Relationships among superantigen toxin gene profiles, genotypes, and pathogenic characteristics of *Staphylococcus aureus* isolates from bovine mastitis

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ABSTRACT

Staphylococcus aureus is one of the major etiological agents of bovine mastitis, harboring a wide variety of staphylococcal superantigen (SAg) toxin genes. The SAg toxin genes are reported to be closely associated with the pathogenicity of the *Staph. aureus* causing the bovine mastitis. This study was conducted to investigate SAg toxin gene profiles and to assess the relationships among SAg toxin genes, genotypes of *Staph. aureus*, and their pathogenic properties. A total of 327 quarter milk samples were collected from bovine mastitis cases for isolation and identification of pathogens. In total, 35 isolates were identified as *Staph. aureus*, and the prevalence of *Staph. aureus* in milk samples was 13.6% (35/256). Polymerase chain reaction (PCR) and randomly amplified polymorphic DNA (RAPD) assays were used to detect the SAg toxin genes and to genotype *Staph. aureus* strains isolated from milk samples of bovine mastitis in 10 dairy herds located in Ningxia, China, respectively. The results showed that among the *Staph. aureus* isolates ($n = 35$), 71.4% ($n = 25$) of isolates carried at least one SAg toxin gene. In total, 18 SAg genes and 21 different gene combination patterns were detected among these isolates. The most common SAg genes in *Staph. aureus* isolates were *sei*, *sen*, and *seu* (44.0% each), followed by *seo*, *tst*, and *etB* (28.0% each), *etA* (24.0%), *sem* and *sep* (16.0% each), *seb*, *sec*, *sed*, and *sek* (12.0% each), and *sea* and *seh* genes (8.0% each); the *seg*, *sej*, and *ser* genes were present in 4.0% of the isolates. Three gene combinations were found to be related to mobile genetic elements that carried 2 or more genes. The egc-cluster of the

seg-sei-sem-sen-seo genes, located on the pathogenicity island Type I ν Sa β , was detected in 16% of isolates. Interestingly, we observed 6 RAPD genotypes (I to VI) in *Staph. aureus* isolates, and 2 of these genotypes were strongly associated with the severity of bovine mastitis; there was a close relationship between the RAPD genotypes and SAg genes. Isolates of RAPD type III were more frequently associated with clinical and subclinical mastitis, whereas strains of type VI were mostly related to subclinical mastitis. In addition, SAg genes were related to severity of bovine mastitis. We conclude that an obvious relationship exists among RAPD genotypes, SAg toxin genes, and severity of bovine mastitis.

Key words: *Staphylococcus aureus*, bovine mastitis, superantigen toxin genes, PCR

INTRODUCTION

Staphylococcus aureus is a global pathogen causing several serious diseases in humans and animals. This microorganism is also one of the most common etiological agents of bovine mastitis, which results in huge economic losses to the dairy industry due to the reduced milk quality and production (Deb et al., 2013; Gomes and Henriques, 2016). Importantly, the enterotoxins produced by *Staph. aureus* are common causes of food poisoning globally (Li et al., 2015; Sabouni et al., 2014). *Staphylococcus aureus* has a strong capability to produce a wide variety of enterotoxins, including toxic shock syndrome toxin 1 (TSST-1), Panton-Valentine leukocidin (PVL), exfoliative toxins A and B (ETA and ETB), and staphylococcal enterotoxins (SE) including classical enterotoxins (SEA to SEE) with proven emetic activity and newly described enterotoxins, such as staphylococcal-like proteins (SEIL, SEIQ, SEG to SER) without emetic activity. To date, 23 serologically different staphylococcal enterotoxin and staphylococ-

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cal-like proteins have been recognized (Pinheiro et al., 2015; Podkowik et al., 2016). Collectively, these toxins are known as staphylococcal superantigens (**SAg**) because of their superantigenic activity. These SAg toxin genes have been reported to be associated with the virulence factors in bovine IMI, and these toxins may have an important role in the pathogenesis of bovine mastitis (Aman and Adhikari, 2014; Reyes-Robles et al., 2016). Recent studies have reported that SAg toxin genes are associated with mobile genetic elements such as plasmids, pathogenicity islands, and prophages, suggesting the horizontal transfer of these genes among the bacteria (Malachowa and DeLeo, 2010; Alibayov et al., 2014). In addition, staphylococcal food poisoning, resulting from the consumption of contaminated food with staphylococcal enterotoxins, is one of the most common food-borne illnesses (Suzuki et al., 2015; Corredor Arias et al., 2016).

Considerable genetic heterogeneity in natural populations of *Staph. aureus* has been documented, and a small number of clonal types not only cause the majority of bovine IMI but also have a wide geographical distribution (Silva et al., 2013; Lundberg et al., 2014). As different pathogenic properties of the pathogens require different strategies toward the prevention and treatment of the disease, research on the genotypes of *Staph. aureus* causing bovine IMI and their pathogenic properties is of particular clinical importance.

