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Research paper

Genotyping of *Brucella melitensis* and *Brucella abortus* strains currently circulating in Xinjiang, China

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ABSTRACT

Brucellosis is a well-known zoonotic disease that can cause severe economic and healthcare losses. Xinjiang, one of the biggest livestock husbandry sectors in China, has gone through increasing incidence of brucellosis in cattle and small ruminants recently. In this paper, 50 *B. melitensis* strains and 9 *B. abortus* strains collected from across Xinjiang area (from 2010 to 2015) were genotyped using multiple locus variable-number tandem-repeat (VNTR) analysis (MLVA) and multi-locus sequence typing (MLST). Based on 8 loci (MLVA-8), 50 *B. melitensis* strains were classified into three genotypes. Genotypes 42 (n = 38, 76%) and 63 (n = 11, 22%) were part of the East Mediterranean group, and one genotype with pattern of 1-5-3-13-2-4-3-2 represents a single-locus variant from genotype 63. MLVA-16 resolved 50 *B. melitensis* strains into 28 genotypes, of which 15 are unique to Xinjiang and 10 are in common with those in adjacent country Kazakhstan and neighboring provinces of China. Minimum Spanning Tree (MST) analysis implies that *B. melitensis* strains collected from across Kazakhstan, Xinjiang and China areas may share a common origin. Nine *B. abortus* strains were sorted into three genotypes by MLVA-8, genotypes 36 (n = 7, 77.8%), 86 (n = 1, 11.1%) and a new genotype with pattern of 4-5-3-13-2-2-3-1. Each *B. abortus* strain showed distinct MLVA-16 genotypes, suggesting that *B. abortus* species may possess more genetic diversity than *B. melitensis*. Using MLST, most *B. melitensis* strains (n = 49) were identified as sequence type ST8, and most *B. abortus* strains (n = 8) were recognized as ST2. Two new sequence types, ST37 and ST38, represented by single strain from *B. melitensis* and *B. abortus* species respectively, were also detected in this study. These results could facilitate the pathogen surveillance in the forthcoming eradication programs and serve as a guide in source tracking in case of new outbreaks occur.

1. Introduction

Brucellosis is a well-known zoonosis that affects productivity in domesticated animals through abortion, delayed growth, reduced milk yield and loss of breeding capacity. Infection in different animal species is normally caused by different species in genus *Brucella*. The primary *Brucella* species in domesticated animals are *B. melitensis* (sheep and goats), *B. abortus* (cattle), *B. ovis* (sheep and goats), *B. suis* (swine) and *B. canis* (dog), although other *Brucella* species in wildlife and marine animals occur (Cloeckaert et al., 2001; Scholz et al., 2008). Brucellosis in livestock has been eradicated in most of the industrialized countries, but it is still a main concern for developing countries in Asia, Africa, Central and Southern America (Chen et al., 2013; Kilic et al., 2011; Shevtsov et al., 2015; Tay et al., 2015).

Xinjiang is located in the northwest of China, and is the biggest provincial district, covering one sixth of the country (1,660,000 km^2). According to national statistics data (http://data.stats.gov.cn) in 2014, there were 38.8 million small ruminants (sheep and goat) being raised in Xinjiang, making it the second largest small ruminants rearing sector after Inner Mongolia. And it also contained 3.8 million cattle, more than 60% of which were raised in the middle and north area including prefectures Ili, Tarbaghatay and Altay. Bovine and small ruminant brucellosis had been endemic in Xinjiang between 1960s and 1980s. Surveillance data showed that average seropositive rates in cattle and sheep were 18.4% and 4.5%, respectively, with a seropositive rate of 27.9% in veterinarians and farm workers in animal product manufacture. In the 1980s, massive prevention measures commenced to control the zoonosis in

Abbreviations: MLVA, Multiple locus variable-number tandem-repeat (VNTR) analysis; TR, Tandem repeat; MLST, Multi-locus sequence typing; ST, Sequence type; HGDI, Hunter & Gaston diversity index; MST, Minimum Spanning Tree.

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domestic animals. Brucellosis incidence declined gradually in the following ten years. By the 1990s, only 0.19% of cattle and 0.09% of small ruminants were seropositive for brucellosis. However, from the beginning of new millennium, prevalence of brucellosis was growing again in Xinjiang, with a sharp increase in incidence observed in 2007–2008. In recent years, annual brucellosis outbreaks in domestic animals increased dramatically, with a total of 495 outbreaks reported in 2013, 629 outbreaks in 2014 and 927 outbreaks at the end of 2015 according to *Chinese Official Veterinary Bulletin* (http://www.moa.gov.cn). In Xinjiang, incidence of human brucellosis increased at a similar pace. A total of 297 human brucellosis cases were reported in 2005, this number went up to 3909 cases (17.3 cases per 100,000 people) in 2013 (http://www.chinacdc.cn), supporting the findings of increase in brucellosis in livestock.

In the past twenty years, livestock production mode has changed greatly. The dominant household practices of livestock rearing are being replaced by a more intensive livestock farming system. In Xinjiang, around 70% of dairy cattle and 40% of small ruminants are raised in large scale factory farms, in which prevention measures are implemented efficiently and incidence of brucellosis is normally constrained to a low level (http://www.xjxmt.gov.cn/). But in rural areas, high incidence was still observed in household backyard cattle or sheep, likely due to the common nomadic and mixed cattle-small ruminant pasturing that makes the disease control programs less effective. Infections in peasants and livestock farmers in rural areas represented 80% of total human brucellosis cases, implicating that the primary risk factor for human infection was from the loosely organized herds in countryside (Adal et al., 2013; Zhu et al., 2014). In Xinjiang, most human cases of brucellosis were caused by *B. melitensis* species, consistent with the local distribution of household and small-scale of small ruminants flocks (Zhang, et al., 2015). There were few reported human brucellosis cases caused by *B. abortus* species in recent years.

