Research Square

Preprints are preliminary reports that have not undergone peer review.
They should not be considered conclusive, used to inform clinical practice,
or referenced by the media as validated information.

Construction and Evaluation of a Reference Strain System for Brucella melitensis Biovar 2 and Genomic Characteristic Analysis

Wenqiang Yang

917461182@qq.com

Zhuhai Center for Disease Control and Prevention

Jiahan Wu

Zhuhai Center for Disease Control and Prevention

Yanmei Fang

Zhuhai Center for Disease Control and Prevention

Xuling Liu

Zhuhai Center for Disease Control and Prevention

Ying Cui

Zhuhai Center for Disease Control and Prevention

Changwen Ke

Guangdong Provincial Center for Disease Control and Prevention

Shigang Yao

Zhuhai Center for Disease Control and Prevention

Jie Wu

Zhuhai Center for Disease Control and Prevention

Huitao Huang

Zhuhai Center for Disease Control and Prevention

Yixiong Lin

Zhuhai Center for Disease Control and Prevention

Xiaoling Deng

Guangdong Provincial Center for Disease Control and Prevention

Wenyan Huang

Zhuhai Center for Disease Control and Prevention

Research Article

Keywords: B. melitensis, reference strain, strain evaluation, genomic characteristics

Posted Date: October 28th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-10986830/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License

Additional Declarations: No competing interests reported.

Abstract

Objective To establish a candidate national reference strain of *Brucella melitensis* biovar 2 according to relevant technical standards using a *Brucella* strain preserved in the local biological sample repository in Zhuhai City.

Methods In accordance with the Technical Evaluation Standard for National Pathogenic Microorganism Strains (WS/T 812–2022), morphological identification of the candidate Brucella strain was performed via colony observation, Gram-staining and transmission electron microscopy. Species-level identification was carried out using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS), biochemical assays, serum agglutination tests and phage lysis assays. Whole-genome sequencing was applied for biovar typing, virulence- and resistance-associated gene profiling. Physiological characteristics were further characterized by antimicrobial susceptibility testing and strain stability assessment.

Results Strain ZH25-Bru-002 (abbreviated as ZH25 throughout the text) was a Gram-negative bacillus. It grew well on modified Brucella agar and blood agar at 37°C, with cell dimensions of (0.3–0.4) μm × (0.6–0.8) μm. MALDI-TOF-MS identified it as *B. melitensis* with a score of 2.366. The strain was positive for oxidase, catalase and urease, and negative for hydrogen sulphide production. Plaque formation was observed in the Bk-phage lysis test. Agglutination titer for serum A was higher than that for serum M. Among the 12 tested antibiotics, the strain was resistant only to erythromycin (MIC > 32 μg/mL). Whole-genome sequencing together with 16S rRNA and *rpoB* gene typing confirmed its identity as *B. melitensis* biovar 2. Following serial in-vitro passaging to obtain the 1st-generation (G1), 5th-generation (G5), and 10th-generation (G10) derivatives of ZH25, colony morphology, biochemical profiles and biovar features remained stable. The strain has been deposited in the Pathogenic Microorganism Repository of Guangdong Provincial Center for Disease Control and Prevention and assigned a national preservation accession number.

Conclusion The *B. melitensis* biovar 2 strain obtained in this study exhibits typical phenotypic and genotypic features of the pathogen. It qualifies as a reference strain for brucellosis surveillance, vaccine evaluation and diagnostic reagent development. The strain has passed review by the national repository and obtained the official preservation number NPRC(S) 01.0114.

Introduction

Brucella spp. are facultative intracellular Gram-negative coccobacilli. They are the causative agents of brucellosis, a Category B notifiable infectious disease in China as stipulated by the Law of the People's Republic of China on the Prevention and Treatment of Infectious Diseases. Brucella represents one of the most widespread and harmful zoonotic pathogens worldwide. To date, 12 classical species have been identified within the genus Brucella. Among them, *B. melitensis* exhibits the highest virulence to humans. It predominates in human brucellosis globally and constitutes the overwhelmingly dominant species associated with brucellosis epidemics in China[1, 2]. It has been reported that more than 500

000 new human brucellosis cases occur annually worldwide, imposing a substantial burden on public-health systems across countries[3]. In livestock, brucellosis induces abortion, stillbirth and impaired reproductive performance in ruminants such as cattle, goats and sheep. According to the Food and Agriculture Organization of the United Nations (FAO), brucellosis causes an estimated annual economic loss of 200 million to 600 million US dollars in animal husbandry. This pathogen therefore poses multifaceted threats to public-health security, livestock-industry development and socioeconomic stability[4].

In China, historical brucellosis foci were largely confined to traditional pastoral regions in northern provinces. Nevertheless, over the past two decades, brucellosis has exhibited a distinct spatiotemporal shift characterized by the southward spread of the disease[5–7]. The annual growth rate of reported human brucellosis cases in non-pastoral southern provinces has reached 31.5%, with continuously expanding endemic areas, which poses a new challenge to national brucellosis control[8]. As a key hub for cross-provincial circulation of livestock products in southern China and a core component of the Guangdong-Hong Kong-Macao Greater Bay Area, Guangdong Province has witnessed an epidemiological transition in recent years: brucellosis is no longer predominantly imported, but presents a mixed pattern of imported cases and local sporadic infections, with locally-acquired cases increasing year-on-year. *B. melitensis* is the dominant circulating species in this province[9].Internationally accepted reference strains of *B. melitensis*, such as 16M and ATCC 23457, were originally isolated from European and American foci. Their genetic backgrounds differ substantially from indigenous strains circulating in southern China, limiting their suitability as national reference materials for local brucellosis prevention and control. Zhuhai, located on the western bank of the Pearl River Estuary, is a densely-connected node city within the Greater Bay Area with intensive human and commodity mobility. Reported human brucellosis cases have kept rising since 2010, and seroprevalence among high-risk livestock-exposed workers peaks at 9.2%[10]. *B. melitensis* accounts for most local clinical infections and places substantial pressure on regional public-health preparedness.

Based on the Technical Evaluation Standard for National Pathogenic Microorganism Strains (WS/T 812-2022), this study performed systematic identification and characterization of a *B. melitensis* biovar 2 strain preserved in the Zhuhai biological sample repository. The establishment of this reference strain provides indigenous core reference materials for pathogen-based surveillance of brucellosis, development of clinical diagnostic reagents, and evaluation of vaccine immunogenicity in southern China.

1 Materials and Methods

1.1 Strain information The candidate reference strain ZH25 used in this study was obtained from the preserved strain stock in the biorepository of Zhuhai Center for Disease Control and Prevention. *B. melitensis* is classified as a Group 2 pathogenic microorganism under the Regulations on the Administration of Biosafety of Pathogenic Microorganism Laboratories. All live-bacterial manipulations and cultivation procedures in this work were performed in a biosafety level-3 (BSL-3) laboratory.

1.2 Bacterial culture, serial passage and storage The original bacterial suspension was transported to the BSL-3 laboratory and streaked onto blood agar or modified Brucella agar medium with an inoculating loop, followed by incubation at 37 °C for 48–72 h. Single colonies were picked to obtain the primary stock, designated generation 1 (G1). Each subsequent round of single-colony streaking represented one additional generation, so that the next passage was recorded as generation 2 (G2). Serial in-vitro passaging was performed following this rule. Bacterial lawns from each generation were harvested, resuspended in 30% (v/v) sterile glycerol solution, and stored at −80 °C.

1.3 Observation of colony morphology The test strain was inoculated onto modified Brucella agar and blood agar, respectively, and incubated at 37 °C for 48 h and 72 h. Colony morphologies on different culture media were examined at the two incubation time-points.