Although a large number of virulence factors have been found in *Staph. aureus* isolates from bovine IMI, very few studies have investigated the relationships between subtypes of *Staph. aureus* isolates from bovine IMI and their pathogenic properties. Thus, the present study was carried out to determine SAg toxin genes profiles and randomly amplified polymorphic DNA (**RAPD**) genotypes of *Staph. aureus* isolates and to analyze the relationships among SAg toxin genes, genotypes, and pathogenic characteristics of *Staph. aureus* isolates from bovine mastitis in the dairy herds of Ningxia province of China.

MATERIALS AND METHODS

Bacterial Isolates

Previously isolated *Staph. aureus* isolates ($n = 35$) from cases of bovine mastitis (Wang et al., 2016) were used in the present study. A total of 327 quarter milk samples were collected from cows with clinical and subclinical mastitis between March and October 2013, which came from 10 herds in 4 geographic regions (Yinchuan, Wuzhong, Shizuishan, and Zhongwei) of Ningxia province (Figure 1), located in northwest China. The

majority of cows in the study herds were Chinese Holstein and were housed in freestall barns with straw, sawdust, or sand as bedding. The average size of the herds was 253 cows, and their average yield was 8,540 kg of milk per lactation. A total of 2,527 cows were involved in the epidemiological investigation of bovine mastitis. Among them, healthy cows accounted for 50.3% (1,272/2,527), the prevalence of clinical mastitis was 13.2% (333/2,527), and the incidence of subclinical mastitis at the cow and quarter level were 36.5% (922/2,527) and 17.8% (1,794/10,108), respectively. Bacterial isolates were identified based on morphological, biochemical, and molecular methods as described previously (Wang et al., 2016). Briefly, after incubation on blood agar, the isolates produced grayish-white or yellow colonies with distinct zones of hemolysis. They were gram-positive cocci and produced catalase, coagulase, and DNase. All the isolates were confirmed by using the PCR amplification of 16S rRNA gene specific to *Staph. aureus*.

DNA Isolation and Purification

Each *Staph. aureus* isolate was grown overnight at 37°C in brain heart infusion broth (Oxoid, Basingstoke, UK). The genomic DNA of the isolate was extracted and purified using TIANamp Bacteria DNA Kit (Tiangen Biotech, Beijing, China) according to the manufacturer's instruction.

Detection of SAg Toxin Genes

The presence of genes encoding enterotoxins (*se*), TSST-1(*tst*), Panton-Valentine leukocidin (*PVL*), and exfoliative toxins (*etA* and *etB*) in the entire set of *Staph. aureus* isolates ($n = 35$) was tested by PCR assays. The SAg toxin genes, primer sequences, and the expected size of PCR products are shown in Table 1. The reaction mixture (25 μ L) for each PCR contained 50 ng of genomic DNA, 20 pmol of each primer, and 12.5 μ L of 2 $\times$ Taq PCR MasterMix (Tiangen Biotech). Amplification was carried out with the following cycling conditions: a predenaturation step at 94°C for 3 min, followed by 30 cycles of amplification (denaturation at 94°C for 30 s, annealing for 30 s at specific temperature, and extension at 72°C for 1 min), and ending with a final extension at 72°C for 5 min. All PCR-amplified products were characterized by 0.8% agarose gel electrophoresis in 1 $\times$ Tris-acetate-EDTA buffer, followed by staining with Gel Red and visualization under UV light. The *Staph. aureus* strain ATCC29213 was used as the positive control and the negative control (only sterile water) was used in each PCR reaction.

Figure 1. Map of China showing Ningxia province and indicating the 4 geographic regions from where bovine milk samples were collected.

Table 1. The primers for PCR amplification of staphylococcal superantigen (SAg) toxin genes in *Staphylococcus aureus* isolates

