	-	+	+	-	-	-	-	-	+	-	-	+	+
B.suis bv.1 (S2)	-	+	+	-	-	-	+	+	+	-	-	-	+
B.melitensis TZ	-	-	+	+	+	+	+	+	+	+	-	-	+

Note: +, Positive. -, Negative. [a]Concentration of Thionin blue (a = 1:25 000, b = 1:50 000, c = 1:100 000). [b]Concentration of basic fuchsin (b = 1:50 000, c = 1:100 000)

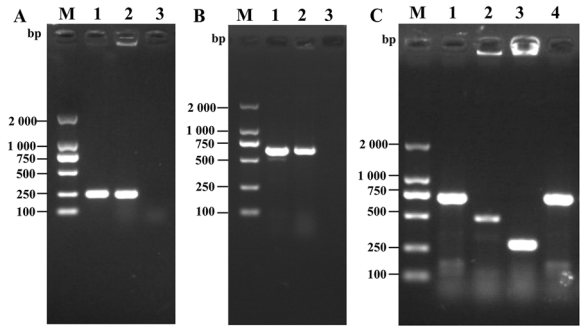

Fig. 3 Isolation and genotyping of *Brucella* from sheep blood cell lysates. **A** Identification of *Brucella* isolates by BCSP31 gene PCR amplification. M, 2,000 bp DNA ladder. Lane 1, *B. melitensis* M5-90 strain. Lane 2, *B. melitensis* TZ strain. Lane 3, *E. coli* ATCC25922 strain. **B** Identification of *Brucella* isolates by OMP 25 gene PCR amplification. M, 2000 bp DNA ladder. Lane 1, *B. melitensis* M5-90 strain. Lane 2, *B. melitensis* TZ strain. Lane 3, *E. coli* ATCC25922 strain. **C** Identification of Brucella species by AMOS typing. M, 2000 bp DNA ladder. Lane 1, *B. melitensis* M5-90 strain. Lane 2, *B. abortus* A19 strain. Lane 3, *B. suis* S2 strain. Lane 4, *B. melitensis* TZ strain

that the allele spectrum of the ST was 3-2-3-2-1-5-3-8-2, and the MLST sequence typing was ST8. The sequence type is the most common in Northwest China (Cao et al. 2018b, c, a). Thus, the isolated *Brucella* strain was named *B. melitensis* TZ. These data suggest that *B. melitensis* TZ belongs to *B. melitensis* ST8, an epidemic genotype in northwest China. Therefore, the results indicated that *B. melitensis* existing in central Gansu Province is genetically stable.

B. melitensis TZ phylogenetic closely related to *B. melitensis* QH61

The whole-genome was sequenceed to determine the genomic characteristics and genetic evolution of *B. melitensis* TZ, and a phylogenetic tree was constructed. The whole-genome sequence analysis demonstrated that the genome was 3,311,814 bp and contented 57.21% of G + C. Two circular chromosomes were identified in *B. melitensis* TZ with 2,126,166 bp and 1,185,648 bp in chromosomes I and II, respectively (Fig. 4A, B). The G + C contents are 57.15% and 57.34%, respectively. The proportion of the predicted coding nucleotide sequence of *B. melitensis* TZ was approximately 86.03% (2,849,142 bp). The copy numbers of small RNAs (sRNAs), tRNAs, and rRNAs were 17, 55 and 9 (3 of 5S_rRNA, 3 of 16S_rRNA, and 3 of 23S_rRNA), respectively. Moreover, this strain has 82 tandem repeats, 54 microsatellite DNA, and 12 microsatellite DNA. In addition, the databases 217 (6.59%) of VFDB, 12 (0.36%) of ARDB, 131 (3.97%) of CAZy, 2793 (84.84%) of IPR, 1526 (46.35%) of SWISS-PROT, 2648 (80.43%) of COG, 5 (0.15%) of CARD, 2077 (63.09%) of GO, 2 161 (65.64%) of KEGG, 3 287 (99.84%) of NR. The complete genome sequence of *B. melitensis* TZ has been submitted to GenBank with accession numbers CP115176-CP115177. The phylogenetic tree, based on the similarity and difference of genotype and phenotype between species, could reflect the evolutionary relationship of the species. The results indicated that *B. melitensis* TZ has a close relationship with *B. melitensis* QH61, an epidemic *Brucella* strain in neighboring provinces of Qinghai (Fig. 4C). This result indicated that the prevalence of *Brucella* might have a close relationship with the same geographical distribution.