1.4 Gram-staining Pure bacterial cultures were inactivated with 2.5 % glutaraldehyde for 10 min before being transferred out of the biosafety level-3 laboratory. Subsequent staining procedures were performed in a biosafety level-2 laboratory.

1.5 Electron-microscopic examination Six millilitres of bacterial suspension adjusted to 1.0 McFarland turbidity was inactivated using 2.5 % glutaraldehyde. Specimens were prepared and examined by transmission and scanning electron microscopy. All electron-microscopy work was completed by Wuhan Servicebio Technology Co., Ltd.

1.6 MALDI-TOF-MS identification Bacterial suspension adjusted to 1.0 McFarland turbidity was prepared. A volume of 250 µL suspension was thoroughly mixed with 750 µL absolute ethanol, left at room temperature for 5 min, and then transferred out of the BSL-3 laboratory. Detection and quality control were performed following the manufacturer's operating manual for the MALDI-TOF-MS instrument. Fresh single colonies grown on blood agar were sampled with a sterile inoculating loop, and a small amount of bacterial lawn was spotted onto the target plate. Each spot was overlaid with 1 µL of HCCA matrix solution and air-dried at room temperature prior to measurement. Species-level identification was assigned for scores ≥2.0; genus-level identification for scores ranging from 1.7 to 2.0; results with scores below 1.7 were regarded as unreliable.

1.7 Phage lysis assay The test strain was adjusted to 3.3 McFarland turbidity. A 0.1 mL aliquot of the bacterial suspension was mixed gently with molten modified Brucella semisolid agar maintained in a water-bath at 52 °C, and the mixture was poured onto a solidified modified Brucella agar base layer. Once the semisolid agar had solidified, 8 µL each of Tb and Bk phage suspensions were spotted at separate positions on the agar surface. After the phage droplets dried, plates were incubated at 37 °C, and experimental outcomes were read at 48 h.

1.8 Biochemical assays Biochemical characterization consisted of oxidase, catalase, urease and hydrogen sulphide tests, which were performed according to the manufacturer's instructions for the commercial kit.

1.9 Serum agglutination test Reaction zones for serum A and serum M were marked on disposable plates. Twenty-microlitre aliquots of serum A and serum M were separately added into designated zones. Bacterial suspension was prepared in normal saline, and aliquots were mixed with each serum using a disposable inoculating loop. Positive agglutination was defined as visible clumping within 2 min; reactions without clumping were recorded as negative.

1.10 Antimicrobial susceptibility testing Susceptibility testing was carried out using customized microbroth dilution panels for Gram-negative aerobic bacteria (E-Brucella, Fosun Diagnostics, Changsha), following the product instructions. Turbidity indicated bacterial growth (positive), whereas a clear well denoted growth inhibition (negative). Minimum inhibitory concentration (MIC) values of the test strain against each antibiotic were determined. *Escherichia coli* ATCC 25922 served as the quality-control strain, and all quality-control results met acceptable criteria.

1.11 Whole-genome sequencing Next-generation sequencing was performed using Illumina DNA Prep, Tagmentation kits and the NextSeq 550AR platform. Raw sequencing data were processed with the Weiweilai Pathogen Bioinformatics Analysis System (Beijing, China) to generate the complete genomic sequence, together with genomic metrics including genome size and GC content. Phylogenetic trees were constructed based on whole-genome sequences, 16S rRNA and *rpoB* gene sequences. Genotyping was conducted via the MLST-2.0 Server hosted on the CGE website. Housekeeping genes were analysed using the MLST-2.0 scheme, and sequence identity of housekeeping genes was compared between ZH25 and its serial-passage derivatives G1, G5 and G10.

1.12 Genomic comparison and variant analysis Comparative genomic analysis was performed on strain ZH25 together with three reference *Brucella* genomes, including 16M, ATCC 23457, and Ether. Average nucleotide identity (ANI) between every pair of genomes was calculated using skani v0.3.2 and fastANI v1.34 for cross-validation. Average amino-acid identity (AAI) was computed based on bidirectional best-hit protein alignments implemented in mmseqs2. Protein-coding genes were predicted by pyrodigal. The pan-genome was constructed with mmseqs2 under thresholds of 70% amino-acid identity, 70% sequence coverage, and an e-value ≤ 1e-5. Using the ZH25 genome as the reference, genome-wide SNPs and InDels were identified via nucmer and show-snps modules within the MUMmer package. The delta-filter program with parameters -i 90 -l 100 was applied to retain high-quality aligned regions for reliable SNP calling, and equivalent filtering criteria were used for InDel variants. Functional annotation of variant effects was conducted using SnpEff with the GenBank-formatted annotation file of ZH25.

2 Results

2.1 Colony-morphology observation

On blood agar, small colourless and transparent colonies appeared after 48 h incubation at 37°C (Fig. 1A). Following 72 h incubation, colonies turned grey-white with no haemolysis (Fig. 1B). On

modified Brucella agar, typical circular colonies with colourless-translucent appearance, smooth surface and slight central elevation developed at both 48 h and 72 h post-incubation at 37°C (Fig. 1C, D).

2.2 Staining and electron-microscopic morphology

Strain ZH25 showed Gram-negative staining and presented as short bacilli with rounded ends; occasional bipolar staining was observed (Fig. 2A). Under transmission and scanning electron microscopy, the organism exhibited coccobacillary morphology with rounded terminals. No obvious spores, capsules or flagella were detected. Cell dimensions were (0.3–0.4) μm × (0.6–0.8) μm (Fig. 2B, C).

2.3 Biochemical characteristics

The strain yielded positive reactions for oxidase, catalase and urease, whereas hydrogen sulphide production was negative (Fig. 3A-D).

2.4 MALDI-TOF-MS identification

MALDI-TOF-MS analysis returned an identification score of 2.366, assigning the strain to *B. melitensis*(Supplementary Fig. 1).

2.5 Phage lysis and serum agglutination assays

The strain was negative in the Tb-phage lysis test but positive for Bk-phage lysis, indicating that ZH25 belonged to smooth-type *Brucella* and matched the phage-lysis profile of B. melitensis (Fig. 4 AC). A higher agglutination titre was observed with serum A compared with serum M, consistent with the serological pattern (A > M) of B. melitensis biovar 2 (Fig. 4 BC).

2.6 Antimicrobial susceptibility results

Susceptibility profiles were interpreted in accordance with guidelines issued by the Clinical and Laboratory Standards Institute (CLSI). Consistent results were obtained across three independent replicates, and quality-control strain Escherichia coli ATCC 25922 fell within acceptable susceptibility ranges. Strain ZH25 was susceptible to levofloxacin, rifampicin, doxycycline, streptomycin, co-trimoxazole, tetracycline, ciprofloxacin, ceftriaxone, gentamicin, ampicillin and amikacin. It exhibited a resistant phenotype exclusively to erythromycin (MIC > 32 μg/mL).

2.7 Whole-genome sequencing and genotyping analysis

Strains G1, G5 and G10 all possessed a genome size of 3.28 Mb with no plasmids detected. These strains belonged to sequence type ST8 and had a GC content of 57.25 mol%. The 16S rRNA gene was 1485 bp in length, and NCBI database alignment confirmed its affiliation to the genus Brucella. Using the *rpoB* gene of *B. melitensis* biovar 2 strain ATCC 23457 as the reference, phylogenetic analysis placed

ZH25 within the same clade as ATCC 23457, confirming that ZH25 is *B. melitensis* biovar 2 (Supplementary Fig. 2).

