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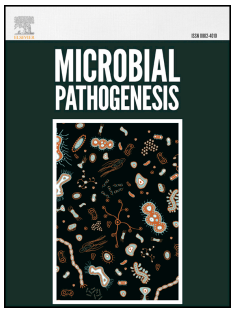

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The diversities of staphylococcal species, virulence and antibiotic resistance genes in the subclinical mastitis milk from a single Chinese cow herd

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ABSTRACT

Staphylococci are the leading pathogens of bovine mastitis which is difficult to control. However, the published data on the prevalence of staphylococcal species, virulence and antibiotic resistance genes in bovine mastitis from China are limited. In this study, 104 out of 209 subclinical mastitis milk samples from a single Chinese dairy herd were cultured-positive for staphylococci (49.8%), which were further identified as coagulase-positive staphylococci (CPS) or coagulase-negative staphylococci (CNS). According to the partial *tuf* and/or 16S rRNA gene sequence, the 28 CPS isolates were confirmed to be *S. aureus* (26.9%), and 76 CNS isolates were assigned to 13 different species (73.1%) with *S. arlettae*, *S. sciuri*, *S. xylosus* and *S. chromogenes* as the dominant species. In the 28 *S. aureus* isolates, the most prevalent general virulence genes were *coa*, *Ig* and *eno* (100%), followed by *hla*

(96.4%), *hlb* (92.9%), *fib* (92.9%), *clfA* (89.3%), *clfB* (85.7%) and *nuc* (85.7%). Both exotoxin and biofilm-associated genes were significantly less prevalent than the previously reported. Although 19 different virulence gene patterns were found, only one was dominant (32.1%). The prevalence of *blaZ* (82.1%) or *mecA* gene (35.7%) was much higher than the previously reported. In the 76 CNS isolates, the virulence genes were significantly less prevalent than that in the *S. aureus* isolates. Among the 4 main CNS species, *S. chromogenes* (n=12) was the only species with high percentage (75%) of *blaZ* gene, while *S. sciuri* (n=12) was the only species with the high percentage (66.7%) of *mecA* gene. The most of antibiotic resistance genes were present as multi-resistance genes, and the antibiotic resistances were attributed by different resistance genes between resistant *S. aureus* and CNS isolates. These data suggest that the prevalence of staphylococcal species, virulence and antibiotic resistance in the mastitis milk from the Chinese dairy herd are different from the previously reported, and that the herd- or farm-based diagnosis of staphylococcal bovine mastitis is required.

Keywords: Subclinical bovine mastitis; Staphylococci; Species; Virulence genes; Antibiotic resistance genes

1. Introduction

Staphylococci are the bacteria most commonly isolated from bovine mastitis [1]. In mastitis diagnosis, staphylococci can be divided into coagulase-positive (CPS) and coagulase-negative (CNS) based on the ability to coagulate rabbit plasma. The major pathogen *S. aureus*, including a broad range of genotypes with distinct pathogenic and epidemiologic characteristics, can cause clinical, but often subclinical mastitis [2]. Although CNS species are traditionally considered as minor mastitis pathogens [3], they have become the dominant pathogens of subclinical or mild clinical infections in many

well-managed dairy herds [4, 5]. This group of staphylococci consists of more than 40 different species and subspecies of which a dozen are commonly found in milk of dairy cows [6, 7]. Although a number of studies have been conducted to identify reservoirs of CNS, the epidemiology CNS mastitis is still unclear, in China in particular [4].

Various virulence factors have been found in *S. aureus* from bovine mastitis, including haemolysins (HLA and HLB), leukocidin, exfoliative toxins (ETA to ETD), staphylococcal enterotoxins (SEs), toxic-shock syndrome toxin-1 (TSST-1), and biofilm formation [8, 9]. Except for their pathogenic role in bovine mastitis, some toxin-producing *S. aureus* strains pose a risk for humans and animals [10]. These toxin genes are mainly located on the mobile genetic elements, and thus can spread among staphylococcal isolates or species [11, 12]. Although these toxin genes were originally identified in *S. aureus* isolates, some of them have also been detected in a variety of CNS species from the mammary glands of cattle and other ruminants [13], which may become a possible reservoir of toxin genes typically identified in *S. aureus*. However, only few studies have so far focused on the virulence factors of CNS isolated from bovine mastitis [3].

S. aureus has the potential to develop resistance to almost all antimicrobial agents [14]. Due to the extensive use of antibiotics as bovine mastitis antibacterial agents, the antimicrobial resistance developed by staphylococci is one of main reasons for low cure rate of mastitis [14, 15]. More importantly, the emergence of methicillin-resistant *S. aureus* (MRSA) strains has become a major public health concern [16]. Furthermore, CNS species tend to be more resistant to antimicrobials than *S. aureus*, and easily develop multi-resistance [17]. Therefore, the investigation into antimicrobial resistance in the staphylococci from dairy cows is important not only for bovine mastitis control, but also for public health. However, the published data on the difference in antibiotic resistance genes

among CNS species are limited, from China in particular [18, 19].

In the light of limited data on the genotypic identification of bovine mastitis staphylococci from China, the objective of this study was to investigate the prevalence of staphylococcal species, virulence and antibiotic resistance in subclinical mastitis milk from a single dairy cow herd.

2. Materials and methods

2.1 Herd and cows

During the period from June 2012 to June 2014, a field study was conducted on a single dairy farm in Jiangsu Province, China. The dairy farm was well-managed and self-contained which had comparable characteristics reflecting the general situation on Chinese middle-sized dairy farms. The herd size was 748 Holstein cows with an average production of 85,000kg of milk/cow per year. On the dairy farm, cows were housed in freestalls with concrete floors and sawdust bedded cubicles. Sawdust bedding was removed 2 to 3 times a day, and replaced by fresh sawdust from a stock stored indoors. Postmilking teat disinfection was practiced by standard iodine dipping. Dry cow therapy was practiced with ampicillin and cloxacillin enzathine injection. Incidence rates of clinical and subclinical mastitis cases during the past two years were 5% and 18%, respectively. The clinical mastitis cows were treated in rotation with kanamycin, cefazolin and compound Chinese medicine consisting of *Flos lonicerae japonicae*, *Radix scutellariae*, *Taraxacum platypecidum*, *Radix glycyrrhizae*, and *Angelica dahurica*. During the period of this study, 6 cohorts of 35 subclinical mastitis cows were randomly selected for milk sample collection.