Gene	Primer sequence ¹ (5'–3')	Product (bp)	Reference
<i>sea</i>	F: GCAGGGAACAGCTTTAGGC R: GTTCTGTAGAAGTATGAAACACG	521	Løvseth et al. (2004)
<i>seb</i>	F: ACATGTAATTTTGATATTCGCACTG R: TGCAGGCATCATGTCATACCA	667	Løvseth et al. (2004)
<i>sec</i>	F: AGATGAAGTAGTTGATGTGTATGG R: CACACTTTTAGAATCAACCG	451	Mehrotra et al. (2000)
<i>sed</i>	F: GTGGTGAAATAGATAGGACTGC R: ATATGAAGGTGCTCTGTGG	385	Løvseth et al. (2004)
<i>see</i>	F: TACCAATTAAGTTGTGGATAGAC R: CTCTTTGCACCTTACCGC	171	Løvseth et al. (2004)
<i>seg</i>	F: CGTCTCCACCTGTTGAAGG R: CCAAGTGATTGTCTATTGTCTG	328	Løvseth et al. (2004)
<i>seh</i>	F: CAACTGCTGATTTAGCTCAG R: GTCGAATGAGTAATCTCTAGG	359	Løvseth et al. (2004)
<i>sei</i>	F: CAACTCGAATTTTCAACAGGTACC R: CAGGCAGTCCATCTCCTG	466	Løvseth et al. (2004)
<i>sej</i>	F: CATCAGAACTGTTGTTCCGCTAG R: CTGAATTTTACCATCAAAGGTAC	142	Løvseth et al. (2004)
<i>sek</i>	F: TAGGTGTCTCTAATAATGCCA R: TAGATATTCGTTAGTAGCTG	293	Omoe et al. (2005)
<i>sel</i>	F: ATGAAAAAAGATTATTATTGTAAATTGTTATTAC R: ATCATCTTTTTGAAATTTTCGACATCTAG	737	Sergeev et al. (2004)
<i>sem</i>	F: ATGAAAAGAATACTTATCATTGTTGTTTATTG R: CTTCAACTTTTCGTCCTTATAAGATATTTT	720	Sergeev et al. (2004)
<i>sen</i>	F: ATAAAAAATATTAATAAAGCTTATGAGATTGTTC R: ACTTAATCTTTATATAAATAACATCAATATG	777	Sergeev et al. (2004)
<i>seo</i>	F: TGTGTAAGAAGTCAAGTGAG R: TCTTTAGAAATCGCTGATGA	214	Omoe et al. (2005)
<i>sep</i>	F: TGATTTATTAGTAGACCTTGG R: ATAACCAACCGAATCACCAG	396	Omoe et al. (2005)
<i>seq</i>	F: GGAAAATACACTTTATATTCACAGTTTCA R: ATTTATTCAGTTTCTCATATGAAATCTC	539	Sergeev et al. (2004)
<i>ser</i>	F: GGATAAAGCGGTAATAGCAG R: GTATTCCAAACACATCTAAC	166	Omoe et al. (2005)
<i>seu</i>	F: ATTTGCTTTTATCTTCAT R: GGACTTTAATGTTTGTCTCTGAT	167	Chiang et al. (2008)
<i>tst</i>	F: GCTTGCGACAACCTGCTACAG R: TGGATCCGTCATTCAATTGTTAT	559	Løvseth et al. (2004)
<i>PVL</i>	F: ATCATTAGGTAAAAATGTCTGGACATGATCCA R: GCATCAAGTGATTGGATAGCAAAAGC	433	McClure et al. (2006)
<i>etA</i>	F: CTAGTGCATTGTATTCAA R: TGCATTGACACCATAGTACT	119	Johnson et al. (1991)
<i>etB</i>	F: ACAAGCAAAAGAATACAGCG R: GTTTTTGGCTGCTTCTCTTG	226	Mehrotra et al. (2000)

¹F = forward; R = reverse.

RAPD-Based Genotyping of *Staph. aureus* Isolates

A PCR-based RAPD analysis was carried out for the genotyping of all the *Staph. aureus* isolates as described in our previous study (Wang et al., 2016).

Relationship Between Genotypes, SAg Genes of *Staph. aureus*, and Severity of Bovine Mastitis

Bovine mastitis was clinically categorized into clinical and subclinical mastitis based on clinical signs of the

disease. Clinical mastitis was defined as the presence of visible abnormalities in the milk (such as watery milk, clots or flakes) and observation of abnormal changes in the udder such as swelling, pain, or heat (Ahmady and Kazemi, 2013; Artursson et al., 2016). Subclinical mastitis was diagnosed when the SCC was greater than 200,000 cells/mL in the milk with little or no overt changes in the milk or udder (Xu et al., 2015; Aslantas and Demir, 2016). The SAg toxin genes and RAPD genotypes of *Staph. aureus* isolates were co-related to the severity of bovine mastitis in terms of epidemiological and clinical data from 10 herds.

RESULTS

Prevalence of SAg Toxin Genes in Staph. aureus Isolates

The presence of 22 SAg genes in *Staph. aureus* isolates was tested by PCR assay, as shown in Table 2. Among the 35 *Staph. aureus* isolates, 25 (71.4%) harbored at least one SAg gene. A total of 18 SAg toxin genes were detected in these isolates; however, *see*, *sel*, *seq*, and *PVL* genes were not detected (Figure 2a and b). The most common SAg genes in the *Staph. aureus* isolates were *sei*, *sen*, and *seu* (44.0% each), whereas *seo*, *tst*, and *etB* (28.0% each) and *etA* (24.0%) genes were carried by approximately one-fourth of the isolates. The *sem* and *sep* genes were PCR amplified in 16.0% of the isolates, whereas *seb*, *sec*, *sed*, and *sek* were detected in 12.0% of the isolates. The *sea* and *seh* genes (8.0% each) and *seg*, *sej*, and *ser* genes (4.0% each) were present only within a few isolates (Table 2).

