

Full Paper

Genetic diversity of *Streptococcus uberis* isolates from dairy cows with subclinical mastitis in Southern Xinjiang Province, China

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Streptococcus uberis is a common cause of dairy cow mastitis throughout the world. The failure to control bovine mastitis caused by *S. uberis* is largely attributed to the little known about the epidemiology of this bacteria, especially strain differences in the same area. To define the local epidemiology of *S. uberis* in the south of Xinjiang, China, we explored the genetic diversity of 28 bovine subclinical mastitis field isolates of *S. uberis*, collected from 3 Chinese farms during 2009 and 2010, which was examined by using pulsed-field gel electrophoresis (PFGE) for clustering of the isolates and multilocus sequence typing (MLST) to assess the relationship between PFGE patterns and to identify genetic lineages. The 28 isolates were grouped into 13 pulsotypes (U1 to U13), and 1 PFGE type (U1) accounted for almost half of the isolates (13/28, 46.4%). This major type was herd specific, indicating either cow-to-cow transmission or infection with isolates from the same environmental reservoirs. The remaining 12 PFGE types of isolates were from different herds, strongly suggesting environmental sources of *S. uberis* infection. All 28 isolates were analyzed by MLST and clustered into 8 sequence types (STs), of which 7 STs were found to be novel, either with 5 new alleles of 6 housekeeping and virulence genes (ST158, ST159) or with different combinations of previously assigned alleles (ST153, ST154, ST155, ST156, ST157). To our knowledge, this is the first report that documents molecular typing studies of bovine isolates of *S. uberis* from southern Xinjiang Province, China, which were shown to represent novel genomic backgrounds of this pathogen.

Key Words—bovine mastitis; genetic diversity; multilocus sequence typing; pulsed-field gel electrophoresis; *Streptococcus uberis*

Introduction

Bovine mastitis is a disease leading to economic

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loss in the dairy industry throughout the world. Mastitis pathogens include two groups: contagious pathogens, including *Staphylococcus aureus* and *Streptococcus agalactiae*, and environmental pathogens, such as *Streptococcus uberis* (Pullinger, 2007). Although mastitis control strategies have successfully been used to reduce the incidence of contagious mastitis, these methods have had limited impact on environmental pathogens. Mastitis caused by *S.uberis* in-

fection was commonly considered the result of environmental exposure to this pathogen (Lopez-Benavides et al., 2007). However, several studies have reported that cow-to-cow transmission of *S. uberis* occurred (Douglas et al., 2000; Phuektes et al., 2001; Zadoks et al., 2003).

The genetic diversity of *S. uberis* has been investigated using several methods based on DNA, aiming to evaluate strain-specific transmission and to improve infection control measures (Khan et al., 2008; Rato et al., 2003; Wieliczko et al., 2002; Zadoks et al., 2003). Pulsed-field gel electrophoresis (PFGE) has been used for typing a broad range of pathogens including bovine and human pathogens. In the same species, PFGE is the most discriminatory and quick method to type the constitution of bacterial genomic DNA. Multi-locus sequence typing (MLST) is a valuable tool used for characterizing isolates of the same species, including bacterial and fungal species. The method relies on the sequencing of internal fragments of certain genes. To our knowledge, 2 MLST schemes have been used to investigate genetic diversity of *S. uberis*, one based on the sequencing of 6 housekeeping genes and virulence genes (Zadoks et al., 2005) and the other based on the sequencing of 7 housekeeping genes (Coffey et al., 2006) (<http://pubmlst.org/suberis/>).

In our study, we performed PFGE and MLST (using the first scheme referred to above) to characterize a collection of field isolates of *S. uberis* from subclinical bovine mastitis occurring in Chinese herds during 2009 and 2010 and to document the genetic diversity and population structure of these isolates, which will be useful in providing further insights into the evolutionary and population genetics of this pathogen.

