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Antimicrobial susceptibility, virulence genes, and randomly amplified polymorphic DNA analysis of *Staphylococcus aureus* recovered from bovine mastitis in Ningxia, China

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ABSTRACT

Staphylococcus aureus is the leading pathogen involved in bovine mastitis, but knowledge about antimicrobial resistance, virulence factors, and genotypes of *Staphylococcus aureus* resulting in bovine mastitis in Ningxia, China, is limited. Therefore, antimicrobial susceptibility, virulence gene, and randomly amplified polymorphic DNA (RAPD) analyses of *Staph. aureus* were carried out. A total of 327 milk samples from cows with clinical and subclinical mastitis in 4 regions of Ningxia were used for the isolation and identification of pathogens according to phenotypic and molecular characteristics. Antimicrobial susceptibility against 22 antimicrobial agents was determined by disk diffusion. The presence of 8 virulence genes in *Staph. aureus* isolates was tested by PCR. Genotypes of isolates were investigated based on RAPD. Results showed that 35 isolates obtained from mastitis milk samples were identified as *Staph. aureus*. The isolates were resistant to sulfamethoxazole (100%), penicillin G (94.3%), ampicillin (94.3%), erythromycin (68.6%), azithromycin (68.6%), clindamycin (25.7%), amoxicillin (11.4%), and tetracycline (5.7%). All of the isolates contained one or more virulence genes with average (standard deviation) of 6.6 ± 1.6. The most prevalent virulence genes were *hlb* (97.1%), followed by *fnbpA*, *hla*, *coa* (94.3% each), *nuc* (85.7%), *fnbpB* (80%), *clfA* (77.1%), and *tsst-1* (40%). Nine different gene patterns were found and 3 of them were the dominant gene combinations (77.1%). *Staphylococcus aureus* isolates (n = 35) were divided into 6 genotypes by RAPD tying, the genotypes III and VI were the most prevalent genotypes. There was great variation in genotypes of *Staph. aureus* isolates, not only among different farms, but also within the same herd in Ningxia province. The study showed a high incidence of *Staph. aureus* with genomic variation of resistance genes, which is matter of great concern in public and animal health in Ningxia province of China.
Key words: bovine mastitis, *Staphylococcus aureus*, antimicrobial susceptibility, virulence gene, RAPD genotype

INTRODUCTION

Bovine mastitis is a major disease affecting the dairy industry worldwide with huge economic loss and decreased animal health (Ahmady and Kazemi, 2013; Deb et al., 2013; Gomes and Henriques, 2016). Although mastitis is often caused by a wide variety of pathogens, such as bacteria, viruses, fungi, mycoplasma, and others, *Staphylococcus aureus* is still described as one of the most frequently isolated etiological agents associated with bovine intramammary infections (Momtaz et al., 2010; Lundberg et al., 2016). Antimicrobial therapy is an important tool in mastitis control programs, but *Staphylococcus aureus* responds poorly to therapy with antimicrobial agents (Saei, 2012; Deb et al., 2013; Xu et al., 2015; Gomes and Henriques, 2016). Antimicrobial resistance of the *Staph. aureus* has been surveilled in many countries and many studies have displayed different results (El Behiry et al., 2012; Saei, 2012; Jagielski et al., 2014; da Costa Krewer et al., 2015; Wang et al., 2015). A large number of virulence factors have been found in *Staph. aureus* originating from bovine mastitis, including clumping factor (*clfA*), fibronectin binding proteins (*fnbpA* and *fnbpB*), hemolysins (*hla* and *hlb*), thermonuclease (*nuc*), coagulase (*coa*), and toxic-shock syndrome toxin-1 (*tsst-1*), which help bacteria to survive and multiply in the mammary gland

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(Xu et al., 2015; Kot et al., 2016). In recent decades, many molecular methods, such as random amplification of polymorphic DNA (**RAPD**) analysis (Chiang et al., 2014), pulsed-field gel electrophoresis (Middleton et al., 2002; Fijałkowski et al., 2014), multilocus sequence typing (Bardiau et al., 2013), RFLP (Puacz et al., 2015), multilocus variable number tandem repeat analysis (SenGupta et al., 2014), *spa* typing, and coagulase gene typing, have been widely used in epidemiological investigations of *Staph. aureus* mastitis in dairy cattle (Zadoks et al., 2011). With the merits of being rapid, able to discriminate, and easy to execute and interpret, RAPD analysis, a PCR-based DNA fingerprint method, has been used successfully to detect polymorphisms in strains of *Staphylococcus* species (Reinoso et al., 2004; Bardiau et al., 2013; Chiang et al., 2014).

It has been well documented that *Staph. aureus* mastitis is an important cause of economic loss to the dairy industry (Middleton and Fox, 2002; Gomes and Henriques, 2016), but there is a paucity of information about antimicrobial resistance, virulence factors, and genotype profiles of *Staph. aureus* isolates in Ningxia province of China; therefore, the aim of the present study was to determine the antimicrobial susceptibility and virulence genes, and to perform the RAPD analysis of *Staph. aureus* isolates.

MATERIALS AND METHODS

Isolation and Identification of Pathogens

Isolation and identification of pathogens were performed as previously described (Bautista-Trujillo et al., 2013; Khichar et al., 2014; Wang et al., 2015). A total of 327 milk samples were collected from the cows with clinical mastitis (n = 92) and subclinical mastitis (n = 235), which originated from 10 herds in 4 geographic regions of the Ningxia province of China (namely, Yinchuan, Wuzhong, Shizuishan, and Zhongwei). The samples were first streaked onto the sheep blood agar and incubated at 37°C for 48 h. The cultures were identified by morphological characteristics (coloring, size, hemolysis), Gram staining, and biochemical tests (production of coagulase, catalase, and DNase; fermentation of glucose, maltose, mannitol; and hydrolysis of esculin). All presumptive *Staph. aureus* isolates were further confirmed by molecular identification. The PCR of 16S rRNA gene of *Staph. aureus* was carried out as described previously (Lange et al., 2015). A pair of primers was designed (primer-1, 5′-TTTTATGGAGAGTTTGATCCTGGC-3′; primer-2, 5′-AGAAAGGAGGTGATCCAGCCG-3′). The PCR program contained initial denaturation at 94°C for 3 min; 30 cycles of denaturation at 94°C for 30 s, annealing at 63°C for 30 s, and primer extension at 72°C for 1 min; and a final extension step at 72°C for 5 min. The *Staph. aureus* ATCC 25923 strain was used as the control strain.