The isolate *B. melitensis* TZ has similar growth kinetics to *B. melitensis* 16 M

Pure culture and cell infection assays were performed to determine the growth kinetics of *B. melitensis* TZ. The growth phenotype of *B. melitensis* TZ in TSB exhibited fast proliferation in the logarithmic growth phase from 8 to 24 h, which was slower than that of the reference strain *B. melitensis* 16 M but similar to that of the representative strain *B. melitensis* (M5-90), and reached a stationary phase at approximately 36 h (Fig. 5A). As intracellular viability is an important indicator of *Brucella* virulence, the survival ability of *B. melitensis* TZ was assessed within HeLa, THP-1 and RAW264.7 cell lines (Fig. 5B, C, and D). The intracellular survival of *B. melitensis* 16 M, *B. melitensis* M5-90, and *B. melitensis* TZ within the three cell lines was similar and showed no significant difference. These data demostrate that *B. melitensis* TZ has growth kinetics similar to those of the *B. melitensis* reference strain.

Discussion

This study aims to assess the effect of the National Brucellosis Prevention and Control Plan (NBPCP) and to guide the further eradication of animal brucellosis. Our epidemiological results demonstrated that from 2019 to 2021, brucellosis in livestock showed a decreasing trend in central Gansu Province. The apparent serological prevalence of *Brucella* at the flock level and individual level declined from 11.32% to 8.26% and 1.17% to 0.57%, respectively. Meanwhile, the livestock population in this area increased to 5.7 million, 0.14 million, 0.48 million, and 0.12 million of sheep, goats, cattle, and yak in 2020, respectively. The implementation of NBPCP in this area has a typical demonstration effect. The vaccine coverage rate and protection duration of immune antibodies are critical factors for controlling and preventing