Materials and Methods

***S. uberis* isolates collection and identification.** This study included 169 milk samples from 169 animals from 3 dairy farms located in southern Xinjiang Province, China. Bacteria were isolated by spreading partial milk onto brain heart infusion (BHI) agar plates overnight at 37°C. *S. uberis* was confirmed by colony morphology, Gram stain, biochemical tests, and PCR amplification of the *gapC* gene. To further identify *S. uberis* species, 16S ribosomal DNA (rDNA) gene sequencing was performed with the following primers: (16S-F: 5'-AGAGTTTGATCATGGCTCAG-3'; 16S-R: 5'-AAGGAGGTGATCCAGCC-3'). Automatic sequencing was

carried out by Sangon Shanghai using the same primers as for amplification. *S. uberis* was identified finally by comparing the sequence data with other sequences of 16S rDNA gene in the GenBank database using BLASTN (<http://www.ncbi.nlm.nih.gov/BLAST/>).

PFGE typing and cluster analysis. PFGE analysis of the 28 *S. uberis* isolates was performed by modifications of previously described methods (Phuektes et al., 2001). In brief, *S. uberis* isolates were incubated in BHI broth (Difco, Houston, TX) to stationary phase at 37°C overnight. The cells were harvested by centrifugation at $13,000 \times g$ for 1 min, washed three times with 1 mol/L NaCl-10 mmol/L Tris-HCl (pH 7.6), then centrifuged and resuspended with 100 μ l of the same solution, mixed with an equal volume of molten 2% (wt/vol) low-melting-temperature agarose (SeaPlaque; FMC Bioproducts, Rockland, ME) and poured into 100 μ l molds. When solidified, plugs were incubated at 37°C for 18 h in lysis buffer (6 mmol/L Tris-HCl [pH 7.6], 1 mol/L NaCl, 100 mmol/L EDTA [pH 7.6], 1% Sarkosyl, and 1 mg of lysozyme/ml). Cells were subsequently transferred into ESP buffer (0.5 mol/L EDTA [pH 9.2], 1% Sarkosyl, and 1 mg of proteinase K/ml), and were incubated at 50°C for 72 h. The plugs were then washed 5 times for 45 min each time with $1 \times$ TE buffer (10 mmol/L Tris-HCl [pH 8] and 1 mmol/L EDTA [pH 8]), and then stored at 4°C in 1 ml of 0.5 mol/L EDTA [pH 8] until used.

A 3-mm slice of each agarose plug was washed 5 times with 100 μ l of $1.2 \times$ restriction endonuclease buffer (MBI Fermentas) for 45 min each time on ice. The slices were then digested in 100 μ l of $1 \times$ fresh restriction endonuclease solution containing 30 U of *Sma*I (MBI Fermentas, Pittsburgh, PA) at 30°C for 18 h.

After incubation, the fragments were separated by a clamped homogeneous electric field device (CHEF DRII, Bio-Rad, Hercules, CA). The restriction fragments were loaded into the wells of 1% (wt/vol) agarose gels in $0.5 \times$ TBE ($1 \times$ TBE is 89 mmol/L Tris-HCl, 89 mmol/L boric acid, and 2 mmol/L EDTA, pH 8.3) at 14°C. Low Range PFG Marker (New England Biolabs, Ipswich, MA) was included as a molecular size standard. The gel was run at 6 V/cm for 20 h with an initial pulse time of 1 s and a final pulse time of 20 s. The gels were stained with ethidium bromide (0.5 mg/L) for 1 h, and photographed under UV light. Strains were defined using the criteria proposed (Tenover et al., 1995). Isolates generating more than three different restriction patterns were assessed as unrelated isolates. Iso-

Table 1. Primers and PCR conditions for MLST of *S. uberis*.