Antimicrobial Susceptibility Tests

The antimicrobial susceptibility test of the isolates was determined by disk diffusion method according to the guideline of the Clinical Laboratory Standards Institute (CLSI, 2013; Wang et al., 2015). A total of 22 antimicrobial agents were used to evaluate the antimicrobial resistance of the isolates, which included penicillin G (10 IU/disk), ampicillin (10 μg/disk), amoxicillin (10 μg/disk), cephalothin (30 μg/disk), cefradine (30 μg/disk), erythromycin (15 μg/disk), azithromycin (15 μg/disk), oxacillin (1 μg/disk), cefoxitin (30 μg/disk), streptomycin (30 μg/disk), gentamicin (10 μg/disk), kanamycin (30 μg/disk), amikacin (30 μg/disk), neomycin (30 μg/disk), clindamycin (2 μg/disk), doxycycline (30 μg/disk), chloramphenicol (30 μg/disk), sulfamethoxazole (30 μg/disk), ofloxacin (5 μg/disk), norfloxacin (10 μg/disk), ciprofloxacin (5 μg/disk), and tetracycline (30 μg/disk). Results were recorded as sensitive, intermediate, and resistant. The *Staph. aureus* ATCC 29213 strain was used as a control.

Detection of Virulence Genes

Staphylococcus aureus isolates (n = 35) were detected by PCR for the presence of virulence genes (Momtaz et al., 2010; Wang et al., 2015; Kot et al., 2016). The target genes, primer sequences, and target fragment of PCR products are given in Table 1. The PCR reactions were performed in a final volume of 25 μL of reaction mixture consisted of 50 ng of genomic DNA, 20 pmol of each primer, and 12.5 μL of 2× Taq PCR MasterMix (Tiangen Biotech, China: 0.1 U of Taq polymerase/μL, 0.5 m*M* dNTP each, 20 m*M* Tris-HCl/pH 8.3, 100 m*M* KCl, 3 m*M* $MgCl_2$). The cycling conditions were the following: an initial denaturation at 94°C for 3 min; 30 cycles of denaturation at 94°C for 30 s, annealing at specific temperature for 30 s, and primer extension at 72°C for 1 min; and a final extension at 72°C for 5 min.

RAPD Typing of Isolates

The RAPD typing of *Staph. aureus* isolates was conducted as described previously (Morandi et al., 2009; Gutiérrez et al., 2011) and all of 35 isolates were subjected to RAPD analysis. For RAPD tying, the specific primer (5′-GTGGATGCGA-3′) was used. The PCR program included an initial denaturation step at 94°C for 4 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 37°C for 45 s and primer exten-

sion at 72°C for 1 min, and a final extension at 72°C for 8 min. The *Staph. aureus* ATCC 29213 strain was used as control.

Statistical Analysis

Data were expressed as absolute numbers and percentages. In case of virulent gene patterns in *Staph. aureus* isolates, the prevalence of virulent genes was given in mean ± standard deviation. Computation was performed using the SPSS (19.0, SPSS Inc., Chicago, IL) statistical software package.

RESULTS

Isolation and Identification of Bovine Mastitis Pathogens

The pathogenic bacteria (68.8%) were isolated from 225 samples of 327 milk samples with clinical and subclinical mastitis in Ningxia province, in which the same type of bacterium (85.3%) was found in 192 milk samples and different species of bacteria (14.7%) existed in 33 milk samples. A total of 258 isolates from milk samples were obtained and identified by morphological and biochemical methods, and were classified into 9 species of bacteria. Coagulase-negative staphylococci (28.3%) was the most prevalent pathogen isolated from bovine mastitis milks in Ningxia province of China, followed by *Streptococcus dysgalactiae* (15.5%), *Staph. aureus* (13.6%), and *Escherichia coli* (10.5%; Table 2). In the present study, 35 isolates from 10 herds in 4 geographic regions were initially identified as *Staph. aureus* by morphological and biochemical methods. The percentages of the *Staph. aureus* in subclinical and clinical mastitis samples were 13.7% (30/219) and 12.8% (5/39), respectively (Table 2). The identification of isolates was confirmed by using the PCR of 16S rRNA gene of *Staph. aureus*. All *Staph. aureus* strains (n = 35) produced a species-specific amplicon of 1,551 bp (Figure 1a and b), in which 5 isolates were from clinical mastitis cases, and the other 30 isolates originated from subclinical mastitis cases.

Antimicrobial Susceptibility Test

The antimicrobial susceptibility test demonstrated that *Staph. aureus* isolates from bovine mastitis cases in Ningxia province had a variable degree of resistance to the antimicrobials by antimicrobial susceptibility tests (Table 3). They were resistant to sulfamethoxazole (100%), penicillin G (94.3%), ampicillin (94.3%), erythromycin (68.6%), azithromycin (68.6%), clindamycin (25.7%), amoxicillin (11.4%), and tetracycline (5.7%), respectively. However, all of them were susceptible to oxacillin (100%), cephalothin (100%), cefoxitin (100%), cefradine (100%), gentamicin (100%), kanamycin (100%), amikacin (100%), neomycin (100%), doxycycline (100%), chloramphenicol (100%), ofloxacin (100%), norfloxacin (100%), ciprofloxacin (100%), and streptomycin (94.3%), respectively.

Detection of Virulence Genes

Eight virulence genes in *Staph. aureus* isolates were found by PCR in the present study, the results indicated that the most prevalent virulence gene was *hlb* (97.1%), followed by *fnbpA*, *hla*, and *coa* (94.3% each), *nuc* (85.7%), *fnbpB* (80%), *clfA* (77.1%), and *tsst-1* (40%), respectively (Table 4). Nine different gene pat-

Table 1. The PCR primers for amplification of *Staphylococcus aureus* virulence genes

Gene	Primer sequence[1] (5′–3′)	Product (bp)	Reference
clfA	F: GGCAACGAATCAAGCTAATACAC R: TTGTACTACCTATGCCAGTTGTC	719	Yang et al. (2012)
fnbpA	F: GCGGAGATCAAAGACAA R: CATCTATAGCTGTGTGG	1,279	Signäs et al. (1989)
fnbpB	F: GGAGAAGGAATTAAGGCG R: GCCGTCGCCTTGAGCGT	812	Jönsson et al. (1991)
hla	F: GGTTTAGCCTGGCCTTC R: CATCACGAACTCGTTCG	534	Salasia et al. (2004)
hlb	F: GCCAAAGCCGAATCTAAG R: CGCATATACATCCCATGGC	833	Salasia et al. (2004)
nuc	F: GCGATTGATGGTGATACGGGT R:AGCCAAGCCTTGACGAACTAAAGC	279	Brakstad et al. (1992)
coa	F: ATAGAGATGCTGGTACAGG R: GCTTCCGATTGTTCGATGC	Variable	Kalorey et al. (2007)
tsst-1	F: GCTTGCGACAACTGCTACAG R: TGGATCCGTCATTCATTGTTAT	559	Løvseth et al. (2004)

[1]F = forward; R = reverse.