2.8 Strain stability

Strains G1, G5 and G10 were resuscitated and cultured on modified Brucella agar plates at 37°C for 72 h. All strains produced colourless, translucent, circular, smooth colonies with slightly raised centres (Supplementary Fig. 3). G1, G5 and G10 were positive for oxidase, catalase and urease, whereas hydrogen sulphide production was negative (Supplementary Fig. 4). Mass-spectrometry identification assigned the three strains to *B. melitensis*, with identification scores of 2.512, 2.508 and 2.565, respectively. Antimicrobial susceptibility was interpreted in accordance with CLSI guidelines. Three independent replicate assays yielded consistent results, demonstrating that all serial passages were resistant only to erythromycin. Whole-genome sequencing revealed that G1, G5 and G10 possessed identical genome sizes of 3.28 Mb with no detectable plasmids, and shared a GC content of 57.25 mol%. The 16S rRNA gene sequences of the three strains exhibited 100.00% identity (Fig. 5A). Average nucleotide identity (ANI) analysis yielded pairwise values of 100.00% for G1 versus G5, G1 versus G10, and G5 versus G10 (Fig. 5B). Sequence alignment of nine housekeeping genes (*aroA*, *cobQ*, *dnaK*, *gap*, *glk*, *gyrB*, *Int_hyp*, *omp25* and *trpE*) showed 100% sequence identity across the three strains (Fig. 5C). Core-genome single-nucleotide polymorphism (cgSNP) phylogenetic analysis against representative domestic and international *Brucella* strains placed G1, G5 and G10 within a single phylogenetic clade (Fig. 5D). Sequence-type (ST) assignment performed via the CGE web server identified all three strains as ST8.

2.9 Virulence and antimicrobial resistance gene profiling

Whole-genome analysis of antimicrobial resistance genes revealed that the reference strain ZH25 harboured 43 resistance-associated genes spanning multiple antibiotic classes (Fig. 6A). Macrolide resistance genes were the most abundant, represented by the RND-family efflux pump genes *bepC*, *bepD*, *bepE*, *bepF* and *bepG*. The strain also harboured *mprF*, yielding a resistome profile consistent with its observed erythromycin resistance phenotype. The strain harboured an extensive set of virulence factors functionally grouped into four classes: secretion systems (T4SS effectors and regulators included), invasion-related factors, adhesion-related factors, and additional virulence regulators (Fig. 6B). Genes for secretion systems, chiefly the type IV secretion system (T4SS), the primary virulence determinant of *Brucella*, represented the largest fraction of these virulence-associated genes. These included the T4SS structural genes *virB1–virB12*, the transcriptional regulator *vjbR*, the TIR-domain-containing effector proteins *btpA* and *btpB*, and the endoplasmic reticulum stress-inducing factor *vceC*, among other key virulence determinants.

2.10 Comparative genomic and variant-site analysis

Pan-genome analysis performed at a 70% identity threshold identified a total of 3 171 gene clusters in reference strain ZH25, consisting of 3 042 core-genome clusters (95.9%), 48 soft-core clusters, 12 shell-

genome clusters and 69 cloud-genome clusters. No strain-specific unique genes were detected in ZH25, which is consistent with the well-documented high genomic conservation within the genus *Brucella* (Fig. 7A). Allelic assignment of ZH25Bru002 was performed across 385 biovar-2-specific SNP sites, where 16M = Ether ≠ ATCC 23457. This strain harbored the bv2 reference (ATCC 23457) allele at 347 sites (90.1%), and the bv1/bv3 reference (16M/Ether) allele at the remaining 38 sites (9.9%) (Fig. 7B). A total of 248 high-quality SNPs were retrieved across the whole genome, comprising 75 synonymous mutations, 119 missense mutations, three nonsense mutations and 51 intergenic SNPs (Fig. 7C). Average nucleotide identity (ANI) between ZH25 and ATCC 23457 reached 99.99%, indicating extremely close genomic relatedness (Fig. 7D). The ZH25 assembly achieved a BUSCO completeness score of 124/124 (100%) (Fig. 7E), demonstrating near-perfect assembly quality that meets the criteria for a high-quality reference genome. Furthermore, 52 tRNA elements were predicted in ZH25 (Fig. 7F); both the copy number and structural features of these genetic elements were consistent with those of the type reference strain.

No mutations were detected within the rifampicin-resistance-determining region (RRDR, residues 517–543) of *RpoB* (1377 aa), and the complete *rpo*B gene sequence of ZH25 was identical to that of the biovar 2 type strain ATCC 23457 (Fig. 8A). Relative to *B. melitensis* strains of other biovars (biovar 1 strain 16M and strain Ether), only three non-critical amino-acid substitutions were identified (aa629, aa985, aa1249); none mapped to the RRDR or to functional domains linked to rifampicin resistance (Fig. 8B).

3 Discussion

Reference strains of *Brucella* represent critical biological resources for brucellosis pathogen surveillance, diagnostic reagent development and vaccine-efficacy evaluation. In this study, systematic phenotypic, antimicrobial-susceptibility and genomic characterizations were performed on a *B. melitensis* strain preserved in the biorepository of Zhuhai Center for Disease Control and Prevention. Biological properties, growth performance and genetic stability were validated in compliance with the national strain-evaluation standard WS/T 812–2022. Multi-dimensional characterization confirmed that reference strain ZH25 exhibits typical phenotypic features of *B. melitensis* biovar 2, with stable traits following serial passages. This strain has passed review by the National Culture Collection Center and obtained the official preservation certificate for national reference strains. It can serve as a national reference strain for *B. melitensis* biovar 2 in China.

Morphology, staining profiles, growth patterns, biochemical reactions, serological agglutination and phage-lysis phenotypes of this strain were all consistent with the taxonomic hallmarks of *B. melitensis*, in agreement with previous phenotypic observations of Chinese *Brucella* isolates[11, 12]. MALDI-TOF-MS identification yielded species-level scores above the cut-off value across all passages, supporting reliable species assignment. Slightly lower scores were obtained for the primary-culture strain relative to passaged derivatives. This discrepancy was presumed to stem from heterogeneous cell populations in primary cultures and batch-to-batch variation in protein extraction, yet it did not compromise species

identification. Molecular identification based on whole-genome sequencing and *rpoB* genotyping further confirmed that ZH25 belongs to *B. melitensis* biovar 2, verifying concordance between conventional phenotypic assays and molecular subtyping[13, 14]. Antimicrobial-susceptibility testing demonstrated that reference strain ZH25 was highly susceptible to first-line clinical agents including doxycycline, rifampicin and streptomycin, and exhibited intrinsic resistance only to erythromycin. This resistance profile matches those reported for most domestic and international *B. melitensis* isolates[15, 16].

The reference strain ZH25 possesses a bipartite-chromosome genome of approximately 3.28 Mb with a GC content of ~ 57%, whose basic genomic features are consistent with those of the well-characterized reference strain 16M[17]. Genomic analyses revealed high intraspecies conservation within *B. melitensis*. The core-genome fraction of ZH25 reached 95.9%, and no strain-specific novel genes were detected, which aligns with findings from previous comparative-genomic studies on *Brucella*[18]. Sequence-type assignment placed ZH25 within ST8, the predominant circulating lineage of *B. melitensis* in China. This observation is concordant with epidemiological data reported from Sichuan regions of China[19]. Resistome analysis indicated that ZH25 remained susceptible to first-line anti-brucellosis therapeutics including doxycycline, rifampicin and streptomycin, reflecting the baseline susceptibility profile typical of *B. melitensis*[15].