Genotyping of SAg Toxin Genes in Staph. aureus Isolates

Twenty-one different combinations of toxin genes were detected in 25 *Staph. aureus* isolates positive for SAg toxin genes. Genotypes carrying the gene pattern *sei-sen-selu* were identified in 2 (8.0%) isolates; the *etA* gene was found in 2 (8.0%) isolates, and 3 (12.0%) isolates were found positive for *etB*, whereas the other genes were confirmed only in individual strains (Table

Table 2. Prevalence of staphylococcal superantigen (SAg) toxin genes in *Staphylococcus aureus* isolates (n = 25)

Virulence determinant	Gene	Prevalence (%)
Classical staphylococcal enterotoxins	<i>sea</i>	2 (8.0)
	<i>seb</i>	3 (12.0)
	<i>sec</i>	3 (12.0)
	<i>sed</i>	3 (12.0)
	<i>see</i>	0
New staphylococcal enterotoxins	<i>seg</i>	1 (4.0)
	<i>seh</i>	2 (8.0)
	<i>sei</i>	11 (44.0)
	<i>sej</i>	1 (4.0)
	<i>sek</i>	3 (12.0)
	<i>sel</i>	0
	<i>sem</i>	4 (16.0)
	<i>sen</i>	11 (44.0)
	<i>seo</i>	7 (28.0)
	<i>sep</i>	4 (16.0)
	<i>seq</i>	0
	<i>ser</i>	1 (4.0)
	<i>seu</i>	11 (44.0)
	<i>tst</i>	7 (28.0)
Panton-Valentine leukocidin	<i>PVL</i>	0
Exfoliative toxin	<i>etA</i>	6 (24.0)
	<i>etB</i>	7 (28.0)

Figure 2. Polymerase chain reaction amplification of staphylococcal superantigen (SAg) toxin genes in *Staphylococcus aureus* isolates. (a) M = molecular size marker, 1 = *etA*, 2 = *sek*, 3 = *sea*, 4 = *see*, 5 = *sec*, 6 = *sel*, 7 = *sen*, 8 = *seh*, 9 = *ser*, 10 = *sem*, 11 = *sei*, 12 = *sed*, 13 = *sej*, 14 = *seb*, 15 = *seo*, 16 = *sep*, 17 = *tst*, 18 = negative control; (b) M = molecular size marker, 1 = *etA*, 2 = *etB*, 3 = *PVL*, 4 = *etB*, 5 = *seb*, 6 = *sej*, 7 = *sei*, 8 = *sen*, 9 = *seo*, 10 = *sep*, 11 = *sel*, 12 = *sed*, 13 = *sec*, 14 = *seb*, 15 = *seo*, 16 = *sep*, 17 = *tst*, 18 = negative control.

Table 3. The staphylococcal superantigen (SAg) toxin genotypes in *Staphylococcus aureus* isolates (n = 25)

SAg toxin gene pattern	Prevalence (%)	Mobile genetic elements
Classical staphylococcus enterotoxin (SE) genes	0	
Classical and new SE genes	8 (32.0)	
<i>sed, sei, sem, sen, seo, seu, sep, tst, etB</i>	1 (4.0)	Type IuSa β , ϕ Sa3n
<i>sed, sei, sem, sen, seo, seu, sep, tst</i>	1 (4.0)	Type IuSa β , ϕ Sa3n
<i>sea, sei, sem, sen, seo, seu, tst</i>	1 (4.0)	ϕ Sa3mu, Type IuSa β
<i>sea, seg, sei, sen, seu</i>	1 (4.0)	ϕ Sa3mu
<i>seb, sec, seh, etA</i>	1 (4.0)	
<i>seb, sec, eta</i>	1 (4.0)	
<i>seb, sek, sec, etA</i>	1 (4.0)	vSa1(SaPI3)
<i>sed, sep</i>	1 (4.0)	ϕ Sa3n
New SE genes	12 (48.0)	
<i>sei, sem, sen, seo, seu, tst</i>	1 (4.0)	Type IuSa β
<i>sei, sen, seo, seu, tst, etB</i>	1 (4.0)	
<i>sen, seu, sep, etB</i>	1 (4.0)	ϕ Sa3n
<i>sek, sei, sen, seo, seu, tst, etA</i>	1 (4.0)	
<i>sei, sen, seu</i>	2 (8.0)	
<i>seh, sek, tst</i>	1 (4.0)	
<i>sej, ser</i>	1 (4.0)	pF5
<i>sei, etB</i>	1 (4.0)	
<i>sei, seu</i>	1 (4.0)	
<i>sen</i>	1 (4.0)	
<i>seo</i>	1 (4.0)	
Other toxin genes	5 (20.0)	
<i>etA</i>	2 (8.0)	
<i>etB</i>	3 (12.0)	

3). Interestingly, 8 (32.0%) *Staph. aureus* isolates contained both classical and new staphylococcal enterotoxin genes, whereas 12 (48.0%) isolates carried only new staphylococcal enterotoxin genes. Five (20.0%) isolates were positive for *etA* and *etB* genes; however, isolates harboring only classical staphylococcal enterotoxin genes were absent.