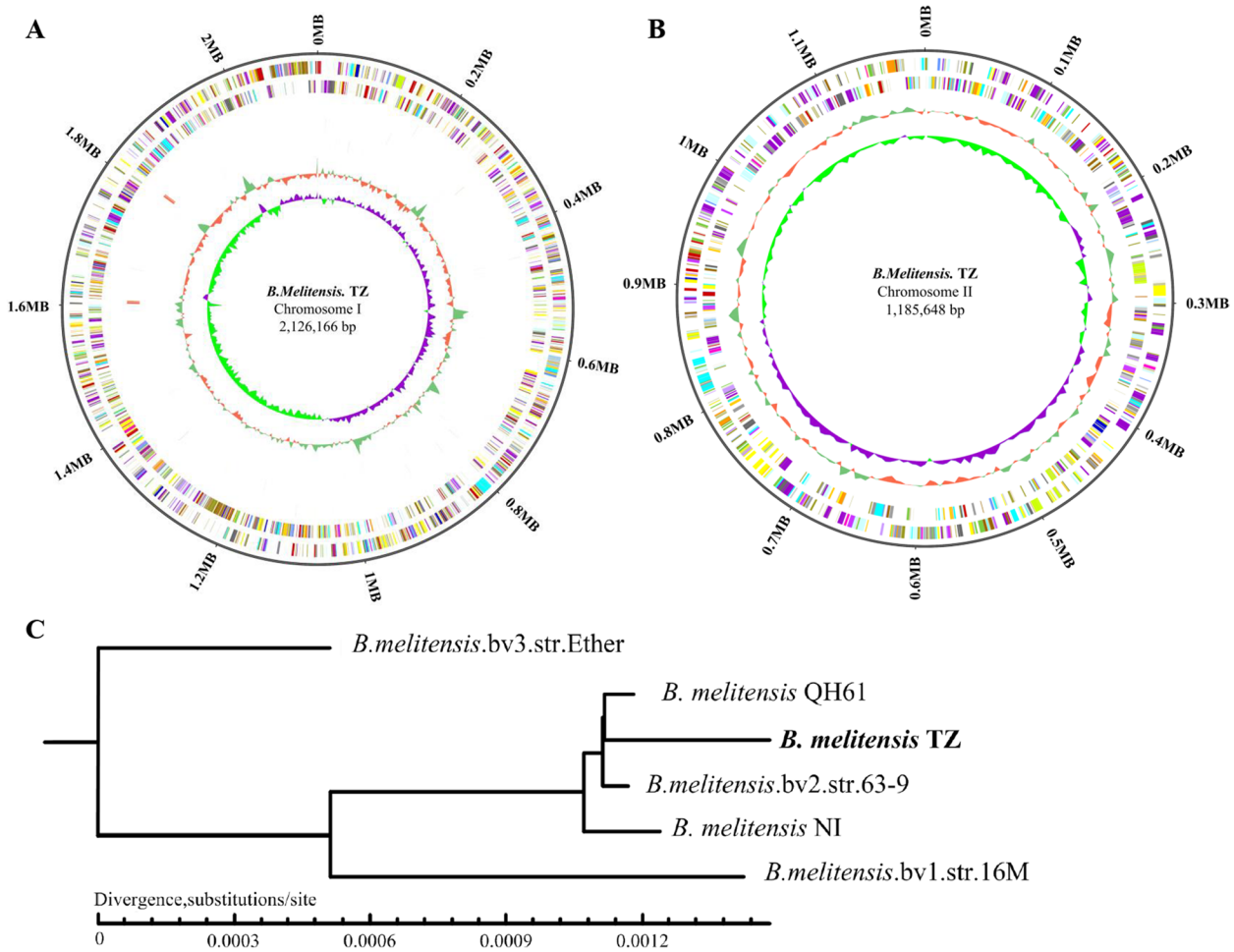

Fig. 4 Circular representation of the whole-genome sequencing of the *B. melitensis* TZ strain isolate. **A** Chromosome I of *B. melitensis* TZ. From outer to inner: genome size, forward strand gene, reverse strand gene, forward strand ncRNA, reverse strand ncRNA, repeat, GC and GC-SKEW. **B** Chromosome II of *B. melitensis* TZ. From outer to inner: genome, size, forward strand gene, reverse strand gene, GC and GC-SKEW. The detailed genome size is numbered outside of the circle. Color refers to the cluster of orthologous groups (COG) classification. **C** Phylogenetic tree of the *B. melitensis* TZ strain and reference strains

animal brucellosis (Beauvais et al. 2016). Thus, vaccination against *Brucella* should be insisted on in the future, and an effective vaccine should be developed without interference with diagnosis. In addition, animal transfer has become more frequent to meet the increasing demand for meat and milk, which could increase the spread of brucellosis due to the lack of essential inspection and quarantine. Therefore, standardized quarantine of the interregional transfer of live animals and pasteurization of animal products are essential to control the spread of brucellosis. Our survey results indicated that animal brucellosis had been stably controlled post-NBPCP, but sporadic and spot distributions existed in the study area. The existing seropositive animals and farms in the study area could be attributed to the different breeding patterns, geographical and climatic factors, and physiological characteristics of *Brucella* spp.

Our research demonstrated that different patterns of management on farms could influence animal brucellosis. For instance, under the guidance of the government animal husbandry policy, intensive large-scale farming became the main management pattern in County M and County G. Consistent with previous research (McDermott et al. 2013; Oseguera Montiel et al. 2015), flock size may be an important risk factor. The lower seroprevalence rate observed in the smaller flock size could be attributed to the feeding and management mode, characterized by proper herd size and lower exposure rate that decreases the disease spread. Therefore, animals breeding in higher stock density farms may have higher exposure opportunities with the infected ones than those breeding in lower stock density farms. In addition, the higher flock-level seroprevalence in County T could be explained by the pattern of mixed breeding. Although

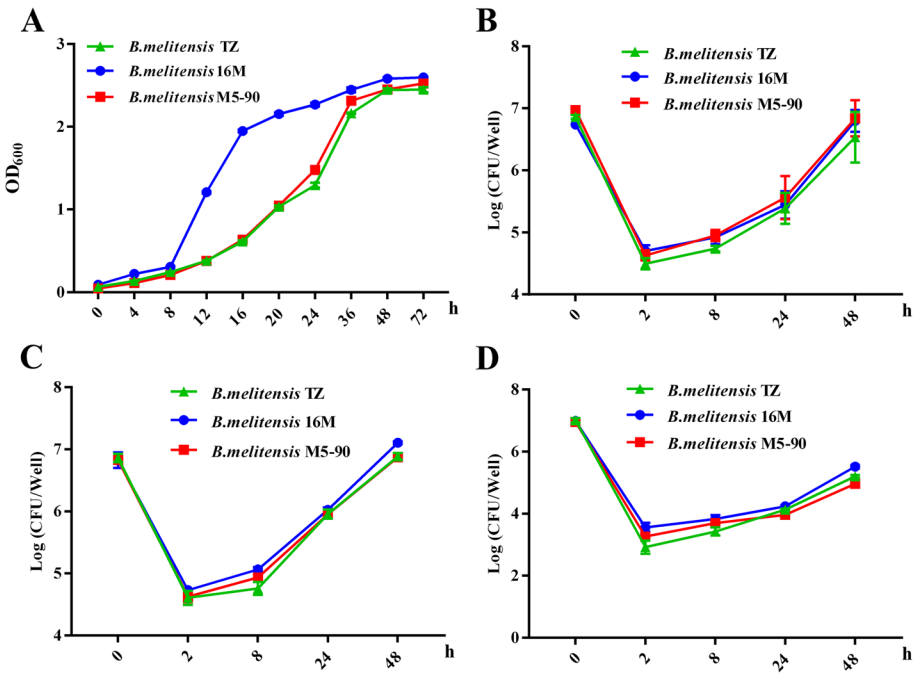

Fig. 5 Growth kinetics of the *B. melitensis* TZ strain in pure culture and infected cell lines. **A** Growth characteristics of *B. melitensis* TZ, *B. melitensis* 16 M and *B. melitensis* M5-90 in TSB. The optical density was obtained every 4 h before 24 h and at 36 h, 48 h, and 72 h with three repetitions. **B** Intracellular replication of *B. melitensis* TZ, *B. melitensis* 16 M and *B. melitensis* M5-90 in infected RAW 264.7 cells. **C.** Intracellular replication of *B. melitensis* TZ, *B. melitensis* 16 M and *B. melitensis* M5-90 in infected THP-1 cells. **D.** Intracellular replication of *B. melitensis* TZ, *B. melitensis* 16 M and *B. melitensis* M5-90 in infected HeLa cells. The level of initial infection was the same for all *B. melitensis* strains as described in the Materials and Methods. At 0, 2, 8, 24, and 48 h postinfection, infected macrophages were lysed, and supernatants were diluted for CFU enumeration