In China, *B. melitensis* biovar 1 and biovar 3 constitute the predominant circulating biovars, whereas reports of biovar 2 isolates remain scarce[19, 20]. Based on 90.1% sequence concordance at biovar-2-specific SNP loci, the present study precisely defined a native Chinese biovar 2 isolate at the genomic level and developed it as a reference strain, filling the gap in domestic native reference genomes for *B. melitensis* biovar 2. Genomic comparison revealed extremely high genetic relatedness between ZH25 and the biovar-2 type strain ATCC 23457, while substantial genetic divergence was observed relative to biovar-1 reference strain 16M. These findings indicate that genetic divergence across biovars of *B. melitensis* markedly exceeds intra-biovar strain-to-strain variation[20, 21]. Previous studies have demonstrated that the overall genome of *B. melitensis* is highly conserved. Biovar-specific traits are not driven by large-scale genomic rearrangements, but are largely determined by discrete variations at a small set of key LPS functional sites, such as those governing O-antigen polysaccharide epitopes[22, 23].

Furthermore, whole-genome resistome annotation identified multiple categories of antimicrobial-resistance-associated genes in ZH25. These were predominantly chromosomal intrinsic efflux-pump and regulatory genes, with no canonical horizontally-acquired resistance determinants detected. This pattern aligns with the intrinsic-resistance genetic background characteristic of *B. melitensis*[16, 24]. Genes linked to macrolide resistance exhibited the highest abundance, which correlates well with the reduced-susceptibility phenotype against erythromycin observed in phenotypic susceptibility assays[25]. Existing evidence indicates that diminished susceptibility to macrolides (erythromycin, azithromycin) represents a common intrinsic trait among clinical *B. melitensis* isolates in China. Stable carriage of these intrinsic resistance genes constitutes the core genetic basis for macrolide tolerance in such strains[16]. No critical rifampicin-resistance-conferring mutations were identified within the *rpoB* gene of ZH25, and no

complete acquired-resistance genes were retrieved, pointing to a low potential risk for drug resistance. This distinguishes ZH25 from previously described *Brucella* isolates harbouring resistance-associated mutations[13, 14]. The complete susceptibility of ZH25 to first-line agents, together with the absence of acquired resistance determinants and of *rpoB* resistance-associated mutations, was confirmed by phenotypic susceptibility testing (three independent replicates; CLSI criteria), indicating a low risk of drug resistance in this national standard strain.

This study accomplished the systematic evaluation of a native southern-China *B. melitensis* biovar 2 reference strain, which has passed national culture-collection review and obtained an official preservation certificate. This strain fills the gap in certified reference materials for *B. melitensis* biovar 2 in China. It provides a locally-sourced reference for regional brucellosis pathogen surveillance, diagnostic reagent development, vaccine efficacy assessment and fundamental pathogenic research. It also bears substantial practical value for enriching China's national *Brucella* reference-strain repository and supporting precise brucellosis prevention and control.

Declarations

Acknowledgments

We sincerely thank all colleagues. W.Y., J.W., Y.F., C.K., J.W., W.H., H.H., designed and supervised the study. W.Y., J.L., X.L., S.Y., L.D., H.H., conducted the experiment enrolled in this study. W.Y., J.W., Y.C., Y.L., analyzed the data and drafted the manuscript. All authors contributed to revising, reading, and approving the submitted version.

This work was supported by the Zhuhai Municipal Medical Science Project (No. 2520009000023).

Ethics approval and consent to participate

This study only used the standard reference bacterial strain of *Brucella melitensis* biovar 2. No-human participants, human tissues or human clinical samples were involved in this research. Therefore, ethics approval and informed consent to participate were not required. Since no human-related experiments were performed, the Declaration of Helsinki was not applicable to this work.

Data Availability Statement

The whole-genome sequence generated in this study for the *Brucella melitensis* biovar 2 reference strain has been deposited in GenBank under the accession number [Accession number JCDVFA000000000]. All data generated and analyzed in this study are available within the published article. No additional raw data are deposited in public repositories.

Consent for publication

Not applicable.

Conflicts of interest

No conflicts of interest.

About the Author

Dr. Wenqiang Yang is engaged in public-health-related work at Zhuhai Center for Disease Control and Prevention. He focuses on pathogen testing in daily routine work. His research interests include population-based monitoring as well as prevention-and-control strategies for newly emerging infectious diseases.

References

1. Liu Z, Wang M, Shi Y, Wang L, Ren X, Li Z, Zheng C. Epidemiological Evolution Profile of Human Brucellosis and Socioeconomic Factor Correlation Analysis - Southern and Northern Areas, China, 1950–2021. China CDC Wkly. 2025;7(14):460–6.
2. Tan Q, Wang Y, Liu Y, Tao Z, Yu C, Huang Y, Yang X, Ying X, Hu Y, Li S. Molecular epidemiological characteristics of Brucella in Guizhou Province, China, from 2009 to 2021. Front Microbiol. 2023;14:1188469.
3. Rubach MP, Halliday JE, Cleaveland S, Crump JA. Brucellosis in low-income and middle-income countries. Curr Opin Infect Dis. 2013;26(5):404–12.
4. Ahad AA, Megersa B, Edao BM. Brucellosis in camel, small ruminants, and Somali pastoralists in Eastern Ethiopia: a One Health approach. Front Vet Sci. 2024;11:1276275.
5. Zhong Z, Yu S, Wang X, Dong S, Xu J, Wang Y, Chen Z, Ren Z, Peng G. Human brucellosis in the People's Republic of China during 2005–2010. Int J Infect Dis. 2013;17(5):e289–92.
6. Kong W. Brucellosis infection increasing in Southern China. Eur J Intern Med. 2018;51:e16–8. 10.1016/j.ejim.2018.03.004. . Epub 2018 Mar 15.
7. Zhu X, Zhao Z, Ma S, Guo Z, Wang M, Li Z, Liu Z. Brucella melitensis, a latent travel bacterium, continual spread and expansion from Northern to Southern China and its relationship to worldwide lineages. Emerg Microbes Infect. 2020;9(1):1618–27.
8. Zhou S, Qin H, Shi Q, Li S, Chen J, Chen Q. Changing Patterns of Epidemiological Characteristics and Spatial-Temporal Clusters of Human Brucellosis Based on County Level - China, 2011–2023. China CDC Wkly. 2025;7(14):453–9.
9. Ye HY, Xing FF, Yang J, Lo SK, Lau RW, Chen JH, Chiu KH, Yuen KY. High index of suspicion for brucellosis in a highly cosmopolitan city in southern China. BMC Infect Dis. 2020;20(1):22.
10. Yong-Yu Z, Ban-Qun Z, Heng-Qiu Z. Analysis on Epidemiological Characteristics of Brucellosis Among Occupational Populations in Zhuhai City in 2010–2012. Practical Prev Med 2013.
11. Di Bonaventura G, Angeletti S, Ianni A, Petitti T, Gherardi G. Microbiological Laboratory Diagnosis of Human Brucellosis: An Overview. Pathogens. 2021;10(12):1623.