2.2 Sample collection

According to the estimation of somatic cell count (SCC) using California mastitis test (CMT), a mammary quarter was considered with subclinical mastitis when the SCC was greater than 250,000/ml in individual quarter foremilk without overt clinical signs [20]. Quarter milk samples were collected aseptically at bimonthly intervals (n=6) from the cohort cows according standard procedures [21]. Samples were transported immediately on ice to the laboratory for bacteriological examination.

2.3 Staphylococcal isolation

Bacteriological culture of milk samples and bacterial identification were done as recommended by the National Mastitis Council [21]. Briefly, 0.1 ml of each milk sample was spread on each sheep blood agar plate and incubated for 24 h at 37 ℃. Phenotypic differentiation of bacterial species was done as previously described [22]. Staphylococci were identified presumptively based on colony morphology, Gram's stain, and catalase test. *S. aureus* was differentiated from other *Staphylococcus spp*. based on morphology, pigmentation, hemolysis, Coa tube test and thermonuclase (Nuc) activity. All of non-*S. aureus* staphylococci were a priori considered as CNS. For milk samples yielding at least 3 CNS colonies, 2 colonies were picked and transferred to self-made tryptone soy agar (TSA) for further identification. When more than one type of CNS colony was present, more colonies were picked. The TSA plates were incubated for 18 h at 37 ℃. The mammary quarter was considered *S. aureus* and CNS infected when the number of bacterial colonies was ≥50 and 250cfu/ml, respectively [23].

2.4 Genotyping of staphylococcal isolates

Each colony was picked from TSA plates and grown overnight at 37 ℃ in LB both.

Staphylococcal genomic DNA was extracted using High Pure PCR Template Preparation Kit (Roche, Shanghai, China) according to the manufacturer's instruction. The concentration of DNA was adjusted to 100ng/μl by addition of deionized water. Staphylococcal isolates were genotypically identified by PCR amplification of *tuf*, *coa* and *nuc* genes as previously described [24]. PCR amplification was performed in 25μl volumes using 50 ng of DNA template, 2.5 U of rTaq DNA polymerase (TaKaRa, Dalian, China), 1 × Taq buffer, 0.25 mM dNTP mix, 1.5 mM $MgCl_2$, 0.1 μmol of each primer. The primer sequences for amplification of *coa* and *nuc* genes are listed in Table 1. The *tuf* gene segments were amplified using the primer pair of 5'-GCCAGTTGAGGACGTATTCT-3' and 5'-CCATTTCAGTACCTTCTGGTAA-3'.

2.5 Identification of staphylococcal species

Staphylococcal species were differentiated first by sequencing the PCR products of partial *tuf* gene segments. PCR products were purified using High Pure PCR Product Purification Kit (Roche) according to the manufacturer's instruction, and submitted to forward and reverse sequencing. The generated sequences were searched against staphylococcal *tuf* gene sequences in GenBank, and a sequence identity of ≥98.0% was used as the rule for speciation [24]. For the non-definitive strains, 16S rRNA genes were amplified with primer pair of 5'-AGAGTTTGATCMTGGCTCAG-3' and 5'-CCGTCAATTCMTTTRAGTTT-3' for sequencing and species discrimination [13].

2.6 Detection of virulence genes

All staphylococcal isolates were tested by PCR for the presence of virulence genes (Table 1). The *coa* gene coding for Coa [25], *spa* gene for protein A [25], *Ig* gene for Ig-binding protein [26], *map* gene for MHC class II analog protein [27] or *bap* gene for biofilm-associated protein [28] was

amplified by PCR as previously described. The other virulence genes were amplified by duplex [28-31] or multiplex PCR [32-35] as previously described. The primer sequences for amplification of virulence genes are listed in Table 1.

2.7 Detection of antibiotic resistance genes

All staphylococcal isolates were also tested by PCR for the presence of antibiotic resistance genes. The *linA* gene conferring resistance to lincosamides was detected by PCR as previously described [36]. The other antibiotic genes were detected by duplex [36] or multiplex PCR [37, 38]. The primer sequences for amplification antibiotic genes are listed in Table 2.

2.8 Detection of antibiotic resistance

All staphylococcal isolates, as well as *S. aureus* reference strain ATCC25923 (ATCC, USA), were tested for antibiotic susceptibilities with the disc diffusion method (Clinical and Laboratory Standards Institute, 2013, CLSI2013) using Mueller-Hinton agar (MHA) plates (OXOID, USA) that contained the following 9 antibiotics: cefoxitin, clindamycin, erythromycin, gentamicin, kanamycin, penicillin, streptomycin, tetracycline and tobramycin. The resistance breakpoints were those proposed for staphylococci in the guidelines of CLSI2013.

3. Results

3.1 Identification of Staphylococci in subclinical mastitis milk

Based on bacteriological examination, 104 out 209 subclinical mastitis milk samples (49.8 %) were cultured-positive for *Staphylococci*. According to the phenotypic and genotypic examination, 28

out 104 staphylococcal isolates were identified as *S. aureus* (26.9%) and the remaining 76 isolates were a priori considered as CNS (Table 3).

3.2 Genotypic identification of staphylococcal species

The partial *tuf* gene sequence analysis showed that the 28 *S. aureus* isolates had 100% sequence similarity to the reference strain (Table 4). By using a sequence identity of ≥98.0% as the rule for speciation, 76 CNS isolates were assigned into 13 different species based on *tuf* and/or 16S rRNA gene sequences. Among these, 70 isolates were identified to the species level (92.1%) according to the partial *tuf* gene sequences only (Table 4). The remaining 6 CNS isolates had 96.9% or 97.5% *tuf* gene similarity to *S. xylosus* or *S. hyicus* reference strain. Among them, 5 isolates were confirmed as *S. xylosus* and another one as *S. hyicus* with 99% 16S rRNA sequence similarity to the reference strain (Table 4). Among the 76 CNS isolates, *S. arlettae*, *S. sciuri*, *S. xylosus* and *S. chromogenes* were the dominant species (a total of 63.2%), followed by *S. epidermidis*, *S. simulans*, *S. equorum*, *S. haemolyticus*, *S. warneri* and other four (Table 4).