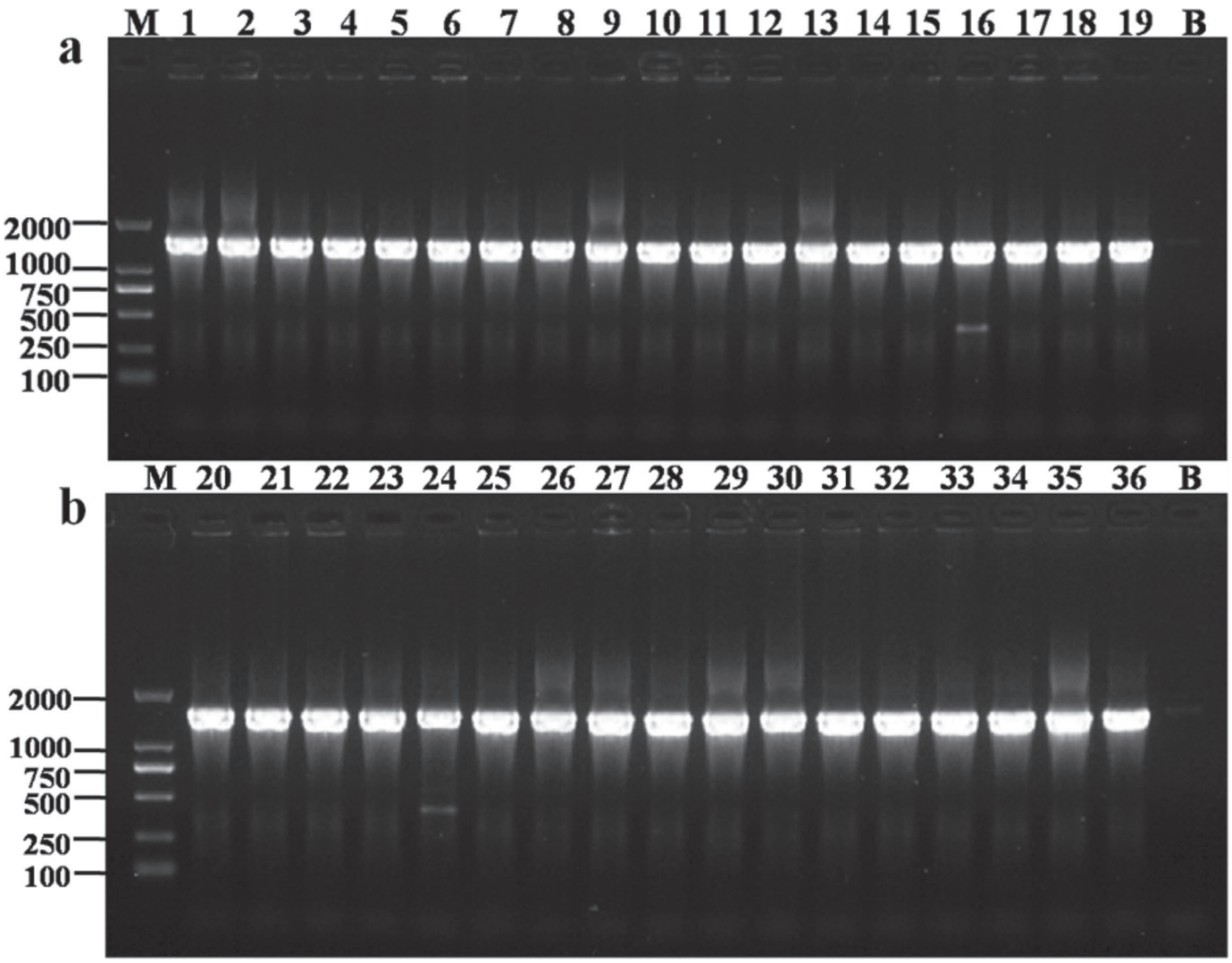

Figure 1. Identification of *Staphylococcus aureus* isolates by 16S rRNA PCR. M = DL2000 marker; B = negative control; lanes 1 to 19 (a) and lanes 20 to 35 (b) = *Staph. aureus* isolates; lane 36 = *Staph. aureus* ATCC25923 (control strain).

terns were found in the *Staph. aureus* isolates and 3 of them were the dominant gene combinations, accounting for 77.1% of the *Staph. aureus* isolates (Table 5). All of the *Staph. aureus* isolates from bovine mastitis contained one or more virulence genes, and the average (SD) was 6.6 ± 1.6.

Table 2. Results of isolation and identification of bovine mastitis pathogens

	Mastitis		Clinical mastitis		Subclinical mastitis	
Pathogen	Isolates (no.)	%	Isolates (no.)	%	Isolates (no.)	%
Staphylococcus aureus	35	13.6	5	12.8	30	13.7
Coagulase-negative staphylococci	73	28.3	7	18.0	66	30.1
Streptococcus agalactiae	9	3.5	—	—	9	4.1
Streptococcus dysgalactiae	40	15.5	5	12.8	35	16.0
Streptococcus uberis	7	2.7	1	2.6	6	2.7
Escherichia coli	27	10.5	5	12.8	22	10.1
Other gram-negative bacilli	14	5.4	4	10.3	10	4.6
Gram-positive bacilli	45	17.3	4	10.3	41	18.7
Yeast	8	3.1	8	20.5	—	—
Total	258	100	39	100	219	100

Table 3. Results of antimicrobial susceptibility tests of *Staphylococcus aureus* isolates (n = 35)

Antibiotics	Resistant, % (no.)	Intermediate, % (no.)	Susceptible, % (no.)
Penicillin G	94.3 (n = 33)	0	5.7 (n = 2)
Ampicillin	94.3 (n = 33)	0	5.7 (n = 2)
Amoxicillin	11.4 (n = 4)	20.0 (n = 7)	68.6 (n = 24)
Oxacillin	0	0	100 (n = 35)
Cephalothin	0	0	100 (n = 35)
Cefoxitin	0	0	100 (n = 35)
Cefradine	0	0	100 (n = 35)
Erythromycin	68.6 (n = 24)	0	31.4 (n = 11)
Azithromycin	68.6 (n = 24)	0	31.4 (n = 11)
Streptomycin	0	5.7 (n = 2)	94.3 (n = 33)
Gentamicin	0	0	100 (n = 35)
Kanamycin	0	0	100 (n = 35)
Amikacin	0	0	100 (n = 35)
Neomycin	0	0	100 (n = 35)
Clindamycin	25.7 (n = 9)	0	74.3 (n = 26)
Doxycycline	0	0	100 (n = 35)
Chloramphenicol	0	0	100 (n = 35)
Sulfamethoxazole	100 (n = 35)	0	0
Ofloxacin	0	0	100 (n = 35)
Norfloxacin	0	0	100 (n = 35)
Ciprofloxacin	0	0	10 (n = 35)
Tetracycline	5.7 (n = 2)	0	94.3 (n = 33)

RAPD Typing of Isolates

The RAPD typing of *Staph. aureus* isolates stated clearly that the 35 *Staph. aureus* isolates from several regions produced distinctive amplicon patterns in the 150- to 1,800-bp range (Figure 2a and b). InfoQuest FP software (Bio-Rad Laboratories, Hercules, CA) was used to do cluster analysis of the isolates, and the genetic relationship cluster map was established (Figure 3). The *Staph. aureus* isolates were clustered into 6 genotypes (I to VI) according to their similarity, (Table 6), 2 of which consisted of 71.4% isolates (25/35), and were prevalent in isolated *Staph. aureus*. Genotypes III and VI were prevalent and observed in all 4 geographic regions studied in Ningxia (Table 6). The results showed that *Staph. aureus* isolates had substantial genotype variation, not only among different farms, but also within the same herd in Ningxia province (Table 7).