12. WS 269–2019. Diagnosis for brucellosis. Natl Health Comm People's Repub China 2019.01.02.
13. Minghui L, Zhiguo L, Dongri P, Hongyan Z, Guozhong T, Hai J. Genetic polymorphism analysis of rpoB gene of *B. melitensis* strains isolated in China. Disease Surveillance 2018.
14. Marianelli C, Ciuchini F, Tarantino M, Pasquali P, Adone R. Molecular characterization of the rpoB gene in Brucella species: new potential molecular markers for genotyping. Microbes Infect. 2006;8(3):860–5.
15. Turkmani A, Ioannidis A, Christidou A, Psaroulaki A, Loukaides F, Tselentis Y. In vitro susceptibilities of Brucella melitensis isolates to eleven antibiotics. Ann Clin Microbiol Antimicrob. 2006;5:24.
16. Ma HR, Xu HJ, Wang X, Bu ZY, Yao T, Zheng ZR, Sun Y, Ji X, Liu J. Molecular characterization and antimicrobial susceptibility of human Brucella in Northeast China. Front Microbiol. 2023;14:1137932.
17. He Y. Analyses of Brucella pathogenesis, host immunity, and vaccine targets using systems biology and bioinformatics. Front Cell Infect Microbiol. 2012;2:2.
18. Anbazhagan S, Himani KM, Karthikeyan R, Prakasan L, Dinesh M, Nair SS, Lalsiamthara J, Abhishek, Ramachandra SG, Chaturvedi VK, Chaudhuri P, Thomas P. Comparative genomics of Brucella abortus and Brucella melitensis unravels the gene sharing, virulence factors and SNP diversity among the standard, vaccine and field strains. Int Microbiol. 2024;27(1):101–11.
19. Li W, Zeng L, Yuan R, Qi T, Liao H, Cao Y, Huang S, Liu Z, Li Z. Genetic diversity atlas of Brucella melitensis strains from Sichuan Province, China. BMC Microbiol. 2025;25(1):21.
20. Huang L, Zhou L, Zhang N, Zhou W, Xu B, Hong J, Zhang W, Zhang Y, Xu K, Bao C, Jiang H, Tan Z, Li J. Limited genomic diversity and convergent adaptation of Brucella melitensis isolated from human in East China, from 2011 to 2024. Front Microbiol. 2025;16:1663555.
21. Vergnaud G, Hauck Y, Christiany D, Daoud B, Pourcel C, Jacques I, Cloeckaert A, Zygmunt MS. Genotypic Expansion Within the Population Structure of Classical Brucella Species Revealed by MLVA16 Typing of 1404 Brucella Isolates From Different Animal and Geographic Origins, 1974–2006. Front Microbiol. 2018;9:1545.
22. Bundle DR, McGiven J. Brucellosis: Improved Diagnostics and Vaccine Insights from Synthetic Glycans. Acc Chem Res. 2017;50(12):2958–67.
23. Stranahan LW, Arenas-Gamboa AM. When the Going Gets Rough: The Significance of Brucella Lipopolysaccharide Phenotype in Host-Pathogen Interactions. Front Microbiol. 2021;12:713157.
24. Dadar M, Alamian S, Brangsch H, Elbadawy M, Elkharsawi AR, Neubauer H, Wareth G. Determination of Virulence-Associated Genes and Antimicrobial Resistance Profiles in Brucella Isolates Recovered from Humans and Animals in Iran Using NGS Technology. Pathogens. 2023;12(1):82.
25. Ayoub H, Kumar MS, Mehta R, Thomas P, Dubey M, Dhanze H, Ajantha GS, Bhilegaonkar KN, Salih HM, Cull CA, Veeranna RP, Amachawadi RG. Exploring genetic determinants of antimicrobial resistance in Brucella melitensis strains of human and animal origin from India. Front Microbiol. 2024;15:1474957.

26. Marianelli C, Ciuchini F, Tarantino M, Pasquali P, Adone R. Genetic bases of the rifampin resistance phenotype in Brucella spp. J Clin Microbiol. 2004;42(12):5439–43.

Figures

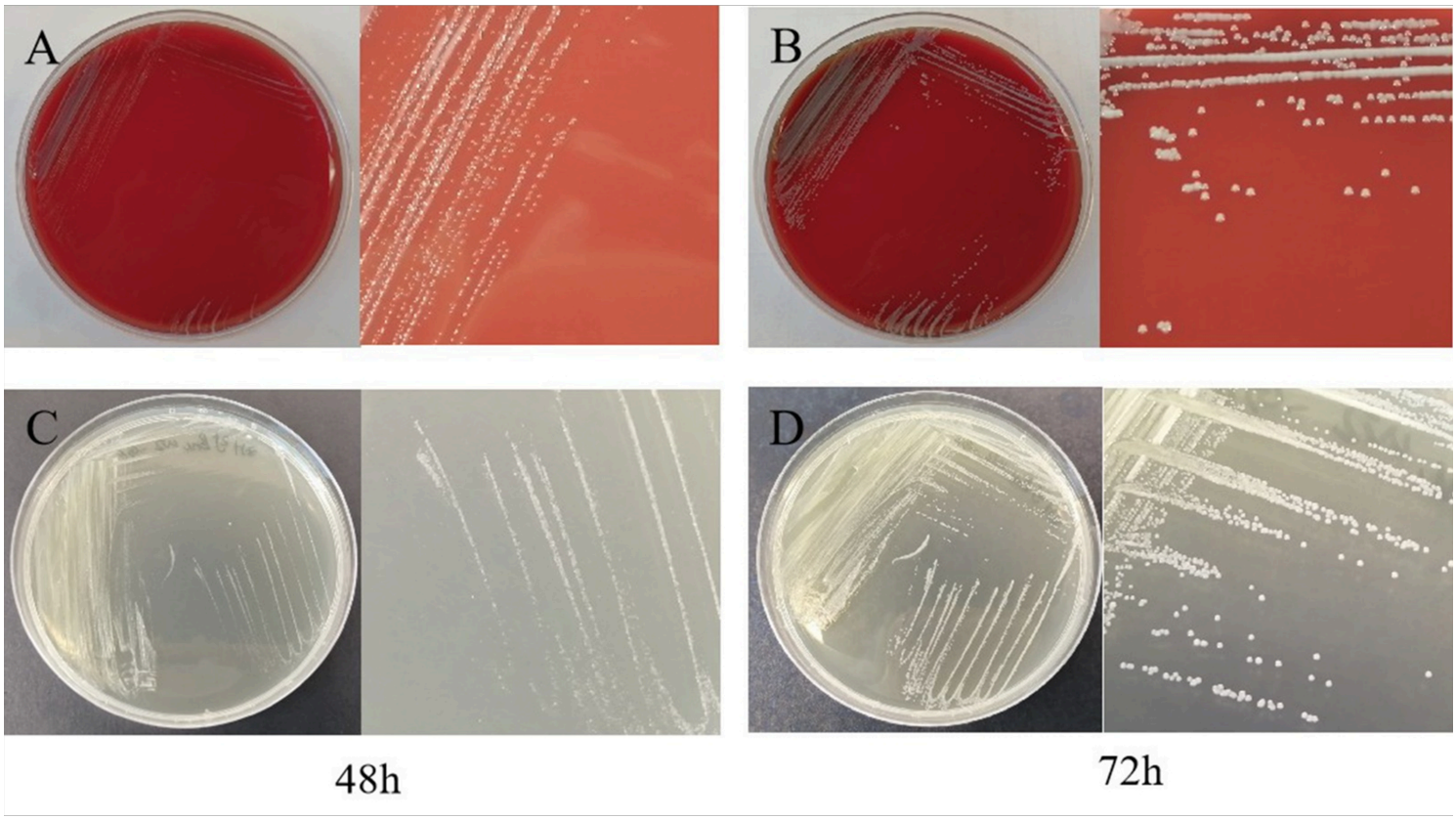


Figure 1

Legend. Colonial morphology of reference strain ZH25. (A, B) Colonial morphology of the strain cultured on blood agar plates for 48 h and 72 h, respectively; (C, D) Colonial morphology of the strain cultured on modified Brucella agar plates for 48 h and 72 h, respectively.