sheep and goats are generally kept and managed in separate flocks, a degree of mixing occurs when frequent animal movements occur among different herds with less restriction.

Moreover, in some farms with a mixture of different species of animals, the risk of brucellosis transmission has also increased. Higher seroprevalence in pastoral and agro-pastoral areas could likely be attributed to larger herd size and a mixture of different flocks. Furthermore, frequent animal trade between provinces with a higher incidence of brucellosis may also increase the chance of spreading brucellosis. Therefore, moderate flock size, standardized breeding patterns, evaluation of animals for purchase, and quarantine of animals are likely to be the optimal solution choices for effectively controlling animal brucellosis.

The seropositive farms in four counties are scattered in the south Qilian Mountain region, middle plain oasis region, and north arid climate region, where the climate is very dry and cold. A previous study showed that Brucella spp. could survive for up to 56 d Plates were placed in 5-10% CO in a suitable environment (Calfee and Wendling 2012). Therefore, livestock housing, feces, and the environment might be contaminated by infected animals. The seropositive herd's pollutants, such as fecal matter, can run off relatively easily and quickly into nearby areas. Contamination of the farm environment with *Brucella* organisms occurs and helps to rapidly spread causative agents in flocks of different sizes. Atypical rough *Brucella* and *B. abortus* biovar 1 was isolated from Himalayan marmot (Yan et al. 2020), which indicated that Himalayan marmot might be a possible source of brucellosis infection in this region. As a result, farm biosecurity measures that include disposal of infected animals and garbage and wild animal control could be helpful to reduce the prevalence and spread of *Brucella* among farms.

Brucella infection in gender is mainly caused by sexual transmission during natural mating, and its prevalence is higher in the genital organs than in other organs, indicating that genital excretion plays a vital role in its spread (Frey et al. 2013). In ruminant livestock, it is well known that brucellosis in primiparous female animals often results in abortion, and *Brucella* is excreted with abortion products during the process (Carvalho Neta et al. 2010). Natural mating is still the main mode of reproduction in livestock production in this region. Most of the investigated farms harbor a certain number of breeding females, which usually have a long reproductive cycle. Consequently, the potential transmission of infection in herds is higher, and the individual-level seroprevalence

of female animals was significantly higher than that of male animals. Therefore, artificial insemination should be a suitable reproduction mode to replace natural mating for farms with corresponding conditions. Meanwhile, a regular *Brucella* testing program should be implemented for breeding animals on farms, and the infected animals should be safely disposed of in a timely manner.

The isolation of *Brucella* is a time-consuming, skilled and risky task for laboratory personnel. The most reliable tissue samples, including spleen, vaginal secretions, genital organs, lymph nodes, and genital organs, are usually selected to improve the isolation rate of *Brucella* (Godfroid et al. 2010). In addition, the isolation rate primarily lies in the number of viable *Brucella* in the collected samples and the growth stage at which most of the surviving bacteria are present (Yagupsky et al. 2019). Considering the potential risk of *Brucella* spread during abortion tissue sample collection and biosecurity implications, seropositive anticoagulant blood samples were selected for bacterial isolation. One *Brucella* strain was isolated (*B. melitensis* TZ*)* from the positive blood samples. To our knowledge, this is the first report of *Brucella* isolation from animal blood. Culture, colony morphology, Gram staining, and biochemical features, including oxidase, catalase, and urease, were performed to identify and confirm the *Brucella* strain. Bacterial culture was performed to determine the genotype and molecular features of the isolated Brucella strain. Furthermore, *Brucella* AMOS-PCR is applied to differentiate *B. melitensis*, *B. abortus,* and *B. suis* (Bricker and Halling 1994). The biochemical testing and serotyping results indicate that the isolate belongs to *B. melitensis* bv 3, the universal biovar isolated from Northwest China.