DISCUSSION

Staphylococcus aureus is one of the leading pathogens causing intramammary infections in dairy cows, and inducing clinical and subclinical mastitis (Middleton and Fox, 2002; Wang et al., 2009). In previous reports, *Staph. aureus* was initially identified on the basis of phenotypic characteristics including morphological features (coloring, size, hemolysis), Gram staining, and biochemical tests (production of coagulase, catalase, and DNase; fermentation of glucose, maltose, mannitol; and hydrolysis of esculin) (Bautista-Trujillo et al., 2013; Khichar et al., 2014). Recently identification of isolates used to be confirmed by molecular techniques, such as the PCR of 16S rRNA gene of *Staph. aureus* (Lange et al., 2015; Rajic-Savic et al., 2015). In the current study, 35 isolates from 327 mastitis milk samples from 10 dairy farms in Ningxia province of China were

Table 4. Prevalence of virulence genes in *Staphylococcus aureus* isolates (n = 35)

Virulence	Gene	No. of isolates (%)
Clumping factor	*clfA*	27 (77.1)
Fibronectin binding protein	*fnbpA*	33 (94.3)
	fnbpB	28 (80.0)
Hemolysin	*hla*	33 (94.3)
	hlb	34 (97.1)
Thermonuclease	*nuc*	30 (85.7)
Coagulase	*coa*	33 (94.3)
Toxic shock syndrome toxin-1	*tsst-1*	14 (40.0)

Table 5. The virulence gene patterns in *Staphylococcus aureus* isolates (n = 35)

Virulence gene	Isolates (%)
nuc, *coa*, *clfA*, *fnbpA*, *fnbpB*, *hla*, *hlb*, *tsst-1*	8 (22.9)
nuc, *coa*, *clfA*, *fnbpA*, *fnbpB*, *hla*, *hlb*	15 (42.9)
nuc, *coa*, *fnbpA*, *fnbpB*, *hla*, *hlb*, *tsst-1*	4 (11.4)
nuc, *coa*, *clfA*, *fnbpA*, *hla*, *hlb*	2 (5.7)
coa, *fnbpA*, *fnbpB*, *hla*, *hlb*, *tsst-1*	1 (2.9)
coa, *clfA*, *fnbpA*, *hla*, *hlb*	2 (5.7)
coa, *fnbpA*, *hla*, *hlb*	1 (2.9)
nuc, *tsst-1*	1 (2.9)
hlb	1 (2.9)
Mean ± SD	6.6 ± 1.6

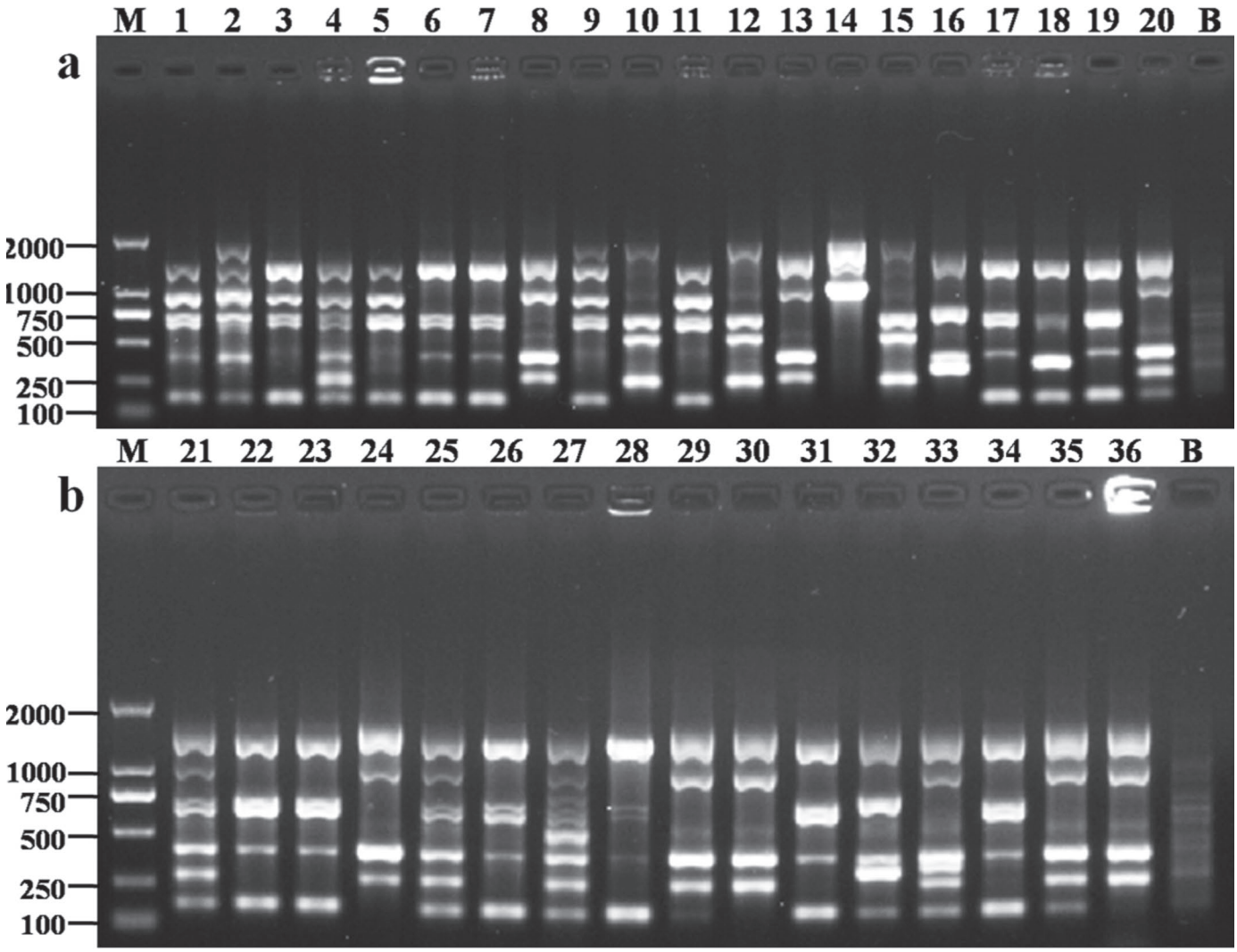

Figure 2. Random amplification of polymorphic DNA (RAPD) tying of *Staphylococcus aureus* isolates. M = DL2000 marker; B = negative control; lanes 1 to 19 (a) and lanes 20 to 35 (b) = *Staph. aureus* isolates; lane 36 = *Staph. aureus* ATCC25923 (control strain).

identified as *Staph. aureus* according to phenotypic characteristics and molecular method.