3.3 Detection of virulence genes in staphylococci

The presence of 37 virulence genes in the staphylococci from subclinical mastitis milk was detected by PCR. These virulence genes could be divided into three functional groups: general virulence factors, exotoxins and biofilm formation. In 28 *S. aureus* isolates, the most prevalent general virulence factor genes were the *coa* for coagulase, *Ig* for Ig binding protein, and *eno* for laminin binding protein (100%), followed by *hla* (96.4%) and *hlb* (92.9%) for hemolysins, *fib* (92.9%) for fibrinogen binding protein, *clfA* (89.3%) and *clfB* (85.7%) for clumping factors, and *nuc* (85.7%) for thermonuclease (Table 5). Among the 12 exotoxin genes tested, only *seg* (14.3%), *sei* (10.7%) and *sea*

(7.1%) for enterotoxins were detected. Among the 10 biofilm-associated genes tested, the most prevalent gene was *spa* (96.4%) for protein A, followed by *fnbB* (75%) for fibronectin binding protein, *icaD* (71.4%) for intercellular adhesion, and *agr-1* (64.3%) *and agr-2* (17.9%) *for* accessory gene regulation (Table 5). Although 19 different gene patterns were found in the 28 *S. aureus* isolates, only one (*Ig, coa, eno, spa, hla, hlb, fib, clfA, clfB, nuc, map, fnbB, icaD, agr-1, cap5*) was the dominant (32.1%) gene combination (Table 6). In the 76 CNS isolates, the most prevalent general virulence gene was *eno* (53.9%), followed by *Ig* (18.4%) and *map* for MHC class II analog protein (11.8%). Among the 12 exotoxin genes tested, only *sei* (5.3%) and *seb* (2.6%) were detected. Among the 10 biofilm-associated genes tested, only *bap* for biofilm-associated protein (10.5%), *agr-2* (3.9%), *fnbA* (2.6%) and *fnbB* (2.6%) genes were detected (Table 5). Although different virulence gene patterns were also present in the 13 CNS species, they consisted of only 1-4 virulence genes (Table 6).

3.4 Detection of antibiotic resistance genes in staphylococci

The presence of 17 antibiotic resistance genes in the staphylococci from subclinical mastitis milk was also detected by PCR. In the 28 *S. aureus* isolates, the most prevalent antibiotic resistance gene was *blaZ* conferring the resistance to penicillin (82.1%), followed by *mecA* to methicillin (35.7%), *aacA-aphD* to aminoglycoside (32.1%), *aac(6')/aph(2")* to streptomycin (28.6%), *tetK* to tetracycline (10.7%), *ermC* to erythromycin and clindamycin and *linA* to lincosamides (7.1%; Table 7). These antibiotic resistance genes were present as 6 different gene combinations containing 1-4 genes (Table 8). In the 76 CNS isolates, the most prevalent antibiotic resistance gene was *linA* (38.2%), followed by *tetK* (34.2%), *blaZ* (30.3%), *aacA-aphD* (21.1%), *msrB* (19.7%), *msrA* (17.1%), *mecA* (17.1%), *ermC* (13.2%), *aac(6')/aph(2")* (10.5%), *ermB* (9.2%), and *tetM* (2.6%; Table 7). These antibiotic resistance

genes were also present as different gene combinations containing 1-7 genes (Table 8).

3.5 Detection of antibiotic resistance in staphylococci

In the 28 *S. aureus* isolates, penicillin resistance was the most frequent resistance phenotype (82.1%), followed by resistance to streptomycin (46.4%), kanamycin and tobramycin (35.7%), cefoxitin and gentamicin (32.1%), erythromycin (14.3%), tetracycline (10.7%), and clindamycin (3.6%). In the 76 CNS isolates, penicillin resistance was also the most frequent resistance phenotype (86.8%), but followed by resistance to erythromycin (48.7%), streptomycin (46.1%), tetracycline (39.5%), clindamycin (30.3%), cefoxitin and kanamycin (27.6%), gentamicin (13.2%), and tobramycin (11.8%). Resistance to 2 or more antibiotics was present in 100% % of the *S. aureus* isolates (n=23) or in 79.4% CNS isolates (n=68). The genes attributed to antibiotic resistances are listed in Table 9.

4. Discussion

This study provides different data on the prevalence of staphylococcal species, virulence and antibiotic resistance genes in subclinical mastitis milk from a Chinese dairy farm. According to the bacteriological examination, 104 out of 209 milk samples were cultured-positive for *Staphylococci* (49.8 %), confirming that staphylococci were the main pathogen of bovine mastitis in China and other countries [19]. Among the 104 staphylococcal isolates, 28 and 76 isolates were further identified as *S. aureus* (26.9%) and CNS (73.1%), respectively, which supported the previous finding that CNS species have become the dominant pathogens of subclinical or mild clinical infections [4, 5].

Molecular identification has been proposed as the gold standard for bovine CNS speciation [39]. Among the proposed molecular methods, although 16S rRNA gene sequencing is widely used, the high degree of sequence similarity between closely related species limits its usefulness for some CNS

speciation. Recently, the partial *tuf* gene sequencing has been proved to be a reliable method for CNS identification [24]. Therefore, in this study we amplified the partial *tuf* genes from 104 staphylococcal isolates from subclinical mastitis milk for staphylococcal speciation. Sequence analysis showed that the 28 *S. aureus* isolates had 100% sequence similarity to the reference strain, confirming the reliability of partial *tuf* gene sequencing for *S. aureus* identification. For 76 CNS isolates, however, only 70 isolates were identified to the species level (92.1%) based on the partial *tuf* gene sequence only (Table 4). The remaining 6 CNS isolates had the highest *tuf* sequence similarity with *S. xylosus* (96.9%) or *S. hyicus* (97.5%) reference strain, which was below the cut-off value (≥98.0%) for speciation [24]. Therefore, we amplified the 16S rRNA genes for further speciation. Among them, 5 isolates were identified as *S. xylosus* and one as *S. hyicus* with 99% sequence identity to the reference strains. These data suggest that, like 16S rRNA gene sequencing, the partial *tuf* gene sequencing alone was insufficient to distinguish some closely related CNS species.