MLST results of the isolates from Qinghai in the 1980s indicated that ST8 of *B. melitensis* was the dominant genotype in small ruminants and humans (Ma et al. 2016). Previous investigations have shown that the *B. melitensis* ST8 genotype is widely spread in Inner Mongolia (Liu et al. 2019) and Xinjiang (Sun et al. 2016). Our MLST analysis results show that the genotype of *B. melitensis* TZ belongs to ST8, the main genotype causing sheep brucellosis in northern China. The results indicate that *B. melitensis* existing in central Gansu Province is relatively stable. Furthermore, sequence and analysis results of *B. melitensis* TZ indicated that the whole genome is 3,311,252 bp. Five stains were used for comparison with *B. melitensis* TZ in this research including *B. melitensis* QH61, *B. melitensis* NI, *B. melitensis* bv. 1 str. 16 M, *B. melitensis* bv. 3 str. Ether and *B. melitensis* bv. 2 str. 63–9. The results reconfirmed that *B. melitensis* TZ is a regnant *Brucella* strain and is highly homologous to *B. melitensis* QH61. Based on the results, the spread of animal brucellosis could be associated with horizontal transmission in neighboring areas.

Growth analysis showed that *B. melitensis* TZ was slower than *B. melitensis* 16 M in the early stage and similar to B. melitensis M5-90 at the exponential phase in TSB medium, and the strains grew to similar titers at 48-72 h. Establishing cell infection is a crucial step of chronic brucellosis infection. The growth kinetics and cell infection results indicated that *B. melitensis* TZ exhibits stable intracellular viability in the three cell lines compared to *B. melitensis* 16 M.

In summary, we assessed the epidemiological variation tendency of livestock brucellosis in central Gansu Province and isolated a *Brucella melitensis* strain from sheep blood cell lysate. To our present knowledge, this research is the first report of epidemiological evaluation post-NBPCP and the first isolation of animal *Brucella* from blood cells. Control and prevention of livestock brucellosis in the northwestern region will be very important for further effectively eradicating animal brucellosis in China.

Conclusion

The present research provides a large-scale serological survey of ruminant livestock brucellosis epidemiology in 4 northwestern counties from 2019 to 2021 to assess the effect of NBPCP. Our study drew three main conclusions: (i) A total of 11,296 sero-samples from 337 farms were collected for testing. The yearly average apparent individual-level seroprevalence of small and large ruminants in the four counties from 2019 to 2021 was 0.89% and 0.52%, respectively. Considering the sensitivity and specificity of the test methods, the average estimated true individual-level seroprevalence of large and small ruminants were below 0.1% and 0.2%, respectively. The brucellosis distribution has shifted from pastoral areas to the agro-pastoral area. The animal brucellosis epidemic in central Gansu Province declined after the NBPCP approach. (ii) *B. melitensis*. TZ was isolated from female Tibetan sheep blood cell lysates. Phonotypical features proved that it belongs to *B. melitensis.* biovar 3. The MLST results indicated that *B. melitensis* TZ belongs to ST8, which demonstrated that the genotypes of the epidemic strain of *Brucella* in central Gansu Province are relatively stable compared with *Brucella* strains isolated in northwest areas. Results of hole genome sequencing and comparative genomics confirmed that *B. melitensis* TZ has a high identity to *B. melitensis* QH61. (iii) The growth kinetics of *B. melitensis* TZ are similar to those of *B. melitensis* 16 M.

Methods

National Brucellosis Prevention and Control Plan (NBPCP)

Brucellosis had a resurgence in China in the mid-1990s, spread widely from northern to southern areas, and reached a historically high level in 2015 (Chen et al.

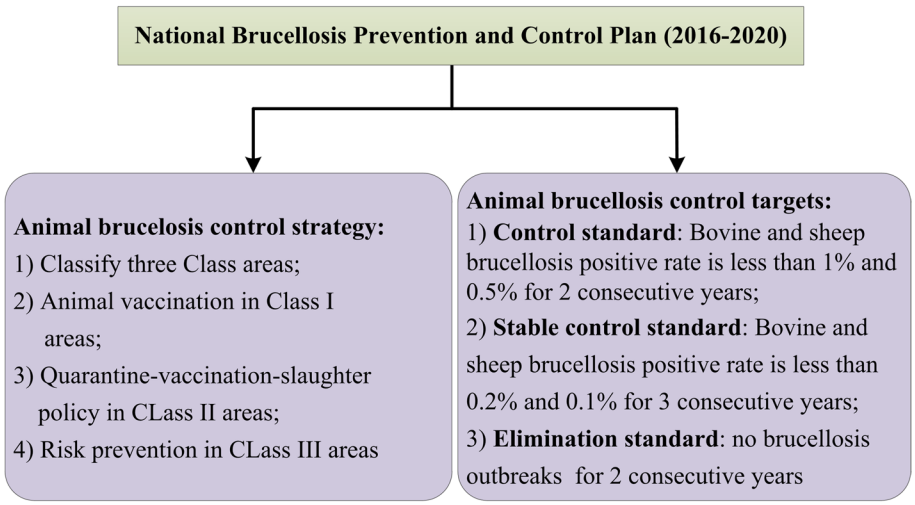

Fig. 6 Control strategy and targets of animal brucellosis in the National Brucellosis Prevention and Control Plan (2016–2020)