Because of the poor treatment effect of antibiotics on infections with *Staph. aureus*, it is extremely important to determine the antibiotic sensitivity of the *Staph. aureus* isolates from bovine mastitis, which can be helpful in choosing the most suitable drug for the treatment of the disease. By recognizing the resistance mechanisms, effective measures to control the disease can be established (Saei, 2012; da Costa Krewer et al., 2015). In this study, the results of antimicrobial resistance tests indicated that *Staph. aureus* isolates from cows displayed differences in the antimicrobial susceptibility patterns. The isolates were resistant to sulfamethoxazole, penicillin G, ampicillin, erythromycin, and azithromycin. Similar findings were reported by El Behiry et al. (2012) and Jagielski et al. (2014). It is strongly recommend that penicillin and ampicillin, along with other β-lactam antibiotics, due to 94% drug resistance, should not be used in the treatment of IMI with *Staph. aureus* in dairy cows in Ningxia province of China.

Previous reports indicated that a large number of virulence factors have been found in *Staph. aureus* originating from bovine mastitis cases, including adhesins, hemolysins, thermonuclease, and coagulase (Momtaz et al., 2010; Xu et al., 2015; Kot et al., 2016). In the present study, we observed that most of the *Staph. aureus* isolates harbored the *clfA*, *fnbpA*, *fnbpB*, *hla*, *hlb*, *nuc*, and *coa* genes (77.1 to 97.1%). The ability of *Staph. aureus* to adhere to extracellular matrix proteins is essential for the colonization and establishment of infections and

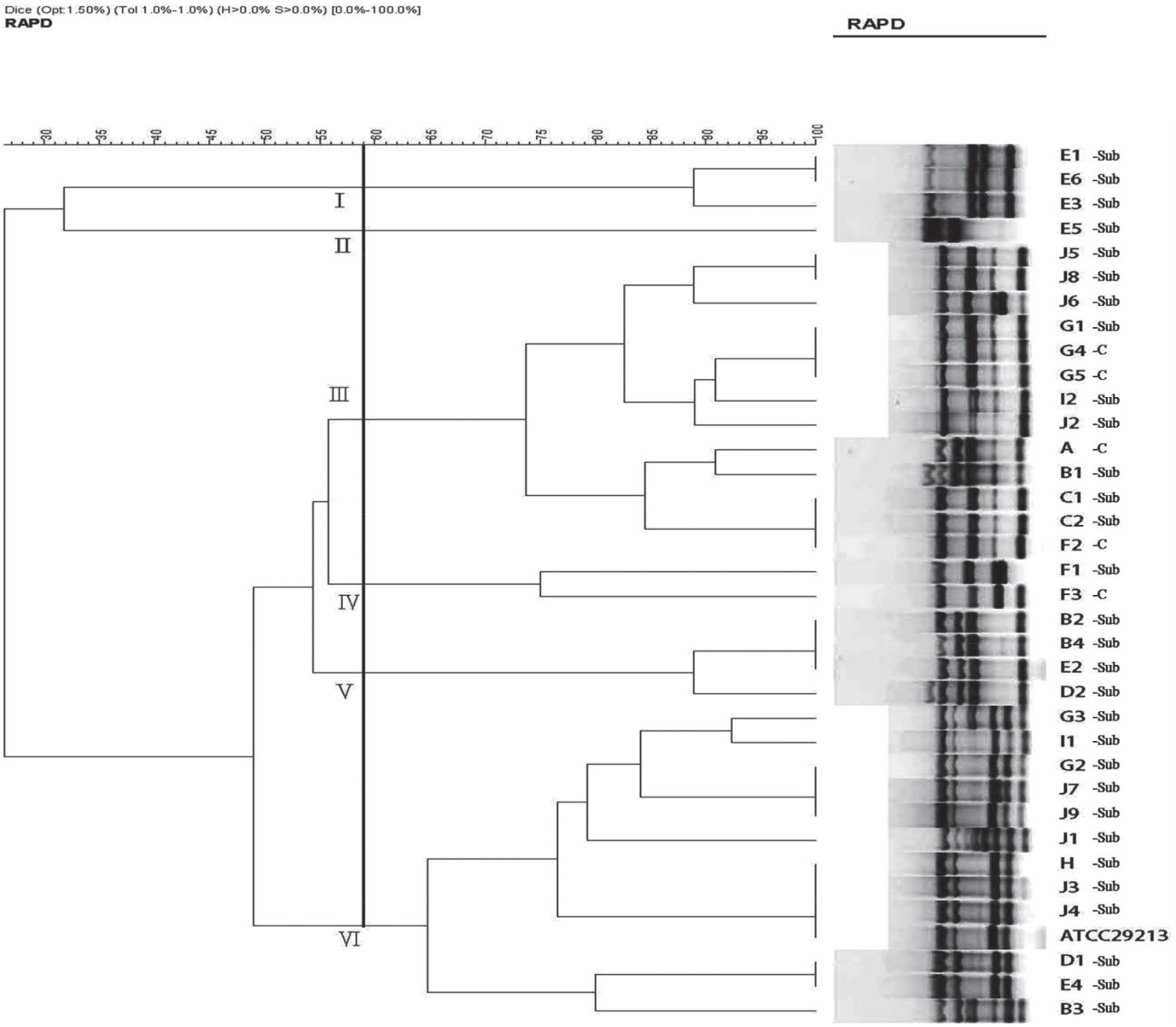

Figure 3. Genetic relationship between *Staphylococcus aureus* isolates (n = 35) from bovine clinical and subclinical mastitis milk, as estimated by clustering analysis of random amplification of polymorphic DNA (RAPD) profiles obtained with the 3 oligonucleotides. The dendrogram was generated by the unweighted pair group method with arithmetic averages. C = clinical mastitis; Sub = subclinical mastitis.

Table 6. Genotype (I to VI) distributions of *Staphylococcus aureus* isolates (n = 35) in different regions of Ningxia

Region	No. of isolates	*Staph. aureus* random amplification of polymorphic DNA typing					
		I	II	III	IV	V	VI
Yinchuan	15	3	1	4	0	4	3
Shizuishan	8	0	0	4	2	0	2
Wuzhong	3	0	0	1	0	0	2
Zhongwei	9	0	0	4	0	0	5
Total	35	3 (8.6%)	1 (2.9%)	13 (37.1%)	2 (5.7%)	4 (11.4%)	12 (34.3%)