More than 20 CNS species have been isolated from bovine milk, and five of them (*S. chromogenes*, *S. simulans*, *S. haemolyticus*, *S. xylosus* and *S. epidermidis*) are considered as the main CNS species [3, 40]. In this study, however, *S. arlettae* and *S. sciuri*, together with *S. chromogenes* and *S. xylosus*, were identified as the dominant CNS species. Moreover, the previously reported main CNS species, *S. haemolyticus* and *S. epidermidis*, were less prevalent, *S. haemolyticus* (3.9%) in particular. These data suggest that the distribution of main CNS species in mastitis milk was different among dairy farms or herds. This was supported by a recent study, in which *S. warneri*, *S. epidermidis* and *S. hyicus* are identified to be the dominant species among 18 CNS species isolated from CMT-positive cow milk [41].

Various virulence factors have been identified in *S. aureus* isolates from bovine mastitis. However,

only few staphylococcal virulence factors have been tested in animal models. Among these, one HLA-positive *S. aureus* isolate has been shown to be most virulent in a mouse model, followed by HLA- and HLB-positive isolates, and HLB-positive isolates. The least virulent isolates are the non-hemolytic *S. aureus* strains, but even they are more virulent than two CNS species tested [42]. In this study, most *S. aureus* isolates harbored the *hla* (96.4%) and/or *hlb* (92.9%) gene, indicating their potential virulence in bovine mastitis. Exotoxins are a special class of virulence factors with several potential functions and public health concern [3, 43]. Although these toxins are originally identified in *S. aureus*, they have also been detected in CNS, including the isolates from bovine milk [41]. In this study, however, only three (*sea*, *seg* and *sei*) or two (*seb* and *sei*) enterotoxin genes were detected in 28 *S. aureus* (32.1%) or 76 CNS isolates (7.9%), which were significantly less prevalent than the previously reported (up to 66%). The possible reason (s) for this could be due to the low prevalence of toxin-producing staphylococcal isolates in the dairy herd, and/or the presence of other toxin genes which were not tested. It is currently accepted that the most important virulence factor of CNS is biofilm formation [44, 45]. Among the biofilm-associated genes identified, the *ica* gene cluster coding for intercellular adhesion proteins has a wide distribution, and thus is traditionally regarded as the most important biofilm-associated gene in human-associated CNS [44, 46]. However, this appears not to be the case for CNS from bovine mastitis milk [3]. In this study, for example, 20 out of 28 *S. aureus* isolates were positive for *icaD* but not *icaA* gene, both of which were not detected in the 76 CNS isolates (Table 5). The *bap* gene encoding biofilm-associated protein has also been identified in biofilm-producing staphylococci from bovine mastitis [47]. Among the 76 CNS isolates in this study, however, only 8 isolates harbored the *bap* gene (10.5%), which was significantly less prevalent than that (~50%) of the previously reported [48]. In addition, the *bap* gene was found only in two CNS

species (*S. xylosus* and *S. equorum*), but not in the 28 *S. aureus* and other CNS species (Table 6). The *eno* gene encoding laminin-binding protein has been shown to be the most frequent (75%) among the CNS isolates from mastitis [49]. This was supported by our investigation, in which 100% of *S. aureus* and 53.9% of CNS isolates were positive for *eno* gene (Table 5). The virulence genes listed above are frequently present in different combinations. In this study, however, although 19 virulence gene combinations were found in the 28 *S. aureus* isolates, only one was the dominant (32.1%). Due to their low prevalence of virulence genes, the virulence gene patterns in the 13 CNS species were much fewer and simpler with only 1-4 virulence genes (Table 6).

The most common resistance mechanism in staphylococci is blactamase production. The reported percentage of penicillin resistance in the CNS isolates from bovine mastitis is variable from 25% to 61%, which is generally higher than that in *S. aureus* (from 7% to 32%) from some European countries [46]. In this study, however, 82.1% of *S. aureus* isolates contained the *blaZ* gene, the prevalence of which was significantly higher than that (30.3%) in the 76 CNS isolates (Table 7). Among the 13 CNS species, *S. epidermidis* was the species with the highest percentage of *blaZ* gene (100%), followed by *S. chromogenes* (75%). Moreover, most of the *blaZ* genes were present as the multi-resistance genes in both *S. aureus* and CNS isolates (Table 8). The higher prevalence of *blaZ* gene may be due to the frequent use of penicillin for dry cow therapy on this dairy farm. In addition, these data also suggest the potential spread of penicillin-resistant staphylococci within the dairy herd. The emergence of MRSA infection in dairy animals is of great concern for livestock and public health [50]. Previous surveys have shown that methicillin resistance is relatively rare in *S. aureus* (from 2.5% to 4%) from mastitis milk [46]. In this study, however, 35.7% of *S. aureus* and 17.1% of CNS isolates were positive for *mecA* gene (Table 7), the prevalence of which was not only much higher than the previously reported

from some European countries, but also higher than the recently reported (15.5%) from China [19]. Among the 4 dominant CNS species, *S. sciuri* was the only species harboring *mecA* gene. As expected, all of the *mecA* genes were present as multi-resistance genes in the *S. aureus* and CNS isolates (Table 8). Once again, these data indicate the potential spread of multi-resistant staphylococci within the dairy herd.

Another interesting finding in this investigation was the difference in antibiotic resistance attributed by different antibiotic resistance genes between the resistant *S. aureus* and CNS isolates. For example, the penicillin resistance was attributed completely by the *blaZ* gene in the resistant *S. aureus* isolates (n=23), compared to 34.8% by the *blaZ* gene and 65.2% by unknown gene in the resistant CNS isolates (n=66; Table 9). In addition, the cefoxitin resistance was attributed mainly (88.9%) by the *mecA* + *blaZ* gene in the resistant *S. aureus* isolates (n=9), compared to 42.9%, 28.6%, 19.0% and 9.5% by the *mecA* gene, unknown gene, *mecA* + *blaZ* gene and *blaZ* gene in the resistant CNS isolates (n=21). The different attributions of antibiotic resistance genes to other antibiotic resistances were also found between the resistant *S. aureus* and CNS isolates (Table 9). These data suggest the existence of different antibiotic resistance mechanisms between the *S. aureus* and CNS from bovine mastitis milk.