2016; Lai et al. 2017). To effectively control and prevent human and animal brucellosis, the National Commission of Health and Family Planning and China's Ministry of Agriculture coissued the "National Brucellosis Prevention and Control Plan (NBPCP) (2016–2020)" in 2016 with specific control measures for brucellosis prevention (Fig. 6). According to the NBPCP, three class areas were classified based on the human brucellosis case rate. Vaccination is suggested to be the major prevention and control measure. The *B. suis* S2 vaccine was widely used to immunize small ruminant livestock orally, and the B. abortus A19 vaccine was administered to large ruminant livestock by subcutaneous injection annually from September to October 2016 until 2020 with more than 90% coverage of vaccination density.

Investigation loci

This study was conducted in four counties located in central Gansu Province, the eastern end of the Hexi Corridor, and the northern part of the Qilian Mountains, among the Huangtu Plateau and Qingzang Plateau of Northwest China, which covers a total area of 32,347 km^2 (Fig. 7) (standard map was downloaded from http://bzdt.ch.mnr.gov.cn/). This area can be divided into three regions: the south Qilian Mountain region, the middle plain oasis region, and the north arid climate region. Meanwhile, a typical representation of farming patterns includes pastoral, agro-pastoral and agriculture regions in this area.

Flock calculation

We constructed a sampling frame containing all the ruminant livestock farms of the four counties. The herd number was calculated considering the assumption of brucellosis of the small and large ruminant flocks with 20% and 30%, respectively. The following formula was used to estimate the herds' number considering the desired precision of 5% and a confidence interval (CI) of 95%. The formula is as follows: $n = P\,(1\text{-}P)\,Z^2/d^2$, where n is the sample size, P is the expected prevalence, Z is 1.96, and d is the absolute precision (Reiczigel et al. 2010).

In addition, the producers' availability to participate voluntarily in the research and distribution in the county should also be considered important factors. Producers were contacted by telephone and offered a certain amount of free flock testing in exchange for the cooperative in the survey. Thus, the number (N) of selected farms was 106, 110, and 121 from 2019 to 2021, respectively, which contained 258 sheep farms, 26 goat farms, 32 cattle farms, and 21 white yak farms. The farms selected were performed as much as possible to cover the main farming area of each county.

Collection of serum samples

The selected animal number of the different farms was confirmed by the herd size. At least 10% of serum samples from the small ruminant flock and 20% from large ruminants were collected for surveying. All samples were collected randomly. The serum sample collection rates in livestock were 14.55%, 29.23%, 23.60% and 23.34% in sheep, goats, cattle and white yak (proportion of the total population), respectively. A total of 11,296 serum samples, including sheep (9669), goats (473), cattle (664), and white yak (490), were collected from April 2019 through June 2021. These samples collected from 4 counties were 3,407 from County L, 3,272 from County M, 1,893 from County G, and 2,724 from County T. The serum samples were selected from animals with an immunization period of 180 days or more to distinguish the antibodies

Fig. 7 Study area and approximate loci for this study

produced by immunization or wild infection. In addition, possible risk factors evaluated on an individual level included time of sample collection, region, species, flock size, animal size, and gender. Possible risk factors evaluated for flocks included time of sample collection, region, species, flock size, and animal size (Elderbrook et al. 2019).

Serological tests

Approximately 5 mL venous blood was extracted and left for 2 h, followed by centrifugation at 3000 rpm for 10 min. All processed serum samples were transferred to the laboratory and stored at -20°C until the test. According to the NBPCP suggestion, the Rose Bengal Plate Test (RBPT) (Cat no: ZC-BRUCELLA-005, QINGDAO SINOVA-HK BIOTECHNOLOGY CO., LTD) was selected as a primary screening method to check specific antibodies to *Brucella* spp. Then, the positive serum samples were reconfirmed by the standard agglutination test (SAT) (Cat no: ZC-BRUCELLA-006, QINGDAO SINOVA-HK BIOTECHNOLOGY CO., LTD). Animals with seropositive results were considered positive, and animals with negative test results were considered negative. Flocks were considered positive, while at least one or more positive animals were confirmed in the flock (McGiven JA et al. 2006).

The flock-level seroprevalence and individual-level apparent seroprevalence were calculated, and the individual-level true seroprevalence was estimated according to the formula (Reiczigel et al. 2010) using a sensitivity of 81% and specificity of 98% (Wang et al. 2016):

$$True\ prevalence = [Apparent\ prevalence + (Specificity - 1)]/[Sensitivity + (Specificity - 1)]$$

95% confidence intervals (CI) were calculated on apparent and estimated true seroprevalence.