Staph. aureus possesses various adhesion genes, such as *clfA*, *fnbpA*, and *fnbB* (Ote et al., 2011; Pereyra et al., 2016). In the current study, *clfA* (77.1%), *fnbpA* (94.3%), and *fnbpB* (80%) were detected in *Staph. aureus* strains isolated from bovine mastitis cases. Staphylococcal hemolysins were identified as important virulence factors that contributed to bacterial invasion and escape from the host immune response (Berube and Bubeck, 2013; Salgado-Pabón et al., 2014; Pereyra et al., 2016). In this study, 94.3% of the total strains had the *hla* gene and 97.1% of the total strains had the *hlb* gene, in agreement with previous reports (Xu et al., 2015; Pereyra et al., 2016). *Staphylococcus aureus* produced an extracellular thermostable nuclease, encoded by the *nuc* gene, whose activity has been used for the rapid and direct detection of *Staph. aureus* (Sahebnasagh et al., 2014; Sudhaharan et al., 2015). The results of this study showed that 85.7% of the *Staph. aureus* isolates contained the *nuc* gene, which was consistent with other studies (Kalorey et al., 2007; Xu et al., 2015). The coagulase gene was one of the most important virulence genes of *Staph. aureus* isolates and the expression of this gene was thought to resist phagocytosis, making the bacteria more virulent (Khoshkharam-Roodmajani et al., 2014; Peetermans et al., 2015). The findings of this study indicated that 94.3% of the *Staph. aureus* isolates harbored the *coa* gene, which was similar to other studies (Khoshkharam-Roodmajani et al., 2014; Moghassem et al., 2015). Toxic shock syndrome toxin 1 (*tsst-1*), a superantigen secreted by *Staph. aureus* in susceptible hosts, was responsible for toxic shock syndrome in humans (Kulhankova et al., 2014; Udo et al., 2016; Zarei Koosha et al., 2016). Currently, we found that 40% of *Staph. aureus* isolates had the *tsst-1* gene, which was in agreement with previous human studies (Udo et al., 2016). However, in a large European studies (Cosandey et al., 2016) involving 10 countries and using 456 *Staph. aureus* isolates, 59 strains were positive for the *tsst-1* gene (12.9%). The prevalence of *tsst-1* gene of *Staph. aureus* isolates was highest in Switzerland (27.9%), whereas in the other countries it varied between 0 and 16.7%.

In the present study, the 35 *Staph. aureus* isolates from 4 geographic regions in Ningxia province of China were grouped into 6 genotypes; 2 particular subtypes (genotype III and genotype VI) were associated with high within-herd prevalence of IMI, which was in accordance with current knowledge (Bardiau et al., 2013). The analysis confirmed that IMI-associated *Staph. aureus* strains are clonal as reported in previous studies (Kock et al., 2013; Vandendriessche et al., 2014). In Morandi et al. (2009) and Costa et al. (2012), genetic heterogeneity was found among multiple isolates from dairy herds, indicating that the *Staph. aureus* population in a single herd can be multiclonal. Furthermore, the genetic diversity of *Staph. aureus* originating from dairy cows can be relatively lower than that in human beings (Reinoso et al., 2004). Rabello et al. (2007) characterized the strains of *Staph. aureus* and investigated that strain heterogeneity within herds and strain homogeneity between herds in different countries. The findings of our experiment proved that the genotype of *Staph. aureus* in milks has distinctive diversity in the *Staph. aureus* population studied and the existence of predominant clones account for most infections within and among herds in Ningxia province.

CONCLUSIONS

A total of 35 isolates obtained from mastitis milk samples in Ningxia province of China were identified as *Staph. aureus* according to phenotypic characteristics and molecular method. The pathogens displayed differences in antimicrobial resistance. The most prevalent virulence genes were *hlb* (97.1%), followed by *fnbpA*, *hla*, and *coa* (94.3% each), *nuc* (85.7%), *fnbpB* (80%), *clfA* (77.1%), and *tsst-1* (40%), respectively. Nine different gene patterns were found, and 3 of them had the

Table 7. Genotype (I to VI) distributions of *Staphylococcus aureus* isolates (n = 35) in different herds

Herd	No. of isolates	*Staph. aureus* random amplification of polymorphic DNA typing					
		I	II	III	IV	V	VI
A	1	0	0	1	0	0	0
B	4	0	0	1	0	2	1
C	2	0	0	2	0	0	0
D	2	0	0	0	0	1	1
E	6	3	1	0	0	1	1
F	3	0	0	1	2	0	0
G	5	0	0	3	0	0	2
H	1	0	0	0	0	0	1
I	2	0	0	1	0	0	1
J	9	0	0	4	0	0	5
Total	35	3 (8.6%)	1 (2.9%)	13 (37.1%)	2 (5.7%)	4 (11.4%)	12 (34.3%)

dominant gene combinations (77.1%). All of the *Staph. aureus* isolates from bovine clinical and subclinical mastitis contained one or more virulence genes, and the average (SD) was 6.6 ± 1.6. The *Staph. aureus* isolates were divided into 6 genotypes by RAPD tying, and genotypes III and VI were the most prevalent genotypes. There was great variation in genotypes in *Staph. aureus* isolates not only among different farms, but also within the same herd in Ningxia province.

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REFERENCES

Ahmady, M., and S. Kazemi. 2013. Detection of the enterotoxigenic genes (sei, sej) in *Staphylococcus aureus* isolates from bovine mastitis milk in the West Azerbaijan of Iran. Comp. Clin. Path. 22:649–654.

Bardiau, M., K. Yamazaki, J. N. Duprez, B. Taminiau, J. G. Mainil, and I. Ote. 2013. Genotypic and phenotypic characterization of methicillin-resistant *Staphylococcus aureus* (MRSA) isolated from milk of bovine mastitis. Lett. Appl. Microbiol. 57:181–186.

Bautista-Trujillo, G. U., J. L. Solorio-Rivera, I. Rentería-Solórzano, S. I. Carranza-Germán, J. A. Bustos-Martínez, R. I. Arteaga-Garibay, V. M. Baizabal-Aguirre, M. Cajero-Juárez, A. Bravo-Patiño, and J. J. Valdez-Alarcón. 2013. Performance of culture media for the isolation and identification of *Staphylococcus aureus* from bovine mastitis. J. Med. Microbiol. 62:369–376.

Berube, B. J., and W. J. Bubeck. 2013. *Staphylococcus aureus* α-toxin: Nearly a century of intrigue. Toxins (Basel) 5:1140–1166.

Brakstad, O. G., K. Aasbakk, and J. A. Maeland. 1992. Detection of *Staphylococcus aureus* by polymerase chain reaction amplification of the nuc gene. J. Clin. Microbiol. 30:1654–1660.

Chiang, Y. C., C. H. Lai, C. W. Lin, C. Y. Chang, and H. Y. Tsen. 2014. Improvement of strain discrimination by combination of superantigen profiles, PFGE, and RAPD for *Staphylococcus aureus* isolates from clinical samples and food-poisoning cases. Foodborne Pathog. Dis. 11:468–477.

Clinical and Laboratory Standards Institute. 2013. Performance Standards for Antimicrobial Disk Susceptibility Tests, CLSI Document M02–A10. Wayne, PA.