Acknowledgements

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Table 1

The PCR primers for amplification of staphylococcal virulence genes in this study

PCR	Gene	Primer set sequences (5'→3')	Product (bp)	Reference
Singular I	*Ig*	CACCTGCTGCAAATGCTGCG	Variable	Seki et al., 1998
		GGCTTGTTGTTGTCTTCCTC		
Singular II	*spa*	CAAGCACCAAAAGAGGAA	Variable	Kalorey et al., 2007
		CACCAGGTTTAACGACAT		
Singular III	*map*	TAACATTTAATAAGAATCAA	940	Peacock et al., 2002
		CCATTTACTGCAATTGT		
Singular IV	*coa*	ATAGAGATGCTGGTACAGG	Variable	Kalorey et al., 2007
		GCTTCCGATTGTTCGATGC		
Singular V	*bap*	CCCTATATCGAAGGTGTAGAATTG	971	Simojoki et al, 2012
		GCTGTTGAAGTTAATACTGTACCTGC		
Duplex I	*cap5*	ATGACGATGAGGATAGCG	880	Moore et al., 2001
		CTCGGATAACACCTGTTGC		
	cap8	ATGACGATGAGGATAGCG	1147	
		CACCTAACATAAGGCAAG		
Duplex II	*etA*	GCAGGTGTTGATTTAGCATT	93	Mehrotra et al., 2000
		AGATGTCCCTATTTTTGCTG		
	etB	ACAAGCAAAAGAATACAGCG	226	
		GTTTTTGGCTGCTTCTCTTG		
Duplex III	*hla*	GGTTTAGCCTGGCCTTC	550	Booth et al., 2001
		CATCACGAACTCGTTCG		
	hlb	GCCAAAGCCGAATCTAAG	840	
		CGCATATACATCCCATGGC		
Duplex IV	*icaA*	CCTAACTAACGAAAGGTAG	1315	Simojoki et al, 2012
		AAGATATAGCGATAAGTGC		
	icaD	AAACGTAAGAGAGGTGG	381	
		GGCAATATGATCAAGATAC		
Multiplex I	*nuc*	GCGATTGATGGTGATACGGTT	280	Brakstad et al., 1992;
		AGCCAAGCCTTGACGAACTAAAGC		Lina et al., 2003
	agr-1	ATGCACATGGTGCACATGC	439	
		GTCACAAGTACTATAAGCTGCGAT		
	agr-2	ATGCACATGGTGCACATGC	572	
		TATTACTAATTGAAAAGTGCCATAGC		
	agr-3	ATGCACATGGTGCACATGC	321	
		GTAATGTAATAGCTTGTATAATAATACCCAG		
	agr-4	ATGCACATGGTGCACATGC	657	
		CGATAATGCCGTAATACCCG		
Multiplex II	*fib*	CTACAACTACAATTGCCGTCAACAG	404	Tristan et al., 2003
		GCTCTTGTAAGACCATTTTCTTCAC		
	clfA	ATTGGCGTGGCTTCAGTGCT	292	
		CGTTTCTTCCGTAGTTGCATTTG		
	clfB	ACATCAGTAATAGTAGGGGGCAAC	205	
		TTCGCACTGTTTGTGTTTGCAC		
	fnbA	GTGAAGTTTTAGAAGGTGGAAAGATTAG	643	
		GCTCTTGTAAGACCATTTTTCTTCAC		
	fnbB	GTAACAGCTAATGGTCGAATTGATACT	524	
		CAAGTTCGATAGGAGTACTATGTTC		
Multiplex III	*eno*	ACG TGCAGCAGCTGACT	302	Tristan et al., 2003
		CAACAGCATYCTTCAGTACCTTC		
	bbp	AACTACATCTAGTACTCAACAACA	575	
		ATGTGCTTGAATAACACCATCATCT		
	ebp	CATCCAGAACCAATCGAAGAC	186	
		CTTAACAGTTACATCATCATGTTTATCTTTG		
	cna	GTCAAGCAGTTATTAACACCAGAC	423	
		AATCAGTAATTGCACTTTGTCCACTG		

Multiplex IV	*sej*	CATCAGAACTGTTGTTCCGCTAG	142	Løvseth et al., 2004
		CTGAATTTTACCATCAAAGGTAC		
	seh	CAACTGCTGATTTAGCTCAG	359	
		GTCGAATGAGTAATCTCTAGG		
	sea	GCAGGGAACAGCTTTAGGC	521	
		GTTCTGTAGAAGTATGAAACACG		
	seb	ACATGTAATTTTGATATTCGCACTG	667	
		TGCAGGCATCATGTCATACCA		
	sec	CTTGTATGTATGGAGGAATAACAA	284	
		TGCAGGCATCATATCATACCA		
Multiplex V	*tsst*	GCTTGCGACAACTGCTACAG	559	Løvseth et al., 2004
		TGGATCCGTCATTCATTGTTAT		
	sed	GTGGTGAAATAGATAGGACTGC	385	
		ATATGAAGGTGCTCTGTGG		
	see	TACCAATTAACTTGTGGATAGAC	171	
		CTCTTTGCACCTTACCGC		
	seg	CGTCTCCACCTGTTGAAGG	328	
		CCAAGTGATTGTCTATTGTCG		
	sei	CAACTCGAATTTTCAACAGGTACC	466	
		CAGGCAGTCCATCTCCTG		

Table 2

The PCR primers for amplification of staphylococcal antibiotic resistance genes in this study