Gene	Primers ^a	Size (nt)	PCR thermocycling parameters
<i>cpn60</i>	GAI III GCI GGI GAY GGI ACI ACI AC YKI YKI TCI CCR AAI CCI GGI GCI TT	600	94°C, 5:00; 40 × (94°C, 1:00; 45.9°C, 2:00; 72°C, 5:00); 72°C, 10:00
<i>gapC</i>	ATGGTAGTTAAAGTTGGTATTAACG TTATTTAGCGATTTTTGCAAAGTAC	1,011	95°C, 5:00; 30 × (95°C, 0:30; 52°C, 1:00; 72°C, 1:00); 72°C, 10:00
<i>oppF</i>	GAA GCG AAG CTT TGG CT GG GCA GCT TCT GCT TCT GTT GA	800	95°C, 4:00; 35 × (95°C, 1:00; 55°C, 1:00; 72°C, 1:00); 72°C, 7:00
<i>pauA</i>	TTC ACT GCT GTT ACA TAA CTT TGT G CCT TTG AAA GTG ATG CTC GTG	976	94°C, 5:00; 35 × (94°C, 1:00; 50°C, 1:00; 72°C, 1:00); 72°C, 5:00
<i>sodA</i>	CCI TAY ICI TAY GAY GCI YTI GAR CC ARR TAR TAI GCR TGY TCC CAI ACR TC	480	95°C, 3:00; 35 × (95°C, 0:30; 37°C, 2:00; 72°C, 1:30); 72°C, 10:00
<i>tuf</i>	AAY ATG ATI ACI GGI GCI CAR ATG GA AYR TTI TCI CCI GGC ATI ACC AT	803	95°C, 3:00; 35 × (95°C, 0:30; 55°C, 0:30; 72°C, 1:00); 72°C, 7:00

^a All primers are shown 5' to 3'. For each gene, the first primer listed is the forward primer, and the second primer is the reverse primer. I = inosine; K = keto (G or T); R = purine (A or G); Y = pyrimidine (C or T). ^bAccession numbers AF421900.1.

lates which had 1 to 3 band differences were considered to be probably related and assigned as different subtypes. Isolates which had identical restriction patterns were considered to be derived from a common parent.

MLST and sequence typing analysis. The DNA of *S. uberis* isolates was extracted as previously described (Coffey et al., 2006) with some modifications. Briefly, 1 ml of overnight broth culture was centrifuged at 13,000 × *g* for 5 min, and the supernatant was removed. The cells were resuspended with 360 µL of TE (10 mmol/L Tris, 5 mmol/L EDTA [pH 7.8]), and 100 µL of lysozyme at 50 mg/ml was added, then incubated at 37°C for 4–5 h. Cells were lysed by the addition of 40 µL of sodium dodecyl sulfate (20% [wt/vol]), and then incubated at 60°C for 15 min. Sodium perchlorate monohydrate (125 µL, 5 mol/L) was added to precipitate cell proteins. An equal volume of chloroform-isoamyl alcohol (24 : 1) was added, and the upper aqueous phase was obtained by centrifugation (13,000 × *g* for 10 min). DNA was precipitated by adding 2 volumes of ice-cold ethanol and holding the mixture at 4°C for 1 h. Precipitated DNA was pelleted by 10 min of centrifugation at 13,000 × *g*. Pellets were washed with 70% ethanol and then air dried. The DNA was resuspended with 50 µL of TE buffer and then stored at –20°C. Six housekeeping and virulence genes were chosen for MLST. Primers for amplification of *cpn60*, *pauA*, *oppF*, *sodA*, and *tuf* were taken from the literature (Zadoks et al., 2005). For *gapC*, DNA sequence data from GenBank were used to design primers. Primer sequences and cycling parameters for DNA amplification by PCR are listed in

Table 1. A 50-µL aliquot of the reaction system contained 0.5 µL of *Taq* polymerase (Biofuture), 1 µL of forward and reverse primers, 5 µL of 10 × *Taq* PCR buffer (Biofuture), 1 µL of deoxynucleoside triphosphate mix (Biofuture), 2 µL of DNA, and 39.5 µL of sterile distilled water. Both directions of each gene fragment were sequenced using PCR primers. Sequences for each gene were assembled by MegAlign. DnaSP version 4.0 (Rozas and Rozas, 1999) was used for descriptive analyses. For each of the six loci, every different sequence data was assigned as a distinct allele. Each unique combination of alleles was assigned as a sequence type (ST) consisting of six integers. The allele numbers at the six loci were in the order: *cpn60*, *gapC*, *oppF*, *pauA*, *sodA*, and *tuf*.