Cosandey, A., R. Boss, M. Luini, K. Artursson, M. Bardiau, F. Breitenwieser, E. Hehenberger, T. Lam, M. Mansfeld, A. Michel, G. Mösslacher, J. Naskova, S. Nelson, O. Podpečan, A. Raemy, E. Ryan, O. Salat, P. Zangerl, A. Steiner, and H. U. Graber. 2016. *Staphylococcus aureus* genotype B and other genotypes isolated from cow milk in European countries. J. Dairy Sci. 99:529–540.

Costa, G. M., L. V. Paiva, H. C. Figueiredo, A. R. Figueira, U. P. Pereira, and N. Silva. 2012. Population diversity of *Staphylococcus aureus* isolated from bovine mastitis in Brazilian dairy herds. Res. Vet. Sci. 93:733–735.

da Costa Krewer, C., E. S. Amanso, G. V. Gouveia, R. L. Souza, M. M. Costa, and R. A. Mota. 2015. Resistance to antimicrobials and biofilm formation in *Staphylococcus* spp. isolated from bovine mastitis in the Northeast of Brazil. Trop. Anim. Health Prod. 47:511–518.

Deb, R., A. Kumar, S. Chakraborty, A. K. Verma, R. Tiwari, K. Dhama, U. Singh, and R. Kumar. 2013. Trends in diagnosis and control of bovine mastitis: A review. Pak. J. Biol. Sci. 16:1653–1661.

El Behiry, A. E., G. Schlenker, I. Szabo, and U. Roesler. 2012. In vitro susceptibility of *Staphylococcus aureus* strains isolated from cows with subclinical mastitis to different antimicrobial agents. J. Vet. Sci. 13:153–161.

Fijałkowski, K., M. Struk, J. Karakulska, A. Paszkowska, S. Giedrys-Kalemba, H. Masiuk, D. Czernomysy-Furowicz, and P. Nawrotek. 2014. Comparative analysis of superantigen genes in *Staphylococcus xylosus* and *Staphylococcus aureus* isolates collected from a single mammary quarter of cows with mastitis. J. Microbiol. 52:366–372.

Gomes, F., and M. Henriques. 2016. Control of bovine mastitis: Old and recent therapeutic approaches. Curr. Microbiol. 72:377–382.

Gutiérrez, D., A. M. Martín-Platero, A. Rodríguez, M. Martínez-Bueno, P. García, and B. Martínez. 2011. Typing of bacteriophages by randomly amplified polymorphic DNA (RAPD)-PCR to assess genetic diversity. FEMS Microbiol. Lett. 322:90–97.

Jagielski, T., E. Puacz, A. Lisowski, P. Siedlecki, W. Dudziak, J. Międzobrodzki, and H. Krukowski. 2014. Antimicrobial susceptibility profiling and genotyping of *Staphylococcus aureus* isolates from bovine mastitis in Poland. J. Dairy Sci. 97:6122–6128.

Jönsson, K., C. Signäs, H. P. Müller, and M. Lindberg. 1991. Two different genes encode fibronectin binding proteins in *Staphylococcus aureus*. The complete nucleotide sequence and characterization of the second gene. Eur. J. Biochem. 202:1041–1048.

Kalorey, D. R., Y. Shanmugam, N. V. Kurkure, K. K. Chousalkar, and S. B. Barbuddhe. 2007. PCR-based detection of genes encoding virulence determinants in *Staphylococcus aureus* from bovine subclinical mastitis cases. J. Vet. Sci. 8:151–154.

Khichar, V., A. K. Kataria, and R. Sharma. 2014. Characterization of *Staphylococcus aureus* of cattle mastitis origin for two virulence-associated genes (*coa* and *spa*). Comp. Clin. Pathol. 23:603–611.

Khoshkharam-Roodmajani, H., J. Sarvari, A. Bazargani, M. R. Kandekar-Ghahraman, A. Nazari-Alam, and M. Motamedifar. 2014. Molecular typing of methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* isolates from Shiraz teaching hospitals by PCR-RFLP of coagulase gene. Iran. J. Microbiol. 6:246–252.

Kock, R., F. Schaumburg, A. Mellmann, M. Koksal, A. Jurke, K. Becker, and A. W. Friedrich. 2013. Livestock-associated methicillin-resistant *Staphylococcus aureus* (MRSA) as causes of human infection and colonization in Germany. PLoS One 8:e55040.

Kot, B., P. Szweda, A. Frankowska-Maciejewska, M. Piechota, and K. Wolska. 2016. Virulence gene profiles in *Staphylococcus aureus* isolated from cows with subclinical mastitis in eastern Poland. J. Dairy Res. 83:228–235.

Kulhankova, K., J. King, and W. Salgado-Pabón. 2014. Staphylococcal toxic shock syndrome: Superantigen-mediated enhancement of endotoxin shock and adaptive immune suppression. Immunol. Res. 59:182–187.

Lange, C. C., M. A. Brito, D. R. Reis, M. A. Machado, A. S. Guimarães, A. L. Azevedo, É. B. Salles, M. C. Alvim, F. S. Silva, and I. R. Meurer. 2015. Species-level identification of staphylococci isolated from bovine mastitis in Brazil using partial 16S rRNA sequencing. Vet. Microbiol. 176:382–388.

Løvseth, A., S. Loncarevic, and K. G. Berdal. 2004. Modified multiplex PCR method for detection of pyrogenic exotoxin genes in staphylococcal isolates. J. Clin. Microbiol. 42:3869–3872.

Lundberg, Å., A. K. Nyman, A. Aspán, S. Börjesson, H. E. Unnerstad, and K. P. Waller. 2016. Udder infections with *Staphylococcus aureus*, *Streptococcus dysgalactiae*, and *Streptococcus uberis* at calving in dairy herds with suboptimal udder health. J. Dairy Sci. 99:2102–2117.

Middleton, J. R., and L. K. Fox. 2002. Influence of *Staphylococcus aureus* strain on mammary quarter milk production. Vet. Rec. 150:411–413.

Middleton, J. R., L. K. Fox, J. M. Gay, J. W. Tyler, and T. E. Besser. 2002. Use of pulsed-field gel electrophoresis for detecting differences in *Staphylococcus aureus* strain populations between dairy herds with different cattle importation practices. Epidemiol. Infect. 129:387–395.

Moghassem, H. R., S. Hosseinzadeh, S. S. Shekarforoush, M. Poormontaseri, and A. Derakhshandeh. 2015. Association between the enterotoxin production and presence of *coa, nuc* genes among *Staphylococcus aureus* isolated from various sources in Shiraz. Iran J. Vet. Res. (Faisalabad) 16:381–384.

Momtaz, H., E. Rahimi, and E. Tajbakhsh. 2010. Detection of some virulence factors in *Staphylococcus aureus* isolated from clinical and subclinical bovine mastitis in Iran. Afr. J. Biotechnol. 25:3753–3758.

Morandi, S., M. Brasca, C. Andrighetto, A. Lombardi, and R. Lodi. 2009. Phenotypic and genotypic characterization of *Staphylococcus aureus* strains from Italian dairy products. Int. J. Microbiol. 2009:501362.