Since 2010, bacterial isolation and identification has been conducted on aborted cattle and small ruminant fetuses collected from across Xinjiang area by our team. To date, more than 50 *Brucella* strains representing different isolation dates and geographic regions have been obtained. Most of these cultures were isolated from small herds in rural areas. To understand their genetic diversity, a multiple locus variable-number tandem-repeat (VNTR) analysis (MLVA) developed in Orsay, France (Le Fleche et al., 2006) was performed on these *Brucella* strains. In addition, multi-locus sequence typing (MLST), a DNA sequence-based typing method (Whatmore et al., 2007) that was previously reported to show high discriminatory ability among different *Brucella* strains, was also applied. Based on these data, a detailed understanding of genetic diversity of circulating *B. melitensis* and *B. abortus* strains in Xinjiang has been achieved, and appropriate prevention measures can be proposed for subsequent eradication programs.

2. Materials and methods

2.1. Brucella strains

A total of 59 *Brucella* strains were obtained from 10 prefectures comprising ~80% of Xinjiang area, with sampling dates spanning six years from 2010 to 2015 (Supplementary table). The species and biotypes of these strains have been characterized by traditional phenotypical tests in accordance with procedures recommended by OIE guidelines. The tests included CO_2 requirement, H_2S production, growth inhibition by fuchsin and thionin at different concentrations (1:50,000 and 1:100,000), agglutination with A and M monospecific antisera, Tbilisi phage lysis and oxidative metabolic assays. All bacterial handling were carried out in Biosafety Level III laboratories in Xinjiang Center for Animal Disease Control (XACDC) and Chinese Animal Health and Epidemiology Center (CAHEC). 50 strains were identified as *B. melitensis* (biovar 1 = 8, 16%; biovar 2 = 2, 4%; biovar 3 = 40, 80%), and 9 as *B. abortus* biovar 3. In terms of host origin, the majority of *B. melitensis* strains (47 strains) were isolated from sheep, and one each from a goat and cow. In addition, a strain isolated from the blood of a brucellosis patient was also included in this study. All nine *B. abortus* strains were isolated from dairy cattle.

2.2. MLVA genotyping and data analysis

MLVA including eight minisatellite loci (panel 1) and eight microsatellite loci (panel 2, subdivided into 2 A and 2B) was performed as previously described with modifications (Le Fleche et al., 2006; Maquart et al., 2009). Briefly, forward primers were synthesized with 6-carboxyfluorescein (FAM) fluorescent label. Sizes of PCR products were evaluated by capillary electrophoresis on an ABI Prism 3130 automated fluorescent capillary DNA sequencer (Applied Biosystems). Genomic DNAs from reference strains, *B. melitensis* 16M and *B. abortus* 544, were used as positive controls to monitor PCR protocol and calibrate the capillary electrophoresis platform. For locus Bruce19 that shows very short repeat unit (3 bp), amplified DNA fragments were sequenced to obtain accurate sizes. PCR conditions were as follows: initial denaturation at 95 °C for 5 min followed by 30 cycles of 94 °C for 30s, 60 °C for 30s and 72 °C for 60s. Fragments sizes were converted to repeat units according to the published allele numbering system (Le Flèche et al. version 3.6 modified in 2013). All data were analyzed using BioNumerics software (version 7.6, Applied Maths, Belgium). Cluster analysis was based on the categorical coefficient and UPGMA (Unweighted Pair Group Method with Arithmetic Mean). Polymorphisms at each locus were quantified using the Hunter & Gaston diversity index (HGDI). Standard minimum spanning tree (MST) was generated based on categorical coefficients together with single and double locus variance priority rules. Web-based MLVA database (http://mlva.u-psud.fr/) was utilized to compare different strains from countries worldwide. For locus Bruce19, a slight difference (one repeat unit) was neglected during epidemiological relevance analysis and trace-back study among different strains.

2.3. MLST genotyping and data analysis

MLST was performed using the method described previously (Whatmore et al., 2007). Briefly, nine genomic loci corresponding to a total of 4396 bps of sequences were selected, including seven housekeeping genes, one outer membrane protein gene and one intergenic fragment. PCR cycling parameters were as follows: 95 °C for 5 min, followed by 30 cycles of 94 °C for 30s, 63 °C for 30s, 72 °C for 60 s, and an elongation step at 72 °C for 10 min. The resultant PCR products were purified and sequenced at Shenggong Biosciences Company (Shanghai, China). The sequence data were edited using EditSeq module of the Lasergene package (version 7). Each allele of the nine loci was given a distinct numerical designation according to previously published MLST sequences in EMBL database (accession number from AM694191 to AM694568). Each unique allelic profile for the nine loci was identified as a sequence type (ST). The assembled sequences of the nine loci were then concatenated, and phylogenetic analyses were conducted using MEGA software (version 5.1) Neighbor-joining tree was constructed using Jukes-Cantor model.

3. Results

3.1. MLVA genotyping

From all investigated *Brucella* strains, allele sizes at sixteen loci were successfully obtained by capillary electrophoresis (CE). Their VNTR units and Hunter & Gaston diversity indexes at each locus were calculated (Supplementary table, Table 1). For the locus Bruce19, direct sequencing was performed on strains showing different sizes of this locus on CE. Only one allele with a repeat unit of 41 (178 bp) was found in *B. melitensis* strains, and two alleles with repeat units of 36 and 43 (163 bp and 184 bp) were detected in *B. abortus* strains. In *B.*