Results

16S rDNA gene sequence analysis for *S. uberis* identification

This study showed that 65.3% of cows were enrolled with subclinical infection in 3 tested farms. Forty-two isolates were identified and further conformed as *Streptococci* using colony morphology, Gram stain, and biochemical tests. In addition, a partial sequence of the *gapC* gene was amplified in all isolates. After sequencing 16S rRNA, the result showed that 28 isolates were *S. uberis*, and the remaining 14 isolates were identified as other *Streptococci* species. The distribution of the 28 *S. uberis* isolates from the 3 farms (code 1, 2, and 3) was as follows: 13 isolates from farm 1, 10 isolates from farm 2, and 5 isolates from farm 3.

All isolates were collected from different cows. Comparative analysis with the 16S rDNA gene sequence of the *S. uberis* reference strain 0140J from the GenBank database showed that the 28 isolates had a maximum identity of 99%, 2 isolates differed by 1 bp, 5 isolates differed by 2 bp, 4 isolates differed by 3 bp, 4 isolates differed by 4 bp, 4 isolates differed by 5 bp, 4 isolates differed by 6 bp, 2 isolates differed by 7 bp, 1 isolate differed by 8 bp and 2 isolates differed by 10 bp.

PFGE profiles

All 28 isolates of *S. uberis* yielded 9–15 well-resolved fragments of 15–300 kb when analyzed by PFGE (Fig. 1). Thirteen distinct PFGE profiles were observed, which were designated type U1 to U13. Each farm had one or more unique sets of patterns. In no instance were identical PFGE types observed for cows from different farms. Out of the 28 isolates, 13 isolates from farm 1 had the PFGE pattern of type U1; 2 of 10 isolates from farm 2 had the pattern of type U2, and the remaining 8 of 10 isolates from farm 2 had different patterns of types U3–U10; 3 of 5 isolates from farm 3 had the pattern of type U11, and the remaining 2 of 5 isolates from farm 3 showed different patterns of types U12 and U13 (shown in Table 2). The PFGE patterns revealed the high level of heterogeneity of *S. uberis*.

Allelic profiles and sequence types of *S. uberis* from southern Xinjiang Province, China

The gene fragments of 6 housekeeping and virulence genes for this MLST scheme were amplified and

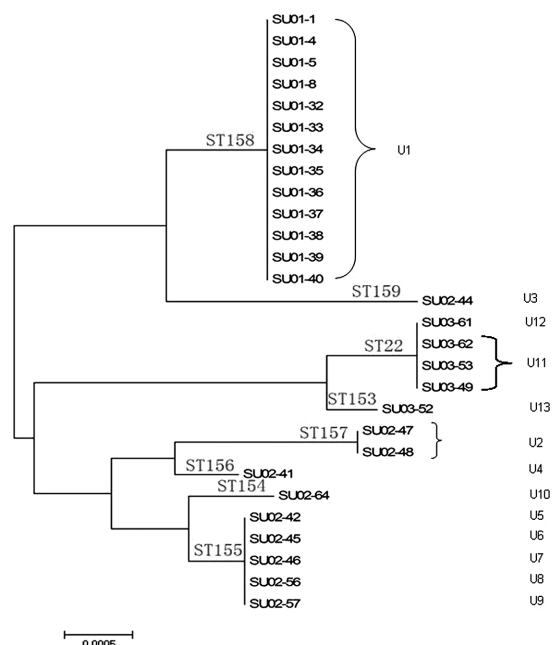


Fig. 2. Dendrogram of the multilocus sequence types (STs). The dendrogram was produced by using MEGA.

sequenced for all 28 isolates confirmed to be *S. uberis*. The fragments based on gene regions with sequence lengths were listed as follows: *cpn60* (517 bp), *gapC* (827 bp), *oppF* (629 bp), *pauA* (821 bp), *sodA* (427 bp), and *tuf* (656 bp). These 28 isolates were clustered into 8 STs, of which 7 STs were found for the first time in this study (Table 2). These STs were novel because they had different combinations of previously assigned alleles (ST153, ST154, ST155, ST156, ST157) or contained 5 new alleles of 6 housekeeping and virulence genes (ST158, ST159). Within the same cluster