Ote, I., B. Taminiau, J. Duprez, I. Dizier, and J. G. Mainil. 2011. Genotypic characterization by polymerase chain reaction of *Staphylococcus aureus* isolates associated with bovine mastitis. Vet. Microbiol. 153:285–292.

Peetermans, M., P. Verhamme, and T. Vanassche. 2015. Coagulase activity by *Staphylococcus aureus*: A potential target for therapy? Semin. Thromb. Hemost. 41:433–444.

Pereyra, E. A., F. Picech, M. S. Renna, C. Baravalle, C. S. Andreotti, R. Russi, L. F. Calvinho, C. Diez, and B. E. Dallard. 2016. Detection of *Staphylococcus aureus* adhesion and biofilm-producing genes and their expression during internalization in bovine mammary epithelial cells. Vet. Microbiol. 183:69–77.

Puacz, E., W. M. Ilczyszyn, M. Kosecka, A. Buda, W. Dudziak, K. Polakowska, T. Panz, A. Białecka, A. Kasprowicz, A. Lisowski, H. Krukowski, V. Cuteri, and J. Międzobrodzki. 2015. Clustering of *Staphylococcus aureus* bovine mastitis strains from regions of Central-Eastern Poland based on their biochemical and genetic characteristics. Pol. J. Vet. Sci. 18:333–342.

Rabello, R. F., B. M. Moreira, R. M. Lopes, L. M. Teixeira, L. W. Riley, and A. C. Castro. 2007. Multilocus sequence typing of *Staphylococcus aureus* isolates recovered from cows with mastitis in Brazilian dairy herds. J. Med. Microbiol. 56:1505–1511.

Rajic-Savic, N., V. Katic, B. Velebit, and S. Colovic. 2015. Characteristics of enterotoxigenic coagulase positive staphylococci isolated from bovine milk in cases of subclinical mastitis. Acta Vet. (Beogr.) 5:250–253.

Reinoso, E., S. Bettera, C. Frigerio, M. DiRenzo, A. Calzolari, and C. Bogni. 2004. RAPD-PCR analysis of *Staphylococcus aureus* strains isolated from bovine and human hosts. Microbiol. Res. 159:245–255.

Saei, H. D. 2012. *Coa* types and antimicrobial resistance profile of *Staphylococcus aureus* isolates from cases of bovine mastitis. Comp. Clin. Pathol. 21:301–307.

Sahebnasagh, R., H. Saderi, and P. Owlia. 2014. The prevalence of resistance to methicillin in *Staphylococcus aureus* strains isolated from patients by PCR method for detection of mecA and nuc genes. Iran. J. Public Health 43:84–92.

Salasia, S. I., Z. Khusnan, C. Lammler, and M. Zschock. 2004. Comparative studies on pheno- and genotypic properties of *Staphylococcus aureus* isolated from bovine subclinical mastitis in central Java in Indonesia and Hesse in Germany. J. Vet. Sci. 5:103–109.

Salgado-Pabón, W., A. Herrera, B. G. Vu, C. S. Stach, J. A. Merriman, A. R. Spaulding, and P. M. Schlievert. 2014. *Staphylococcus aureus* β-toxin production is common in strains with the β-toxin gene inactivated by bacteriophage. J. Infect. Dis. 210:784–792.

SenGupta, D. J., L. A. Cummings, D. R. Hoogestraat, S. M. Butler-Wu, J. Shendure, B. T. Cookson, and S. J. Salipante. 2014. Whole-genome sequencing for high-resolution investigation of methicillin-resistant *Staphylococcus aureus* epidemiology and genome plasticity. J. Clin. Microbiol. 52:2787–2796.

Signäs, C., G. Raucci, K. Jönsson, P. E. Lindgren, G. M. Anantharamaiah, M. Höök, and M. Lindberg. 1989. Nucleotide sequence of the gene for a fibronectin-binding protein from *Staphylococcus aureus*: Use of this peptide sequence in the synthesis of biologically active peptides. Proc. Natl. Acad. Sci. USA 86:699–703.

Sudhaharan, S., L. Vanjari, N. Mamidi, N. Ede, and L. Vemu. 2015. Evaluation of LAMP assay using phenotypic tests and conventional PCR for detection of nuc and mecA genes among clinical isolates of *Staphylococcus* spp. J. Clin. Diagn. Res. 9:DC06–DC09.

Udo, E. E., S. S. Boswihi, and N. Al-Sweih. 2016. High prevalence of toxic shock syndrome toxin-producing epidemic methicillin-resistant *Staphylococcus aureus* 15 (EMRSA-15) strains in Kuwait hospitals. New Microbes New Infect. 12:24–30.

Vandendriessche, S., W. Vanderhaeghen, J. Larsen, R. de Mendonca, M. Hallin, P. Butaye, K. Hermans, F. Haesebrouck, and O. Denis. 2014. High genetic diversity of methicillin-susceptible *Staphylococcus aureus* (MSSA) from humans and animals on livestock farms and presence of SCCmec remnant DNA in MSSA CC398. J. Antimicrob. Chemother. 69:355–362.

Wang, D., Z. C. Wang, Z. T. Yan, J. Y. Wu, T. Ali, J. J. Li, Y. L. Lv, and B. Han. 2015. Bovine mastitis *Staphylococcus aureus*: Antibiotic susceptibility profile, resistance genes and molecular typing of methicillin-resistant and methicillin-sensitive strains in China. Infect. Genet. Evol. 31:9–16.

Wang, S. C., C. M. Wu, S. C. Xia, Y. H. Qi, L. N. Xia, and J. Z. Shen. 2009. Distribution of superantigenic toxin genes in *Staphylococcus aureus* isolates from milk samples of bovine subclinical mastitis cases in two major dairy production regions of China. Vet. Microbiol. 137:276–281.

Xu, J., X. Tan, X. Zhang, X. Xia, and H. Sun. 2015. The diversities of staphylococcal species, virulence and antibiotic resistance genes in the subclinical mastitis milk from a single Chinese cow herd. Microb. Pathog. 88:29–38.

Yang, F. L., X. S. Li, X. W. Liang, X. F. Zhang, G. S. Qin, and B. Z. Yang. 2012. Detection of virulence associated genes in *Staphylococcus aureus* isolated from bovine clinical mastitis milk samples in Guangxi. Trop. Anim. Health Prod. 44:1821–1826.

Zadoks, R. N., J. R. Middleton, S. McDougall, J. Katholm, and Y. H. Schukken. 2011. Molecular epidemiology of mastitis pathogens of dairy cattle and comparative relevance to humans. J. Mammary Gland Biol. Neoplasia 16:357–372.

Zarei Koosha, R., H. H. Mahmoodzadeh, A. E. Mehdizadeh, T. S. Ghorbani, and F. A. A. Imani. 2016. Distribution of *tsst-1* and *mec*A genes in *Staphylococcus aureus* isolated from clinical specimens. Jundishapur J. Microbiol. 9:e29057.