SAg Toxin Genotypes and Mobile Genetic Elements

Three gene combinations related to mobile genetic elements, which contained 2 or more SAg toxin genes, were detected (Table 3). The *egc*-cluster of the *seg-sei-sem-sen-seo* genes, being located on the pathogenicity island Type I vSa β , was detected in 16.0% of the *Staph. aureus* isolates that lacked the *seg* gene. The *seb-selk-selq* gene combination, located in the vSa1(SaPI3) pathogenicity island, was carried by 4.0% of the isolates without the *seg* gene. Additionally, the *sej-ser* gene combination located on the same plasmid (pF5) was present in 4.0% of the isolates. The *sea* gene existing in the phage ϕ Sa3mu was detected in 8.0% of the isolates, and the *sep* gene, related to phage ϕ Sa3n, was found in 16.0% of the *Staph. aureus* isolates.

RAPD Genotypes and Severity of Bovine Mastitis

Based on PCR-based RAPD analyses, the 35 *Staph. aureus* strains isolated from bovine IMI were grouped

into 6 RAPD genotypes (Table 4). Epidemiological data regarding bovine mastitis were obtained from the herds from which quarter milk samples were collected. The 35 *Staph. aureus* strains isolated from milk samples were categorized into subclinical and clinical mastitis based on clinical symptoms of the disease (Table 4). The prevalence of *Staph. aureus* strains belonging to RAPD type III (80.0%) was much higher than that of the RAPD type IV (20.0%) in clinical mastitis; thus, RAPD type III strains were more frequently associated with clinical mastitis. In contrast, RAPD type VI (40.0%) and type III (30.0%) strains were more frequently related to subclinical mastitis.

Table 4. The relationship between randomly amplified polymorphic DNA (RAPD) genotypes and the severity of bovine mastitis in *Staphylococcus aureus* isolates (n = 35) from bovine mastitis

RAPD genotype	Prevalence (%)	Severity	
		Clinical (%)	Subclinical (%)
I	3 (8.6)		3 (10.0)
II	1 (2.9)		1 (3.3)
III	13 (37.1)	4 (80.0)	9 (30.0)
IV	2 (5.7)	1 (20.0)	1 (3.3)
V	4 (11.4)		4 (13.3)
VI	12 (34.3)		12 (40.0)
Total	35 (100)	5 (100)	30 (100)

Table 5. The relationship between randomly amplified polymorphic DNA (RAPD) genotypes and staphylococcal superantigen (SAg) toxin genes in clinical *Staphylococcus aureus* isolates (n = 25)