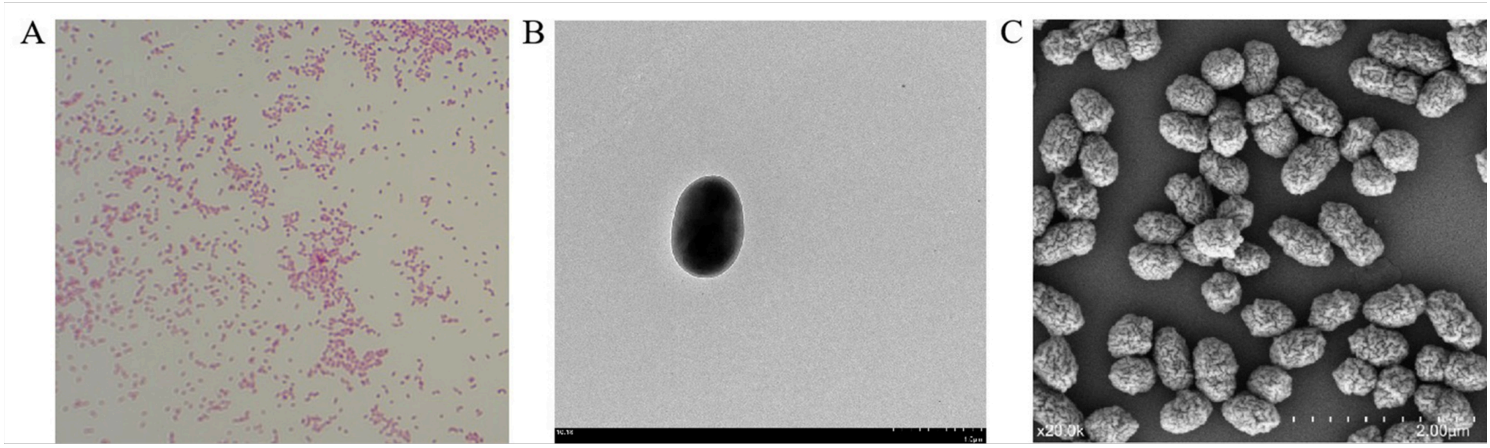


Figure 2

Legend. (A) Gram-staining micrograph (×1000 magnification); (B) Transmission electron micrograph (×10.0 k); (C). Scanning electron micrograph (×20.0 k).

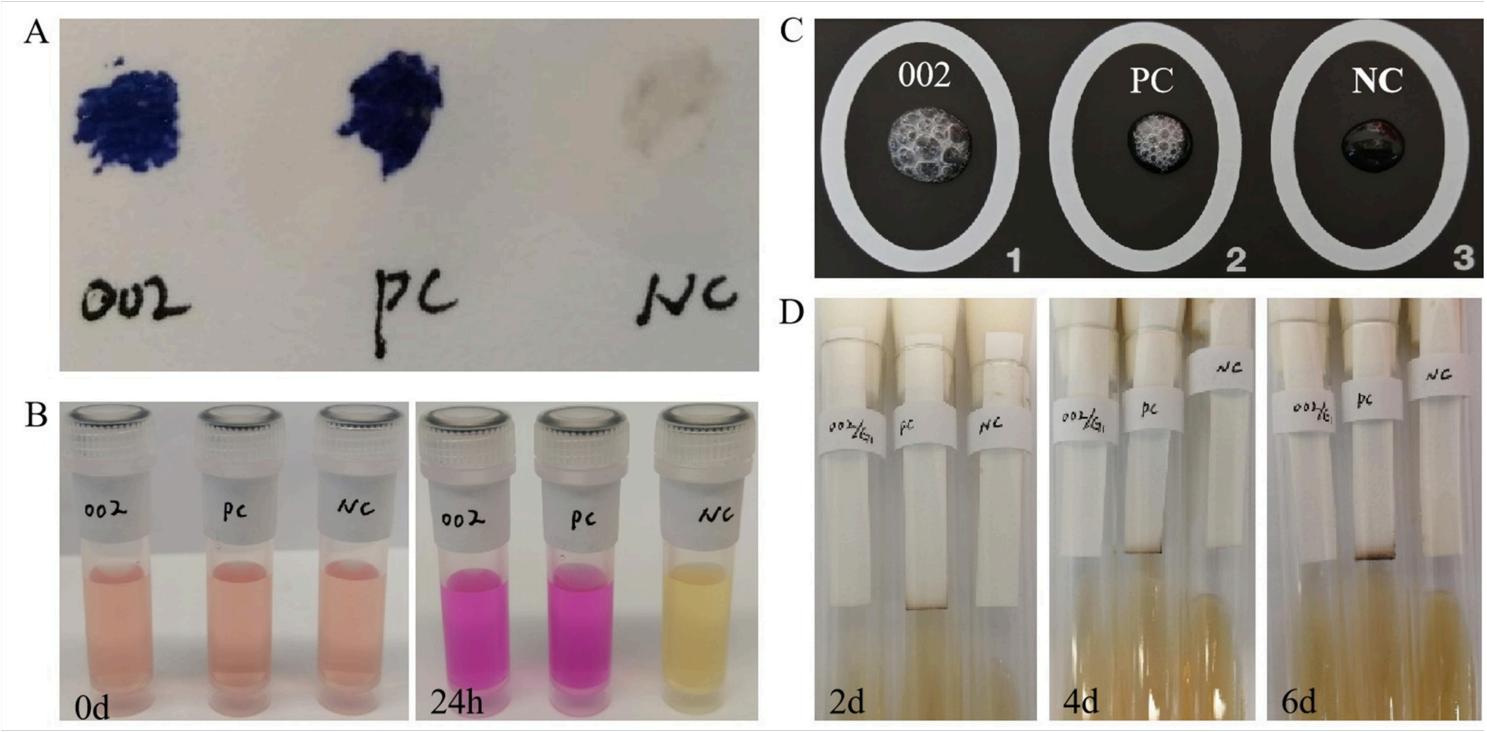


Figure 3

Legend. Biochemical assay results of the strain. (A) Oxidase assay. Reference *Escherichia coli* ATCC 25922 was used as the negative control, and reference *Pseudomonas aeruginosa* ATCC 27853 as the positive control. (B) Urease assay. Reference *Salmonella Typhimurium* ATCC 14028 served as the negative control, and reference *Proteus mirabilis* CMCC 49005 as the positive control. (C) Catalase assay. Reference *Enterococcus faecalis* ATCC 29212 was the negative control, and reference *Listeria monocytogenes* ATCC 19115 the positive control. (D) Hydrogen sulphide production assay. Reference *Salmonella Typhimurium* ATCC 14028 served as the positive control, and reference *Shigella flexneri* CMCC(B) 51572 as the negative control.

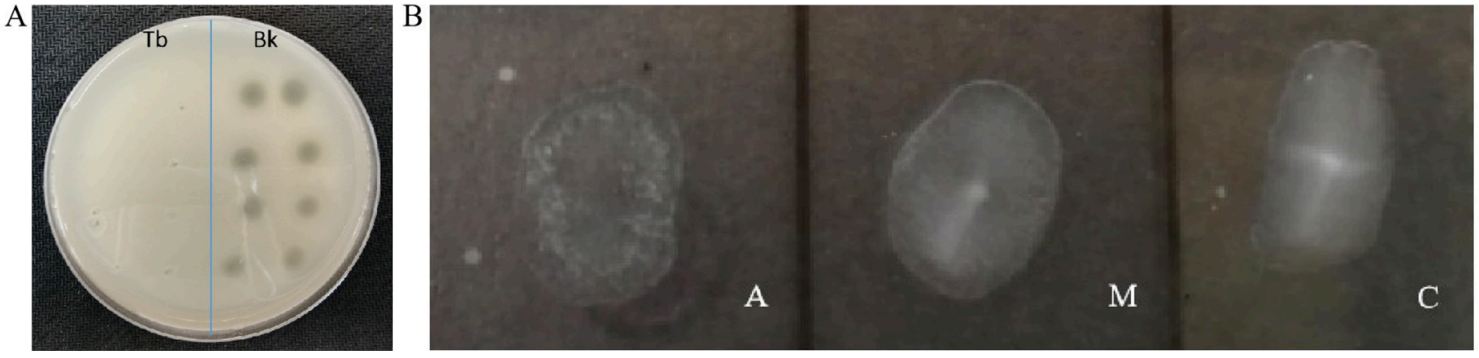


Figure 4

Legend. Phage-lysis and seroagglutination identification. (A) Phage lysis assay using Tb phage and Bk phage; (B) Agglutination assay with species-specific A and M antisera; (C) represents the negative control (normal saline).