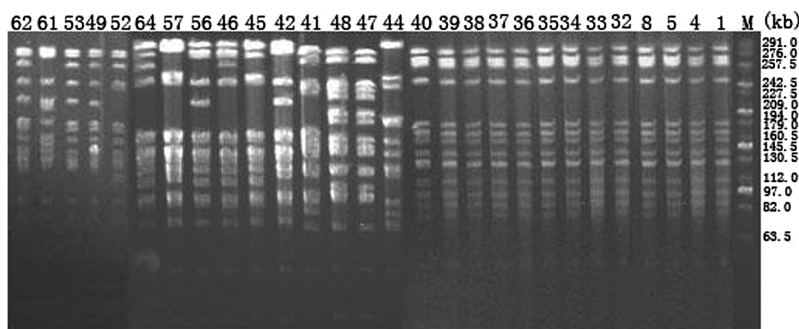


Fig. 1. Pulsed-field gel electrophoresis (PFGE) profiles of *SmaI*-digested genomic DNA from *S. uberis* subclinical mastitis isolates collected from 3 Chinese dairy herds.

M=Low range PFG marker, the remaining lanes include 13 isolates from farm 1 (from right to left, lane 1–40), 10 isolates from farm 2 (lane 44–64), and 5 isolates from farm 3 (lane 52–62).

Table 2. Allelic profiles of *S. uberis* isolates of bovine subclinical mastitis from southern Xinjiang Province, China, assigned by multilocus sequence typing (MLST).

Farm code	Isolate code	PFGE ^a type	Allele						Sequence type (ST)
			<i>cpn60</i>	<i>gapC</i>	<i>oppF</i>	<i>pauA</i>	<i>sodA</i>	<i>tuf</i>	
1	SU01-1	U1	2	31 ^c	4	38 ^c	15	7	158 ^b
	SU01-4	U1	2	31 ^c	4	38 ^c	15	7	158 ^b
	SU01-5	U1	2	31 ^c	4	38 ^c	15	7	158 ^b
	SU01-8	U1	2	31 ^c	4	38 ^c	15	7	158 ^b
	SU01-32	U1	2	31 ^c	4	38 ^c	15	7	158 ^b
	SU01-33	U1	2	31 ^c	4	38 ^c	15	7	158 ^b
	SU01-34	U1	2	31 ^c	4	38 ^c	15	7	158 ^b
	SU01-35	U1	2	31 ^c	4	38 ^c	15	7	158 ^b
	SU01-36	U1	2	31 ^c	4	38 ^c	15	7	158 ^b
	SU01-37	U1	2	31 ^c	4	38 ^c	15	7	158 ^b
	SU01-38	U1	2	31 ^c	4	38 ^c	15	7	158 ^b
	SU01-39	U1	2	31 ^c	4	38 ^c	15	7	158 ^b
	SU01-40	U1	2	31 ^c	4	38 ^c	15	7	158 ^b
2	SU02-41	U4	13	4	1	9	1	4	156 ^b
	SU02-42	U5	6	4	22	9	5	9	155 ^b
	SU02-44	U3	29 ^c	32 ^c	20	39 ^c	1	4	159 ^b
	SU02-45	U6	6	4	22	9	5	9	155 ^b
	SU02-46	U7	6	4	22	9	5	9	155 ^b
	SU02-47	U2	13	4	2	9	6	4	157 ^b
	SU02-48	U2	13	4	2	9	6	4	157 ^b
	SU02-56	U8	6	4	22	9	5	9	155 ^b
	SU02-57	U9	6	4	22	9	5	9	155 ^b
	SU02-64	U10	6	4	22	9	5	4	154 ^b
3	SU03-49	U11	5	4	1	4	1	4	22
	SU03-52	U13	5	4	1	4	1	9	153 ^b
	SU03-53	U11	5	4	1	4	1	4	22
	SU03-61	U12	5	4	1	4	1	4	22
	SU03-62	U11	5	4	1	4	1	4	22

^aPFGE = pulsed-field gel electrophoresis, and the pulsotype of the *S. uberis* isolates is represented by U, followed by the type number. ^bNovel allelic profiles or sequence types (STs). ^cNovel allele.

