J. Dairy Sci. 102:1571–1583
https://doi.org/10.3168/jds.2018-15136

Molecular epidemiology and distribution of antimicrobial resistance genes of *Staphylococcus* species isolated from Chinese dairy cows with clinical mastitis

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ABSTRACT

Staphylococcus species, categorized into *Staphylococcus aureus* and non-*aureus* staphylococci (NAS), are frequent causes of mastitis in dairy cattle around the world. Current treatments using antimicrobials are under increasing scrutiny due to rising prevalence of multi-drug resistance in *S. aureus*. Objectives of this study were to determine: (1) genetic diversity of *Staphylococcus* species isolated from clinical mastitis in cows from large Chinese dairy farms; and (2) prevalence and distribution of antimicrobial resistance genes (ARG) in these isolates. *Staphylococcus aureus* (n = 96) were isolated from 26 herds located in 12 provinces of China, whereas NAS (n = 112) were isolated from 59 herds located in 18 provinces of China. The NAS were identified at the species level using a partial 16S rRNA sequencing method, whereas random amplification of polymorphic DNA (RAPD) PCR was done to determine genetic relationships of isolates. Finally, PCR was used to detect resistance and biofilm formation genes. *Staphylococcus chromogenes* (33%) was the most common NAS species, followed by *Staphylococcus sciuri* (17%) and *Staphylococcus epidermidis* (8%). *Staphylococcus aureus* was grouped in 12 genotypes, of which 2 types represented 56% of isolates. *Staphylococcus chromogenes* (n = 37) clustered into 8 RAPD types, with 2 prevalent types containing 73% of isolates. The most prevalent ARG in *S. aureus* isolates was *blaZ* (95%), followed by *tetM* (33%), *tetK* (31%), *ermT* (26%), and *aacA-aphD* (23%). The *mecA* and *vanA* were detected in 16 and 4% of isolates, respectively. In NAS, *blaZ* (100%), *mecA* (73%), *tetK* (79%), *tetM* (96%), *mphC* (63%), and *msrA* (54%) were frequently detected. Antimicrobial resistance genes *mecA*, *tetK*, *tetL*, *tetM*, *dfrG*, *ermB*, *msrA*, *mphC*, *aadD*, and *aphA3* were more commonly detected in NAS than in *S. aureus*. Biofilm formation genes (*icaA* and *icaD*) were frequently detected in staphylococci isolated from bovine clinical mastitis. The existence of predominant RAPD types in *S. aureus* and *S. chromogenes* isolates across Chinese dairy farms indicated that specific genotypes had disseminated within herds and become more udder-adapted. High prevalence of ARG, especially in NAS, highlighted the risk of selection of multi-drug resistant staphylococci with potential as a reservoir of ARG.

Key words: bovine mastitis, *Staphylococcus aureus*, non-*aureus* staphylococci, random amplification of polymorphic DNA, antimicrobial resistance gene

INTRODUCTION

Bovine mastitis is the most common and economically important disease affecting the dairy industry worldwide (Hogeveen and Van Der Voort, 2017). The incidence rate of clinical mastitis (**CM**) in large Chinese herds is as high as 3.3 cases per 100 cows per month (Gao et al., 2017). *Staphylococcus* species, including *Staphylococcus aureus* and NAS, are one of the most important and prevalent etiological agents of bovine CM (Olde Riekerink et al., 2008; Gao et al., 2017). Though regarded as pathogens with minor clinical incidence, NAS have recently been identified as relatively common bovine mastitis pathogens in many countries, including China (Sampimon et al., 2011; Waller et al., 2011; Gao et al., 2017). In our previous epidemiology study (Gao et al., 2017), NAS (described as CNS in that manuscript) were isolated from 11% of 3,288 CM milk samples from 63% of 161 dairy herds. Although risk factors differ among NAS species (Condas et al.,

Received May 31, 2018.
Accepted October 11, 2018.
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2017), they are usually reported as a single group and usually not identified to the species level.