PCR	Target gene	Primer set sequences (5'→3')	Product (bp)	Reference
Singular	*linA*	GGTGGCTGGGGGGTAGATGTATTAACTGG	323	Lina et al.,
		GCTTCTTTTGAAATACATGGTATTTTTCGATC		1999
Duplex	*msrA*	GGCACAATAAGAGTGTTTAAAGG	940	Lina et al.,
		AAGTTATATCATGAATAGATTGTCCTGTT		1999
	msrB	TATGATATCCATAATAATTATCCAATC	595	
		AAGTTATATCATGAATAGATTGTCCTGTT		
Multiplex I	*mecA*	AAAATCGATGGTAAAGGTTGGC	532	Strommenge
		AGTTCTGCAGTACCGGATTTGC		r et al., 2003
	aacA-aphD	TAATCCAAGAGCAATAAGGGC	227	
		GCCACACTATCATAACCACTA		
	vatA	TGGTCCCGGAACAACATTTAT	268	
		TCCACCGACAATAGAATAGGG		
	vatB	GCTGCGAATTCAGTTGTTACA	136	
		CTGACCAATCCCACCATTTTA		
	vatC	AAGGCCCCAATCCAGAAGAA	467	
		TCAACGTTCTTTGTCACAACC		
Multiplex II	*ermA*	AAGCGGTAAACCCCTCTGA	190	Duran et al.,
		TTCGCAAATCCCTTCTCAAC		2012
	ermB	CTATCTGATTGTTGAAGAAGGATT	142	Strommenge
		GTTTACTCTTGGTTTAGGATGAAA		r et al., 2003
	ermC	AATCGTCAATTCCTGCATGT	299	
		TAATCGTGGAATACGGGTTTG		
	tet K	GTAGCGACAATAGGTAATAGT	360	
		GTAGTGACAATAAACCTCCTA		
	tetM	AGTGGAGCGATTACAGAA	158	
		CATATGTCCTGGCGTGTCTA		
Multiplex III	*blaZ*	ACTTCAACACCTGCTGCTTTC	173	Duran et al.,
		TGACCACTTTTATCAGCAACC		2012
	aac (6')/	GAAGTACGCAGAAGAGA	491	
	aph (2'')	ACATGGCAAGCTCTAGGA		
	aph (3')-IIIa	AAATACCGCTGCGTA	242	
		CATACTCTTCCGAGCAA		
	ant (4')-Ia	AATCGGTAGAAGCCCAA	135	
		GCACCTGCCATTGCTA		

Table 3

Phenotypic and genotypic identification of CPS and CNS isolates from subclinical mastitis milk

Species	No. of isolates (%)	Coa^+ isolates (%)		Nuc^+ isolates (%)		Cat^+ isolates (%)
		Phenotypic	Genotypic	Phenotypic	Genotypic	
S. aureus	28 (26.9)	27 (96.4)	28 (100)	28 (100)	24 (85.7)	28 (100)
CNS	76 (73.1)	0 (0)	0 (0)	1 (1.3)	0 (0)	76 (100)
Total	104	27	28	29	24	104

Table 4

Staphylococcal speciation according to partial *tuf* and/or 16S rRNA gene sequences (n=104)

Species	Isolates (%)	Similarity with reference (%)	Similarity within species (%)	Similarity with other species (%)	Reference Sequence
S. aureus	28 (26.9)	100	100	88.9-95.4	HM352919
S. arlettae	12 (11.5)	99.5-99.8	99.5-100	89.0-93.7	EU652781
S. sciuri	12 (11.5)	99.2-100	99.2-100	85.9-92.1	HM352948
S. xylosus	12 (11.5)	96.9[a]-100	96.7-100	87.7-96.9	HM352950
S. chromogenes	12 (11.5)	98.5-99.3	99.3-100	86.5-95.8	EU652790
S. epidermidis	7 (6.7)	99.8-100	99.8-100	88.2-95.8	AF298800
S. simulans	6 (5.8)	99.5-99.8	99.5-100	87.3-92.7	EU652822
S. equorum	6 (5.8)	99.5-99.8	99.8-100	86.2-92.3	EU652795
S. haemolyticus	3 (2.9)	99.8-100	99.8-100	87.2-95.8	HM032764
S. warneri	2 (1.9)	99.8-100	99.8	87.7-95.5	AF298806
S. hyicus	1 (1.0)	97.5[b]	100	85.8-96.0	JX436514
S. saprophyticus	1 (1.0)	99.3	100	88.2-97.2	AF298804
S. succinus	1 (1.0)	99.8	100	88.0-94.8	EU652824
S. muscae	1 (1.0)	99.5	100	86.5-94.3	EU652807

[a, b]: confirmed by16S rRNA gene sequencing with a sequence similarity of 99%

Table 5

Prevalence of virulence genes in staphylococci from subclinical mastitis milk

Function	Virulence	Gene	Isolates (%)	
			S. aureus (n=28)	CNS (n=76)
General virulence factors	Thermonuclease	*nuc*	24 (85.7)	0 (0)
	Coagulase	*coa*	28 (100)	0 (0)
	IG-binding protein	*Ig*	28 (100)	14 (18.4)
	Hemolysins	*hla*	27 (96.4)	0 (0)
		hlb	26 (92.9)	0 (0)
	Clumping factors	*clfA*	25 (89.3)	0 (0)
		clfB	24 (85.7)	0 (0)
	Bone sialoprotein binding protein	*bbp*	0 (0)	0 (0)
	Fibrinogen binding protein	*fib*	26 (92.9)	0 (0)
	Elastin binding protein	*ebp*	11 (39.3)	0 (0)
	Collagen binding protein	*cna*	3 (10.7)	0 (0)
	MHC class II analog protein	*map*	22 (78.6)	9 (11.8)
	Capsular polysaccharides	*cap5*	13 (46.4)	0 (0)
		cap8	11 (39.3)	1 (1.3)
Superantigens (exotoxins)	Toxic shock syndrome toxin-1	*tsst-1*	0 (0)	0 (0)
	Enterotoxins	*sea*	2 (7.1)	0 (0)
		seb	0 (0)	2 (2.6)
		sec	0 (0)	0 (0)
		sed	0 (0)	0 (0)
		see	0 (0)	0 (0)
		seg	4 (14.3)	0 (0)
		seh	0 (0)	0 (0)
		sei	3 (10.7)	4 (5.3)
		sej	0 (0)	0 (0)
	Exfoliative toxins	*etA*	0 (0)	0 (0)
		etB	0 (0)	0 (0)
`Biofilm formation	Intercellular adhesion	*icaA*	0 (0)	0 (0)
		icaD	20 (71.4)	0 (0)
	Fibronectin binding proteins	*fnbA*	0 (0)	2 (2.6)
		fnbB	21 (75)	2 (2.6)
	Accessory gene regulation	*agr-1*	18 (64.3)	0 (0)
		agr-2	5 (17.9)	3 (3.9)
		agr-3	0 (0)	0 (0)
		agr-4	0 (0)	0 (0)
	Protein A	*spa*	27 (96.4)	0 (0)
	Laminin binding protein	*eno*	28 (100)	41 (53.9)
	Biofilm-associated protein	*bap*	0 (0)	8 (10.5)