Isolation of *Brucella*

A total of 27 positive blood samples (including 23 from sheep, two from goats, one from cattle, and one from white yak) collected in 2021 that tested anti-positive by RBPT and SAT were collected and confirmed by cELISA (Wuhan Keqian Biology Co., Ltd) (Wuhan, China) for *Brucella* isolation. Briefly, 200 µL blood samples were mixed with 0.2% Triton X-100 in equal amounts and then incubated for 10 min. Then, a 200 µL mixture was cultured on *Brucella* selective supplement media composed of medium base (OXOID, England), 5% heat-inactivated horse serum, 1% dextrose, cycloheximide (100 mg/L), vancomycin (20 mg/L), nalidixic acid (5 mg/L), polymyxin B sulfate (5000 IU/L), bacitracin (25,000 IU/L), and nystatin (100,000 IU/L) (Oxoid, UK). Plates were placed in 5-10% CO_2 at 37°C for 3-15 d, and suspected bacterial clones were identified.

Biovar typing and AMOS-PCR

The suspected four bacterial clones were subcultured for purity on tryptic soy agar (TSA) with 5% heat-inactivated horse serum. The isolate was identified based on standard strain identification protocols (Yang et al. 2020). The isolate was primarily tested for modified Ziehl–Neelsen (MZN) and Gram staining, followed by biochemical examination for hydrogen sulfide and urease production, as well as CO_2 and growth ability on specific media plates containing basic thionin and fuchsin. Then, agglutination assays were performed with monospecific antisera: A (Cat no: ZC-BRUCELLA-010), M (Cat no: ZC-BRUCELLA-011), and R (Cat no: ZC-BRUCELLA-009). The reference bacteriophages Tb (Cat no: ZC-BRUCELLA-012) and Bk_2 (Cat no: ZC-BRUCELLA-013) were used for *Brucella* typing according to the instructions (all reagents and reference phages were purchased from QINGDAO SINOVA-HK BIOTECHNOLOGY CO., LTD) (Qingdao, China). A bacterial DNA extraction kit (TIANGEN, Beijing) was selected to extract the total genomic DNA. The purity and concentrations of DNA were detected using a NanoDrop™ 2000 spectrophotometer, and the preparation was stored at -20°C.

To identify *Brucella* spp. at the molecular level, ascertained by a genus-specific *Brucella* PCR assay targeting a 224 bp fragment of BCSP31 and a 628 bp fragment of the OMP25 gene, were performed to ascertain the specific gene according to previous primers and protocols (Sun et al. 2016). The representative strains of *E. coli* ATCC25922 and *B. melitensis* M5-90 were selected as negative and positive controls for the specificity test. Species identification of the isolate was performed by Abortus Melitensis Ovis Suis (AMOS) PCR assay according to the PCR protocols described in previous research (Bricker et al. 1995). Amplification protocols refer to the above procedure. *B. melitensis* M5-90, *B. abortus* A19, and *B. suis* S2 were positive controls for the test. Primers were synthesized at Tsingke Biotechnology Co., Ltd. (Beijing, China).

MLST Genotyping

The confirmed 1 *Brucella* strain was subjected to MLST genotyping analysis. In total, nine specific genes (*dnaK*, *gyrB*, *trpE*, *aroA*, *cobQ*, *gap*, *glk*, *OMP25* and *int-hyp*) were selected for multilocus sequencing typing (MLST) (Whatmore et al. 2007). Genetic sequencing of the PCR products was performed by Tsingke Biotechnology Co., Ltd. (Beijing, China). The MLST online tool (http://pubmlst.org/perl/mlstanalyse/mlstan-alyse.=pubmlst) was used to analyze the alleles.

Genome sequencing and assembly

The *B. melitensis* TZ strain was subjected to whole genome sequencing. Briefly, the PacBio Sequel II and DNBSEQ platforms were used to sequence the genome of *B. melitensis* TZ at the Beijing Genomics Institute (BGI, Shenzhen, China). Four SMRT cells *Zero-Mode Waveguide* sequencing arrays were performed to generate the subread set. PacBio subreads (length < 1000 bp) were removed. The Canu program and GATK (https://www.broadinstitute.org/gatk/) were used for self-correction and making single-base corrections, respectively.

Genome component prediction

Glimmer3 (http://www.cbcb.umd.edu/software/glimmer/) with hidden Markov models was used to predict the genes in the *B. melitensis* TZ genome. Meanwhile, tRNAscan-SE (Lowe et al. 1997), RNAmmer, and Rfam database were selected to identify the tRNA, rRNA and sRNAs. Tandem Repeat Finder (http://tandem.bu.edu/trf/trf.html) was used to analyze the tandem repeat annotation, and microsatellite DNA and minisatellite DNA were chosen by the length and number of repeat units. Genomicis land analysis was performed by the Genomic Island Suite of Tools (GIST) (http://www5.esu.edu/cpsc/bioinfo/software/GIST/) with the IslandPath-DIOMB, SIGI-HMM, and IslandPicker methods. The PHAge Search Tool (PHAST) web server (http://phast.wishartlab.com/) and CRISPRFinder were used to predict the prophage regions.