RAPD genotype	SAg toxin genotypes	
	Gene patterns	Prevalence (%; no./total)
I	<i>seb</i> , <i>sec</i> , <i>seh</i> , <i>etA</i> <i>seb</i> , <i>sec</i> , <i>sek</i> , <i>etA</i> <i>seb</i> , <i>sec</i> , <i>etA</i>	<i>seb</i> (100, 3/3), <i>sec</i> (100, 3/3), <i>seh</i> (33.3, 1/3), <i>etA</i> (100, 3/3), <i>sek</i> (33.3, 1/3)
II	<i>seo</i>	<i>seo</i> (100, 1/1)
III	<i>etA</i> <i>sed</i> , <i>sep</i> <i>sen</i> <i>sei</i> , <i>sen</i> , <i>seu</i> <i>seh</i> , <i>sek</i> , <i>tst</i> <i>sed</i> , <i>sei</i> , <i>sem</i> , <i>sen</i> , <i>seo</i> , <i>sep</i> , <i>seu</i> , <i>tst</i> , <i>etB</i> <i>sej</i> , <i>ser</i> <i>sei</i> , <i>etB</i>	<i>etA</i> (12.5, 1/8), <i>sed</i> (25.0, 2/8), <i>sep</i> (25.0, 2/8), <i>sen</i> (37.5, 3/8), <i>sei</i> (37.5, 3/8), <i>seu</i> (25.0, 2/8), <i>seh</i> (12.5, 1/8), <i>sek</i> (12.5, 1/8), <i>tst</i> (25.0, 2/8), <i>sem</i> (12.5, 1/8), <i>seo</i> (12.5, 1/8), <i>etB</i> (25.0, 2/8), <i>sej</i> (12.5, 1/8), <i>ser</i> (12.5, 1/8)
IV	<i>etA</i>	<i>etA</i> (100, 1/1)
V	<i>sei</i> , <i>sen</i> , <i>seo</i> , <i>seu</i> , <i>tst</i> , <i>etB</i> <i>sei</i> , <i>sem</i> , <i>sen</i> , <i>seo</i> , <i>seu</i> , <i>tst</i> <i>sea</i> , <i>sei</i> , <i>sem</i> , <i>sen</i> , <i>seo</i> , <i>seu</i> , <i>tst</i> <i>sed</i> , <i>sei</i> , <i>sem</i> , <i>sen</i> , <i>seo</i> , <i>sep</i> , <i>seu</i> , <i>tst</i>	<i>sei</i> (100, 4/4), <i>sen</i> (100, 4/4), <i>seo</i> (100, 4/4), <i>seu</i> (100, 4/4), <i>tst</i> (100, 4/4), <i>etB</i> (25.0, 1/4), <i>sem</i> (75.0, 3/4), <i>sea</i> (25.0, 1/4), <i>sed</i> (25.0, 1/4), <i>sep</i> (25.0, 1/4)
VI	<i>sei</i> , <i>seu</i> <i>etB</i> <i>etB</i> <i>etB</i> <i>sea</i> , <i>seg</i> , <i>sei</i> , <i>sen</i> , <i>seu</i> <i>sei</i> , <i>sen</i> , <i>seu</i> <i>sen</i> , <i>sep</i> , <i>seu</i> , <i>etB</i> <i>sei</i> , <i>sek</i> , <i>sen</i> , <i>seo</i> , <i>seu</i> , <i>tst</i> , <i>etA</i>	<i>sei</i> (50.0, 4/8), <i>seu</i> (62.5, 5/8), <i>etB</i> (50.0, 4/8), <i>sea</i> (12.5, 1/8), <i>seg</i> (12.5, 1/8), <i>sen</i> (50.0, 4/8), <i>sep</i> (12.5, 1/8), <i>sek</i> (12.5, 1/8), <i>seo</i> (12.5, 1/8), <i>tst</i> (12.5, 1/8), <i>etA</i> (12.5, 1/8)

Association of RAPD Genotypes with SAg Toxin Genes

We assessed the association of RAPD genotypes with SAg toxin genes in the *Staph. aureus* strains and the results are shown in Table 5. In 3 clinical *Staph. aureus* isolates of RAPD genotype I and that harbored 3 gene patterns with 5 genes in tandem, all of the isolates were positive for *seb*, *sec*, and *etA* genes (100% each); therefore, the *seb*, *sec*, and *etA* genes were more frequently associated with RAPD genotype I. In addition, 8 isolates were found to be associated with RAPD genotype III with 8 genes patterns that carried 14 genes. In these *Staph. aureus* isolates, the *sei* and *sen* genes were present in 37.5% of the isolates; thus, these genes were more frequently associated with RAPD genotype III. The RAPD genotype V with 4 genes patterns, carrying 10 genes in tandem, was related to 4 *Staph. aureus* isolates. All of these *Staph. aureus* isolates contained the *sei*, *sen*, *seo*, *seu*, and *tst* genes (100% each), and 75% of the isolates carried the *sem* gene; thus, the *sei*, *sen*, *seo*, *seu*, *sem*, and *tst* genes were more frequently associated with the RAPD genotype V. Moreover, 8 *Staph. aureus* isolates were found to be related to RAPD genotype VI, exhibiting 8 genes patterns with 11 genes. Among these *Staph. aureus*, 62.5% of the isolates carried the *seu* gene, and half of the isolates possessed the *sei*, *sen*

and *etB* genes; therefore, the *seu*, *sei*, *sen*, and *etB* genes were more frequently related to RAPD genotype VI.

SAg Toxin Genotypes and Severity of Bovine Mastitis

As shown above, *Staph. aureus* isolates with RAPD genotype III were more frequently associated with clinical mastitis, whereas isolates with RAPD genotypes VI and III were predominantly related to subclinical mastitis (Table 4). The *sei* and *sen* genes were more frequently related to RAPD genotype III, whereas the *seu*, *sei*, *sen*, and *etB* genes were more frequently related to RAPD genotype IV (Table 5). Therefore, we can conclude that the *sei* and *sen* genes were more frequently related to clinical mastitis, and the *seu*, *sei*, *sen*, and *etB* genes were mostly related to subclinical mastitis (Table 6).