Table 6

The virulence gene patterns in staphylococci from subclinical mastitis milk

Species	Isolates (%)	Virulence genes	Species	Isolates (%)	Virulence genes
S. aureus	9 (32.1)	*Ig, coa, eno, spa, hla hlb, fib, clfA, clfB, nuc, map, fnbB, icaD, agr-1, cap5*	*S. arlettae*	5 (41.7)	*map*
(n=28)	2 (7.1)	*Ig, coa, eno, spa, hla, hlb, fib, clfA, clfB, nuc,icaD, ebp, agr-2, cap8, seg, sei*	(n=12)	4 (33.3)	*No*
	1 (3.6)	*Ig, coa, eno, spa, hla, hlb, fib, clfA, clfB, nuc, map, fnbB, icaD, ebp, agr-1, cap5*		1 (8.3)	*Eno, Ig, map*
	1 (3.6)	*Ig, coa, eno, spa, hla, hlb, fib, clfA, clfB, nuc, icaD, ebp, agr-2, cap8, seg*		1 (8.3)	*Ig, map, seb*
	1 (3.6)	*Ig, coa, eno, spa, hla, hlb, fib, clfA, clfB, nuc, map, fnbB, ebp, agr-1, cna*		1 (8.3)	*eno, Ig, map, seb*
	1 (3.6)	*Ig, coa, eno, spa, hla, hlb, fib, clfA, clfB, nuc, fnbB, icaD, agr-1, cap5*	*S. sciuri*	5 (41.7)	*eno*
	1 (3.6)	*Ig, coa, eno, spa, hla, hlb, fib, clfA, clfB, nuc, map, fnbB, ebp, agr-1*	(n=12)	4 (33.3)	*eno, sei*
	1 (3.6)	*Ig, coa, eno, spa, hla, hlb, fib, clfA, clfB, nuc, map, fnbB, ebp, cap8*		1 (8.3)	*eno, Ig*
	1 (3.6)	*Ig, coa, eno, hla, hlb, fib, clfA, clfB, nuc, map, icaD, agr-1, cap8, sea*		1 (8.3)	*eno, map*
	1 (3.6)	*Ig, coa, eno, spa, fib, clfA, clfB, nuc, map, fnbB, icaD, agr-1, cap5*		1 (8.3)	*agr-2*
	1 (3.6)	*Ig, coa, eno, spa, hla, hlb, fib, clfA, clfB, nuc, map, fnbB, cap8*	*S. xylosus*	5 (41.7)	*eno, bap*
	1 (3.6)	*Ig, coa, eno, spa, hla, hlb, fib, clfA, clfB, nuc, icaD, agr-2, cap8*	(n=12)	5 (41.7)	*eno*
	1 (3.6)	*Ig, coa, eno, spa, hla, hlb, fib, clfA, clfB, map, fnbB, icaD, cap5*		1 (8.3)	*bap*
	1 (3.6)	*Ig, coa, eno, spa, hla, hlb, fib, clfA, map, fnbB, ebp, cap8, cna*		1 (8.3)	*No*
	1 (3.6)	*Ig, coa, eno, spa, hla, hlb, fib, clfB, nuc, map, fnbB, ebp, agr-1*	*S.*	4 (33.3)	*eno, Ig*
	1 (3.6)	*Ig, coa, eno, spa, hla, hlb, nuc, icaD, ebp, agr-2, cap8, seg, sei*	*chromogenes*	3 (25)	*No*
	1 (3.6)	*Ig, coa, eno, spa, hla, hlb, fib, clfA, map, fnbB, agr-1, cap8*	(n=12)	2 (16.7)	*Ig*
	1 (3.6)	*Ig, coa, eno, spa, hla, clfA, nuc, map, icaD, agr-1, cap8,sea*		2 (16.7)	*eno, Ig, fnbA*
	1 (3.6)	*Ig, coa, eno, spa, hla, hlb, fib, clfB, map, fnbB, ebp, cna*		1 (8.3)	*eno*
S. haemolyticus (n=3)	2 (66.7)	*eno, Ig*	*S. epidermidis*	4 (57.1)	*eno*
	1 (33.3)	*eno*	(n=7)	3 (42.9)	*No*
S. warneri (n=2)	1 (50)	*eno, agr-2*	*S. simulans*	5 (83.3)	*No*
	1 (50)	*No*	(n=6)	1 (16.7)	*fnbB*
S. saprophyticus (n=1)	1 (100)	*eno, agr-2*	*S. equorum*	3 (50)	*No*
S. succinus (n=1)	1 (100)	*eno*	(n=6)	1 (16.7)	*bap, cap8*
S. hyicus (n=1)	1 (100)	*No*		1 (16.7)	*bap*
S. muscae (n=1)	1 (100)	*No*		1 (16.7)	*eno, fnbB*

Table 7

Prevalence of antibiotic resistance genes in staphylococci from subclinical mastitis milk

Antibiotic resistance	Genes	No. of isolates (%)	
		S. aureus (n=28)	CNS (n=76)
Methicillin	*mecA*	10 (35.7)	13 (17.1)
Aminoglycoside	*aacA-aphD*	9 (32.1)	16 (21.1)
Streptogramin A	*vatA, B, C*	0 (0)	0 (0)
Erythromycin	*ermA*	0 (0)	0 (0)
and clindamycin	*ermB*	0 (0)	7 (9.2)
	ermC	2 (7.1)	10 (13.2)
Tetracycline	*tetK*	3 (10.7)	26 (34.2)
	tetM	0 (0)	2 (2.6)
Macrolide	*msrA*	0 (0)	13 (17.1)
(erythromycin)	*msrB*	0 (0)	15 (19.7)
Lincosamides	*linA*	2 (7.1)	29 (38.2)
Penicillin	*blaZ*	23 (82.1)	23 (30.3)
Streptomycin	*aac(6')/Aph(2")*	8 (28.6)	8 (10.5)
Kanamycin	*aph(3')-□a*	0 (0)	0 (0)
Tobramycin	*ant(4')-Ia*	0 (0)	0 (0)

Table 8

The antibiotic resistance gene patterns in staphylococci from subclinical mastitis milk