Gene annotation and protein classification

The blast alignment tool of best hit abstracted was used for gene function annotation. Seven databases, Clusters of Orthologous Groups (COG), Kyoto Encyclopedia of Genes and Genomes (KEGG), Gene Ontology (GO), Non-Redundant Protein Database (NR), TrEMBL, Swiss-Prot and EggNOG, were selected for general function

annotation. The core dataset in Virulence Factors of Pathogenic Bacteria (VFDB), Antibiotic Resistance Genes Database (ARDB), Carbohydrate-Active enZYmes Database, and Comprehensive Antibiotic Resistance Database (CARD) was selected to identify the resistance gene and virulence factors.

Comparative genomics and phylogenetic analysis

B. melitensis bv.1.str.16 M, *B. melitensis* bv.2.str.63–9, *B. melitensis* bv.3.str. Ether, *B. melitensis* NI, and *B. melitensis* QH61 were selected to construct the gene family by multisoftware. The phylogenetic tree was constructed by TreeBeST using the NJ method (http://treesoft.svn.sourceforge.net/viewvc/treesoft/trunk/treebest/).

In vitro growth assay and cell infection assay

To determine the growth kinetics of *B. melitensis* TZ, the growth curve was compared with the reference strain *B. melitensis* 16 M and representative strain *B. melitensis* M5-90 in TSB. Cultures were diluted tenfold in fresh TSB medium into three replicates when OD600 = 0.1 cultured at 37°C at 180 rpm. Aliquots of these cultures were taken, and their OD_{600} absorbance was measured every 4 h for 72 h.

To assess the intracellular survival of *B. melitensis* TZ, RAW264.7, HeLa, and THP-1 cells were cultured in cell culture medium containing 10% fetal bovine serum (FBS) (Gibco®, USA) at 37°C under an atmosphere of 5% CO_2. Cells were numbered and seeded on 24-well plates at a density of 5×10^4 cells per well for 24 h before the infection assay. The three *Brucella* strains (*B. melitensis* 16 M, *B. melitensis* M5-90, and *B. melitensis* TZ) were grown in TSB for 24 h and diluted in cell culture medium. A multiplicity of infection (MOI) of 100:1 was used to infect RAW264.7 and THP-1 cell lines and 500:1 for HeLa cells (Larsen et al. 2013). Then, the 24-well plates were centrifuged at $400 \times g$ for 7 min and incubated at 37°C for 1 h.

The infected cells were processed with PBS and incubated for 1 h to kill extracellular bacteria with medium containing 50 μg/mL gentamicin. The cell samples were lysed with 0.2% Triton X-100 for 10 min and then plated on TSA plates to account for the live bacteria at 2, 8, 24, and 48 h post-infection (PI.). All the cell infection assays were presented as the averages from triplicate infected samples from an independent experiment.

Statistical analysis

Data were transferred to Microsoft Excel (Microsoft Excel 2010, Redmond, USA). The apparent and estimated true seroprevalence and 95% confidence intervals (CI) were calculated using Wilson-Score intervals by statistical software SPSS 22.0, and graphics were generated using GraphPad Prism 7.0. Statistical analysis of bacterial growth characteristics in TSB and survival ability in macrophages were expressed as OD_{600} and the mean log CFU ± the standard deviation (SD), respectively. Student's unpaired *t* test was used for statistical analysis. The differences between groups were analyzed by analysis of variance (ANOVA) followed by Tukey's honestly significant difference posttest. $P<0.05$ and $P<0.01$ were set as the thresholds for statistical significance. The significance is indicated by an asterisk in the figures (*, $P<0.05$. **, $P<0.01$).

Abbreviations

OIE	World Organization for Animal Health
CDC	China Center for Disease Control
NBPCP	National Brucellosis Prevention and Control Plan
RBT	Rose Bengal Plate Test
SAT	Standard Agglutination Test
MLST	Multilocus Sequencing Typing
PCR	Polymerase Chain Reaction
TAE	Tris-acetic Acid-EDTA
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
CFU	Colony forming unit
MOI	Multiplicity of Infection
P	Probability Value
Rpm	Revolutions Per Minute
PBS	Phosphate-buffered saline
PI	Postpost-infection

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Not applicable.

Authors' contributions

LZF and ZJX conceived and designed the project. FYP and WJJ performed the epidemiology survey; ZGY, ZFD, GCY, ZJD, GKX and DY conducted the isolation and cell infection assay, HCC provided materials. FYP and LZF processed and analyzed data and wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

Investigation data used in the study are available on reasonable request from the corresponding author (E-mail: lzf6789@mail.hzau.edu.cn).

Declarations

Ethics approval and consent to participate

Not applicable.

Competing interests

The authors declare no competing interests. Author Huanchun Chen was not involved in the journal's review or decisions related to this manuscript.

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