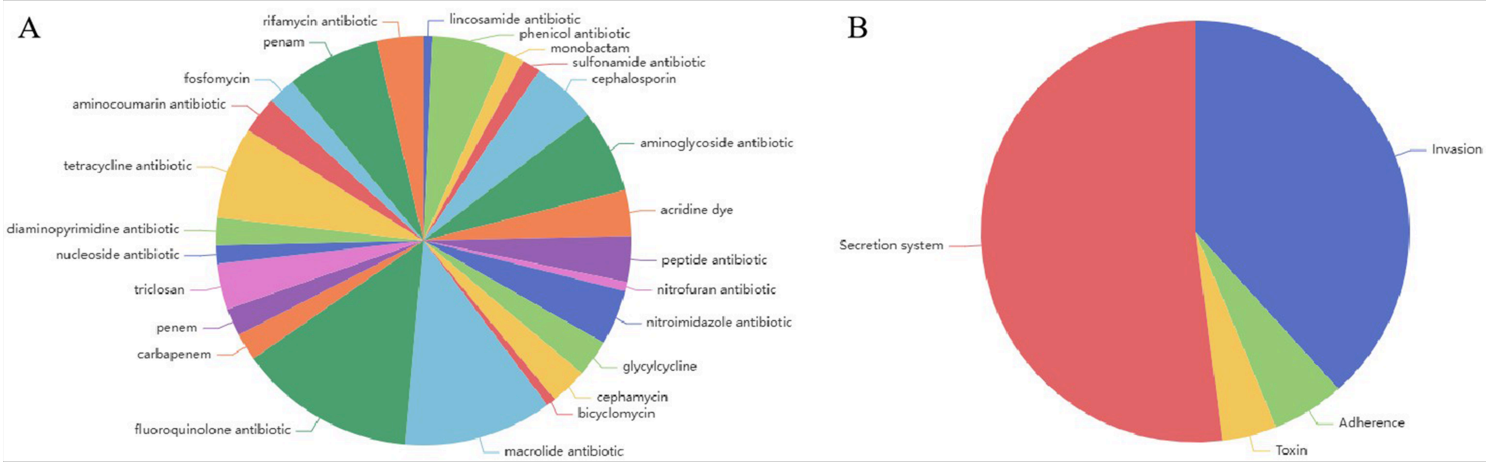


Figure 5

Legend. Strain-stability evaluation. (A) 16S rRNA-based strain stability analysis; (B) Results of average nucleotide identity (ANI) analysis; (C) Similarity analysis of housekeeping genes; (D) cgSNP-based phylogenetic tree.

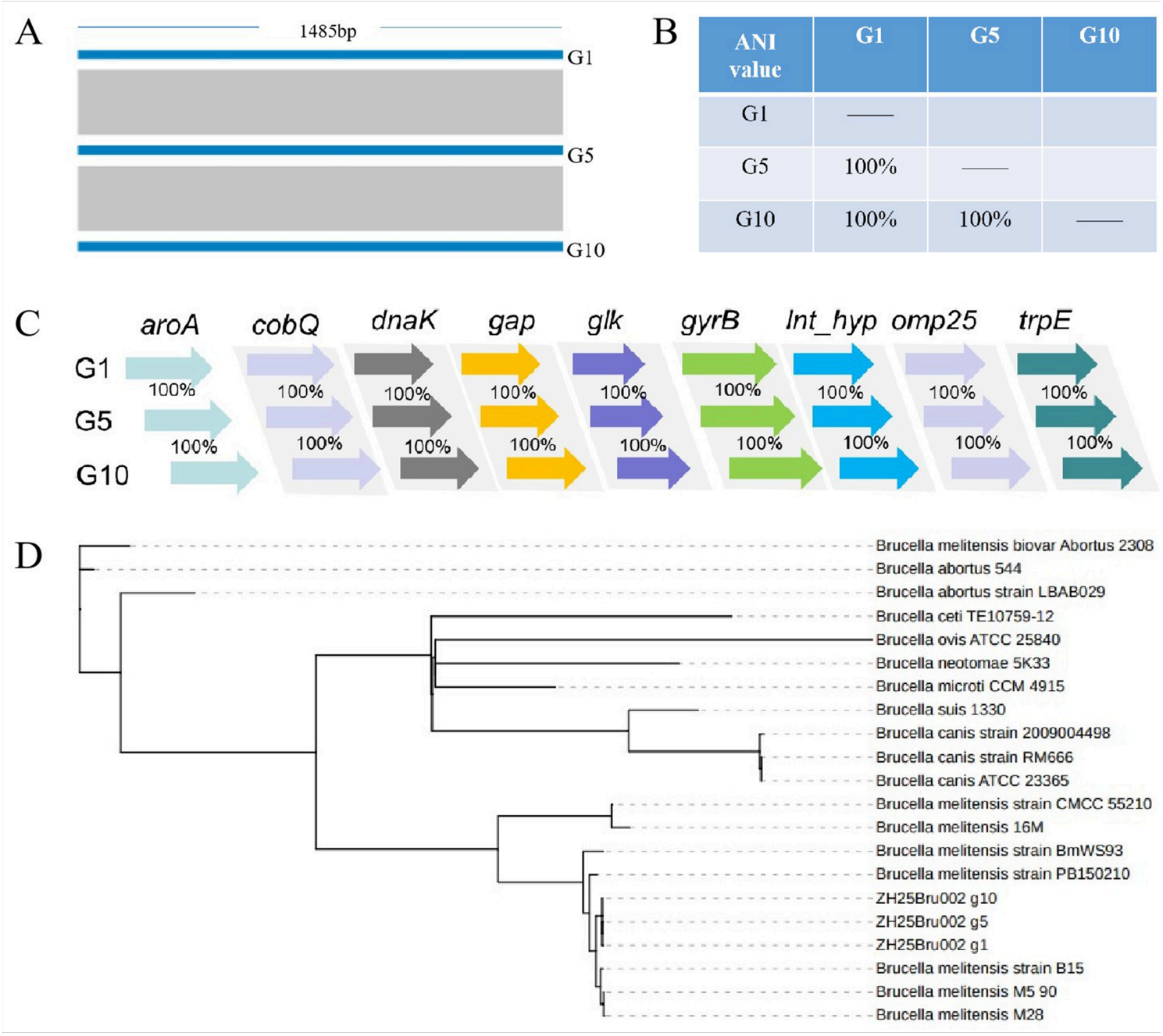


Figure 6

Legend. Profiles of antimicrobial-resistance and virulence-associated genes. (A) Antimicrobial-resistance genes; (B) Virulence-associated genes.