DISCUSSION

Staphylococcus aureus is a pathogen that causes several serious diseases in both humans and animals worldwide. This organism has the capacity to produce a variety of enterotoxins, collectively known as SAg. In the present study, we found that among 35 *Staph. aureus* strains isolated from milk samples of bovine mas-

titis cases in Ningxia province of China, 71.4% of the isolates harbored at least one of the SAg genes. These findings were in accordance with the reports from Italy (Vimercati et al., 2006) and Japan (Katsuda et al., 2005). However, these results are slightly contradicted by the study of Karahan et al. (2009), in which it was reported that 30% of *Staph. aureus* isolates were positive for staphylococcal enterotoxin-encoding genes in Turkey. Up to 92.6 and 95.0% of *Staph. aureus* isolates contained at least one SAg gene in Belgium (Ote et al., 2011) and Switzerland (Fournier et al., 2008), respectively. These discrepancies may reflect the considerable variation in the prevalence of the staphylococcal enterotoxin-encoding genes among different geographical locations (Oliveira et al., 2011; Artursson et al., 2016). It is worth noting that approximately three-fourths of the *Staph. aureus* isolates tested in the current study carried one or several SAg toxin-encoding genes, which may indicate that *Staph. aureus* associated with IMI might pose a high risk for human health due to the presence of these SAg toxins genes in milk; therefore, considerable attention should be paid to the food safety of dairy products.

In this study, all *Staph. aureus* isolates tested negative for *see*, which is in agreement with other studies conducted in Belgium (Ote et al., 2011), China (Xu et al., 2015), and Switzerland (Fournier et al., 2008). None of the isolates tested contained the *seq* gene, which is in concordance with the study conducted in Spain (Fueyo et al., 2005). The present study demonstrated that the distribution of different SAg genes in *Staph. aureus* isolates was highly heterogeneous, which coincided with the recent studies from Belgium (Ote et al., 2011) and Australia (McMillan et al., 2016).

It is well established that the pathogenicity of *Staph. aureus* strains relies on their repertoire of virulence factors. It has been documented that SAg toxin genes are related to mobile genetic elements, including pathogenicity islands, prophages, and plasmids (Malachowa and DeLeo, 2010; Alibayov et al., 2014), which may

suggest that SAg toxin genes can be transferred horizontally between staphylococcal strains. It is commonly observed that *Staph. aureus* strains hold more than one staphylococcal enterotoxin gene (Fijałkowski et al., 2014; Dunyach-Remy et al., 2016). In the present study, some gene combinations related to mobile genetic elements (consisting of 2 or more SAg toxin genes) were detected.

The *egc*-cluster located on the pathogenicity island Type I *uSa*β, carrying the *seg-sei-sem-sen-seo* gene pattern, was detected in 16.0% of the *Staph. aureus* isolates, which was detected less frequently than in previous studies. In a study conducted in Belgium, the *egc*-cluster was found in 31.4% of *Staph. aureus* isolates (Ote et al., 2011), and in a study in Spain (Fueyo et al., 2005), 45.2% of the isolates contained the *egc*-cluster. In addition, the *egc*-cluster found without 1 (*seg*) or 2 (*sem* and *seo* or *seg* and *sem*) genes in the present study. Similarly, the study of Omoe et al. (2005) also demonstrated the *egc*-cluster lacking 1 (*seo*) or 2 (*sem* and *sen*) genes.

The gene cluster harboring *sec*, *tst*, and *sel* genes is located on the pathogenicity island *SaPI*bov (Fournier et al., 2008). In a Spanish study, the cluster of *tst*, *sec*, and *sel* was found in 13.1% of isolates (Fueyo et al., 2005); however, in the current study, the cluster of *sec*, *tst*, and *sel* genes was not detected in any isolate. The *sel* gene was not amplified by PCR in any of the *Staph. aureus* isolates in the current study, and the *sec* and *tst* genes were detected separately. The *sec* gene was detected in 12% of the *Staph. aureus* isolates, whereas the *tst* gene was harbored by 28.8% of the isolates, a finding compatible with the study in Belgium (Ote et al., 2011) but higher than the data reported from some other countries (Karahan et al., 2009; Piccinini et al., 2010).

Similar to other findings, in our study the *seb-selk-selq* gene combinations without the *seq* gene, which was located on the *uSa*1 (*SaPI*3) pathogenicity island, was tested. The *sej-ser* gene combination present on the

Table 6. The relationship between staphylococcal superantigen (SAg) toxin genotypes of *Staphylococcus aureus* isolates (n = 25) and the severity of bovine mastitis

RAPD ¹ genotype	Major SAg toxin genotypes detected	Types of mastitis	
		Clinical (%)	Subclinical (%)
I	<i>seb</i> , <i>sec</i> , <i>etA</i>		3 (10.0)
II	<i>seo</i>		1 (3.3)
III	<i>sei</i> , <i>sen</i>	4 (80.0)	9 (30.0)
IV	<i>etA</i>	1 (20.0)	1 (3.3)
V	<i>sei</i> , <i>sem</i> , <i>sen</i> , <i>seo</i> , <i>seu</i> , <i>tst</i>		4 (13.3)
VI	<i>sei</i> , <i>sen</i> , <i>seu</i> , <i>etB</i>		12 (40.0)
Total		5 (100)	30 (100)

¹Randomly amplified polymorphic DNA.

plasmid (pF5) was found; the *sea* gene in the phage ϕ Sa3mu was detected; the *sep* gene located on the phage ϕ Sa3n was identified; and *seh* gene present in the antibiotic resistance staphylococcal cassette chromosome *mec* (SCC*mec*) was detected (Omoe et al., 2005; Nazari et al., 2014; Xu et al., 2015).