(I, II or III), isolates had the same or different STs: cluster I included 13 isolates of ST158 and 1 isolate of ST159; cluster II included 4 isolates of ST22 and 1 isolate of ST153; cluster III included 1 isolate of ST154, 5 isolates of ST155, 1 isolate of ST156 and 2 isolates of ST157.

The genetic relationships between the STs were further investigated using Molecular Evolutionary Genetics Analysis (MEGA). Six gene sequences of every strain were joined in the order *cpn60-gapC-oppF-pauA-sodA-tuf* and used to construct phylogenetic trees (Fig. 2).

Some different pulsotypes shared the same ST (Table 2). Type U5–U9 shared the same ST155, and type U11–U12 shared the same ST22. Meanwhile, the same

pulsotype had a single ST: 13 isolates from farm 1 belonging to a single ST (ST158) had an identical PFGE type (U1), and 2 isolates from farm 2 belonging to ST157 had the same PFGE type (U2). Additionally, some different pulsotypes shared different STs, including U3 (ST159), U13 (ST153), U4 (ST156), and U10 (ST154).

Discussion

Streptococcus uberis is a well-recognized worldwide bovine pathogen in dairy herds and is considered one of the principal causative agents of mastitis (Leigh, 1999; Shim et al., 2004). It has been noted that the relative importance of transmission mechanisms

and control measures differs between herds and geographical areas (LeBlanc et al., 2006). However, studies on the molecular characterization of field *S. uberis* isolates from China are still not well-documented and are of utmost importance for a more efficient control of bovine mastitis caused by this pathogen.

Our aim in this study was to investigate if there was population diversity of *S. uberis* in subclinical bovine mastitis cases and to assess if subclinical bovine mastitis in Xinjiang was associated with a limited number of dominant types, which is important information for the implementation of targeted mastitis control programs. In addition, we aimed to document the genotypic properties of this species in a region not yet surveyed, contributing to increased knowledge about the evolutionary and population genetics of this pathogen by using MLST.

Analyses of the Xinjiang collection of *S. uberis* field isolates by both MLST and PFGE clearly demonstrated a genetically diverse population. There was a particular clone of *S. uberis*, ST158/U1, that predominated in the Xinjiang data set, suggesting that possible transmission between cows or acquisition from a common source did occur. Additionally, there were a few cases of diverse clones being isolated from different cows, suggesting that the epidemiology and pathophysiology of *S. uberis* infections may be strain specific.

The combined use of MLST and PFGE was a valuable approach to achieve our aims. The prevalence of *S. uberis* varied between herds. Close to half (46.4%; $n=13/28$) of *S. uberis* isolates from farm 1 were clustered into 1 large MLST group, cluster I, of which the pulsotype is type U1, strongly suggesting that cow-to-cow transmission occurred for farm 1. Despite that, other heterogeneous PFGE types were detected on farm 2 and farm 3, which suggests that several environmental sources might have been the source of infections.

Related isolates, according to the PFGE data, were seen to share the same ST; however, PFGE and MLST did not correlate in all cases, even in the same farm. Isolates of unrelated PFGE types U5–U9 were found to share the same ST (ST155; Fig. 2), which, importantly, is a novel type. Additionally, U11 and U12 shared ST22, which is a known type. This result was expected because PFGE is more sensitive to microvariation on genome and, therefore, often has more discriminatory power than MLST, which detects variation that accumulates slowly in housekeeping genes (Maiden et al.,

1998).

The occurrence of an identical ST between China and other countries was detected. ST22 is the only known type which has been reported in other countries. The remaining 7 STs found in the present work are unique and were found for the first time in this collection, which would be expected because information regarding mastitis isolates from Asian countries is lacking in the MLST database.

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