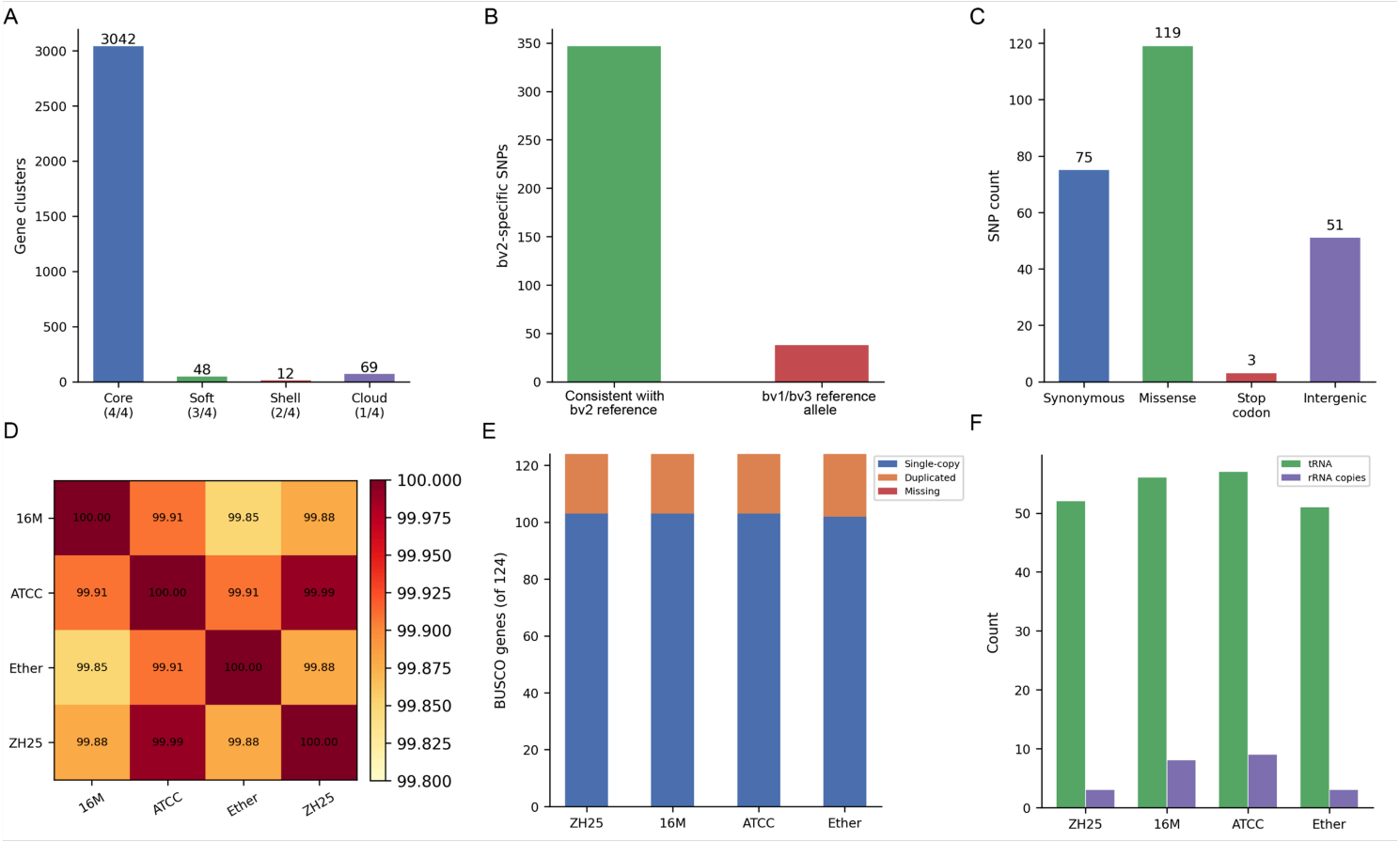


Figure 7

Legend. Genomic feature analysis of reference strain ZH25. (A) Pan-genome composition of the strain calculated under a 70 % identity threshold; (B) Statistics for specific SNP-site concordance between reference strain ZH25 and type strain ATCC 23457; (C) Functional-effect classification and annotation of 248 high-quality genome-wide SNPs; (D) Genomic ANI comparison matrix between ZH25 and reference type strains; (E) BUSCO completeness assessment of genome assembly; (F) Predicted statistics of tRNA and rRNA genetic elements.

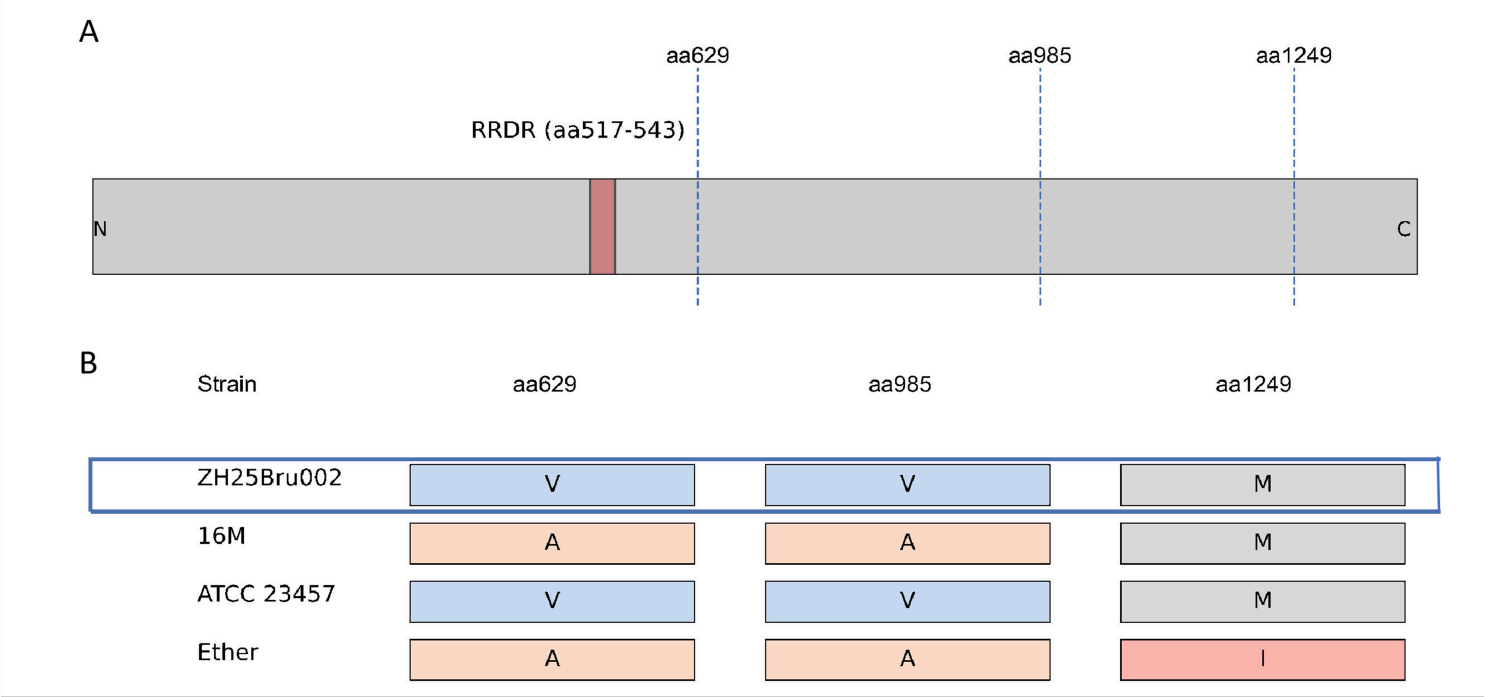


Figure 8

Legend. Comparative analysis of mutation sites within the*rpoB* gene. (A) Schematic diagram of the *RpoB* protein (1377 aa). The red segment denotes the rifampicin-resistance-determining region (RRDR, aa 517–543) of *Brucella*; (B) Amino-acid sequence alignment showing variant sites across four strains.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryFigure2.tif
- SupplementaryFigure3.tif
- SupplementaryFigure4.tif