The exfoliative toxins A and B are related to staphylococcal scalded-skin syndrome (Handler and Schwartz, 2014). In the current study, *etA* was found in 24% of *Staph. aureus* isolates and *etB* was discovered in 28% of the isolates, in contrast to other reports. Although the *etA* gene was detected in 18.3% of the isolates in Belgium, other studies from Europe showed that none of the *Staph. aureus* isolates recovered from bovine IMI harbored *etA* and *etB* (Piccinini et al., 2010; Ote et al., 2011). In a recent Chinese study, all of the 28 *Staph. aureus* isolated from subclinical mastitis were negative for both the *etA* and *etB* genes (Xu et al., 2015).

Panton-Valentine leucocidin (PVL) is encoded by 2 cotranscribed genes, *lukS-PV* and *lukF-PV* (*lukS/F-PV*), which are located on a prophage and cause leukocyte destruction and tissue necrosis (Bhatta et al., 2016). The presence of PVL-encoding genes in *Staph. aureus* is reported to be associated with increased disease severity (Shariati et al., 2016). In the present study, none of the *Staph. aureus* isolate carried the *lukS/F-PV* genes, which was in accordance with studies reported from Spain (Fueyo et al., 2005) and Italy (Parisi et al., 2016); in contrast, 0.4% of the *Staph. aureus* isolates were positive for both *lukS-PV* and *lukF-PV* genes in Belgium (Ote et al., 2011).

Notably, a close relationship is reported between genotypes of *Staph. aureus* and the severity of bovine mastitis. The epidemiological data on 92 *Staph. aureus* strains isolated from cows in Ireland indicated that RAPD type VII and RAPD type V isolates were more likely to be linked to clinical mastitis (Fitzgerald et al., 2000). In Switzerland, genotype B was confirmed to be associated with high contagiousness and increased pathogenicity, whereas the other types (C, OG) were found to be related to IMI of single cows (Fournier et al., 2008). In the current study, RAPD type III strains was more frequently associated with clinical mastitis, whereas RAPD types VI and III strains were more frequently related to subclinical infections; therefore, we concluded that genotypes of *Staph. aureus* are strongly associated with the severity of the bovine mastitis.

Furthermore, a strong association has been documented between RAPD type and SEC/TSST-1 production among Irish *Staph. aureus* isolates from cows with IMI (Fitzgerald et al., 2000). In Ireland, 87.5% of the RAPD type VII isolates coproduced TSST-1 and SEC, and 16% of the tested isolates from Irish herds

produced TSST-1. In Switzerland, genotypes B and C were clearly different in their virulence gene patterns. Genotype B was related to *sed* (84%), *sej* (80%), and *sea* (77%) genes, whereas genotype C was related to *seg* (100%), *sec* (61%), and *tst* (61%; Fournier et al., 2008). In the present study, our results suggested that RAPD type V was strongly associated with the *tst* gene. Among RAPD type V strains, all isolates (100%) were positive for the *tst* gene; however, none of these isolates contained the *sec* gene. Among 35 *Staph. aureus* isolates from bovine mastitis cases, 20% of the isolates were positive for the *tst* gene. In addition, our findings demonstrated that the *seb*, *sec*, and *etA* genes (100% each) were associated with RAPD type I; *sei* and *sen* genes (37.5% each) were related to RAPD type III; and *seu* (62.5%), *sei*, *sen*, and *etB* genes (50.0% each) were related to RAPD type VI; therefore, this might suggest a close relationship between genotypes of *Staph. aureus* and SAg toxin genes.

In the current study, RAPD type III strains (80.0%) were associated with clinical mastitis, whereas RAPD types VI (40.0%) and III strains (30.0%) were related to subclinical infections. The *sei* and *sen* genes (37.5% each) were related to RAPD type III, whereas the *seu* (62.5%), *sei*, *sen*, and *etB* genes (50.0% each) were related to RAPD type IV; therefore, we conclude that the *sei* and *sen* genes were more frequently related to clinical mastitis, and the *seu*, *sei*, *sen*, and *etB* genes were more frequently related to subclinical mastitis. The results of the present study strongly supported the conclusion that SAg toxin genotypes are related to the severity of bovine mastitis.

CONCLUSIONS

Our results indicate that a relationship exists among RAPD genotypes, SAg toxin genes, and severity of bovine mastitis. The findings of the present work revealed that the genotypes of *Staph. aureus* strains isolated from bovine mastitis cases in Ningxia, China, were closely related to SAg toxin gene patterns and pathogenic characteristics of the pathogens.

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