Species	Isolates (%)	Gene combinations
S. aureus (n=28)	10 (35.7)	*blaZ, mecA*
	7 (25)	*blaZ, aacA-aphD, aac(6')/aph(2")*
	5 (17.9)	No
	2 (7.1)	*blaZ, ermC, linA, tetK*
	2 (7.1)	*blaZ*
	1 (3.6)	*blaZ, aacA-aphD, aac(6')/aph(2"), tetK*
	1 (3.6)	*blaZ, aacA-aphD*
S. arlettae (n=12)	4 (33.3)	*msrA, msrB, linA*
	3 (25)	*msrB*
	2 (16.7)	*msrA, msrB, linA, tetK*
	2 (16.7)	*msrA, msrB*
	1 (8.3)	No
S. sciuri (n=12)	6 (50)	*mecA, tetK, linA,aacA-aphD, ermB*
	2 (16.7)	No
	1 (8.3)	*mecA, tetK, tetM, aacA-aphD, aac(6')/aph(2"), ermC*
	1 (8.3)	*mecA, linA, aacA-aphD*
	1 (8.3)	*tetK, tetM, aac(6')/aph(2"), ermC*
	1 (8.3)	*tetK, linA, ermB*
S. chromogenes (n=12)	3 (25)	*blaZ, ermC*
	3 (25)	No
	2 (16.7)	*blaZ, linA, tetK*
	1 (8.3)	*blaZ, aacA-aphD, aac(6')/aph(2")*
	1 (8.3)	*blaZ, aacA-aphD, etK, ermC*
	1 (8.3)	*blaZ, linA*
	1 (8.3)	*blaZ*
S. xylosus (n=12)	8 (66.7)	No
	3 (25)	*tetK*
	1 (8.3)	*linA, tetK*
S. epidermidis (n=7)	1 (14.3)	*blaZ, linA, tetK, aacA-aphD, aac(6')/aph(2"), mecA, ermC*
	1 (14.3)	*blaZ, linA, tetK, aacA-aphD, aac(6')/aph(2")*
	1 (14.3)	*blaZ, linA, tetK, mecA, msrA, msrB*
	1 (14.3)	*blaZ, aacA-aphD, aac(6')/aph(2")*
	1 (14.3)	*blaZ, mecA, msrA, msrB*
	1 (14.3)	*blaZ, msrA, msrB*
	1 (14.3)	*blaZ, linA, tetK*
S. simulans (n=6)	4 (66.7)	No
	1 (16.7)	*linA*
	1 (16.7)	*blaZ*
S. equorum (n=6)	3 (50)	No
	2 (33.3)	*linA*
	1 (16.7)	*tetK*
S. haemolyticus (n=3)	1(33.3)	*aacA-aphD, mecA, ermC, tetK, blaZ, ac(6')/aph(2")*
	1(33.3)	*aacA-aphD, mecA, ermC, tetK, linA*
	1(33.3)	*blaZ*
S. warneri (n=2)	1 (50)	*blaZ, linA, msrA, msrB*
	1 (50)	*blaZ, linA*
S. saprophyticus (n=1)	1 (100)	*linA, tetK, aacA-aphD, aac(6')/aph(2")*
S. succinus (n=1)	1 (100)	*msrA*
S. hyicus (n=1)	1 (100)	*blaZ, ermC*
S. muscae (n=1)	1 (100)	*blaZ*

Table 9

Distribution of antibiotic resistances and antibiotic resistant genes in staphylococci from subclinical mastitis milk

Antibiotic resistance	*S. aureus* (n=28)			CNS (n=76)		
	Phenotypic (%)	Genotype	Genes in isolates (%)	Phenotypic (%)	Genotype	Genes in isolates (%)
Cefoxitin	9 (32.1)	*blaZ*	1 (11.1)	21 (27.6)	*blaZ*	2 (9.5)
		mecA + *blaZ*	8 (88.9)		*mecA*	9 (42.9)
					mecA + *blaZ*	4 (19.0)
					unknown	6 (28.6)
Streptomycin	13 (46.4)	*aacA-aphD*	1 (7.7)	35 (46.1)	*aacA-aphD*	8 (22.9)
		aacA-aphD + *aac(6')/aph(2")*	7 (53.8)		*aac(6')/aph(2")*	1 (2.9)
					aacA-aphD + *aac(6')/aph(2")*	6 (17.1)
		unknown	5 (38.5)		unknown	20 (57.1)
Kanamycin	10 (35.7)	*aacA-aphD*	1 (10)	21 (27.6)	*aacA-aphD*	9 (42.9)
		aacA-aphD + *aac(6')/aph(2")*	8 (80)		*aac(6')/aph(2")*	1 (4.8)
					aacA-aphD + *aac(6')/aph(2")*	7 (33.3)
		unknown	1 (10)		unknown	4 (19)
Gentamicin	9 (32.1)	*aacA-aphD*	1 (11.1)	10 (13.2)	*aacA-aphD*	3 (30)
		aacA-aphD + *aac(6')/aph(2")*	8 (88.9)		*aac(6')/aph(2")*	1 (10)
					aacA-aphD + *aac(6')/aph(2")*	5 (50)
					unknown	1 (10)
Tobramycin	10 (35.7)	*aacA-aphD*	1 (10)	9 (11.8)	*aacA-aphD*	4 (44.4)
		aacA-aphD + *aac(6')/aph(2")*	7 (70)		*aac(6')/aph(2")*	1 (11.1)
					aacA-aphD + *aac(6')/aph(2")*	4 (44.4)
		unknown	2 (20)			
Erythromycin	4 (14.3)	*ermC*	2 (50)	37 (48.7)	*ermB*	7 (18.9)
		unknown	2 (50)		*ermC*	10 (27)
					msrB	2 (5.4)
					msrA + *msrB*	11 (29.7)
					unknown	7 (18.9)
Clindamycin	1 (3.6)	unknown	1 (100)	23 (30.3)	*ermC*	8 (34.8)
					linA	4 (17.4)
					ermB + *linA*	5 (21.7)
					ermC + *linA*	1 (4.3)
					unknown	5 (21.7)
Tetracycline	3 (10.7)	*tetK*	2 (66.7)	30 (39.5)	*tetK*	22 (73.3)
		unknown	1 (33.3)		*tetK* + *tetM*	1 (3.3)
					unknown	7 (23.3)
Penicillin	23 (82.1)	*blaZ*	23 (100)	66 (86.8)	*blaZ*	23 (34.8)
					unknown	43 (65.2)

- We found different distributions of the main coagulase-negative *Staphylococcus* species in subclinical mastitis milk from a Chinese dairy herd.
- We found different virulence gene patterns in the staphylococci from subclinical mastitis milk.
- We found high prevalence of *mecA* gene in both *S.aureus* and CNS isolates from subclinical mastitis milk.