Molecular epidemiological studies have contributed to understanding sources and transmission routes of bovine mastitis pathogens. For *S. aureus*, several typing methods have been used, including ribotyping, random amplification of polymorphic DNA (**RAPD**), pulsed field gel electrophoresis (**PFGE**), multilocus sequence typing, *spa* typing, and multiple-locus variable number tandem repeat analysis methods (Zadoks et al., 2011). The environmental and contagious nature of some NAS species have been similarly explored by strain typing methods such as PFGE, amplified fragment length polymorphism, RAPD (Gillespie et al., 2009; Piessens et al., 2012), and whole-genome sequencing (Naushad et al., 2016). Among them, RAPD analysis has been successfully used to detect polymorphisms of both *S. aureus* and NAS strains from bovine mastitis (Piessens et al., 2012; Wang et al., 2016).

Efficacy of antimicrobial therapy for *S. aureus* CM is regarded as low (Sol et al., 2000). Use of antimicrobials is also increasingly scrutinized in Chinese dairy farms and worldwide due to increasing pressure from human medicine and prevalence of multi-drug-resistant bacterial strains in livestock (Barkema et al., 2015; Tang et al., 2017). Staphylococci can be resistant to antimicrobials, contributing to reduced efficacy of treatment with antibiotics (Barkema et al., 2006). Common antimicrobial resistance genes (**ARG**) detected among bovine *Staphylococcus* isolates included *blaZ* and *mecA* (β-lactam resistance genes); *tetK*, *tetL*, *tetM*, and *tetO* (tetracycline resistance genes); *aacA-aphD*, *aadD*, and *aphA3* (aminoglycoside resistance genes); *ermA*, *ermB*, *ermC*, *ermT*, *msrA*, *mphC*, and *lnuA* (macrolides, lincosamides, and streptogramin B; MLS_B resistance genes); *dfrG* and *dfrK* (trimethoprim resistance genes); and *vanA* and *vanB* (vancomycin resistance genes; Wendlandt et al., 2013; Nobrega et al., 2018). Furthermore, biofilm formation in *Staphylococcus* isolates from mastitis cases was also associated with reduced susceptibility to antimicrobials (Aslantaş and Demir, 2016). The *icaA* and *icaD* genes have been associated with biofilm formation in *S. aureus* and NAS (Vasudevan et al., 2003), whereas *bap* was only identified in *S. aureus* (Cucarella et al., 2004; Aslantaş and Demir, 2016). Antiseptics based on quaternary ammonium compounds (**QAC**) are commonly used for disinfecting barns, milking equipment, and teats of cow as preventive hygienic measures. Resistance of *Staphylococcus* isolates to QAC is associated with *qacA*, *qacB*, and *smr* genes (Bjorland et al., 2001).

Objectives of this study were to (1) identify species of NAS isolated from cows with CM by partial 16S rRNA sequencing (Lange et al., 2015); (2) determine genetic diversity of *Staphylococcus* species; and (3) estimate and compare prevalence of ARG and biofilm formation genes in *S. aureus* and NAS.

MATERIALS AND METHODS

Bacterial Isolates

A total of 208 *Staphylococcus* isolates from cows with CM were used in this study, including 96 *S. aureus* and 112 NAS. Part of the isolates were from a previous study (March 2014 to September 2016) on the incidence of CM in large Chinese dairy herds (Gao et al., 2017), whereas the remainder were collected from October 2016 to March 2017. Herds (≥500 lactating Holstein-Friesian cows) included in this study voluntarily participated in a large dairy farm mastitis program of the Animal Health Department of Boehringer Ingelheim in China. Milk samples were aseptically collected from quarters with visible signs of CM before antimicrobial treatment was initiated.

Non-*aureus* staphylococci isolates originated from 59 farms located in 18 provinces of China, whereas *S. aureus* isolates originated from a subset of these farms (n = 26) located in 12 Chinese provinces (Table 1). Farms in Heilongjiang supplied most *S. aureus* isolates (n = 34). For NAS, the majority of isolates were collected on farms in Hebei (n = 23), Beijing (n = 21), Inner Mongolia (n = 19), and Heilongjiang (n = 17).

Isolation of Bacterial DNA

Genomic DNA of *Staphylococcus* isolates was extracted from overnight culture using a bacterial DNA extraction kit (Transgen, Beijing, China) according to the manufacturer's instructions (http://www.transgen.com.cn/attached/down/EE161-01_2018121015.pdf). Integrity of genomic DNA was tested by resolving DNA extracts on a 0.8% wt/vol agarose gel by electrophoresis. Crude DNA samples were frozen at −20°C.

Species Identification of Isolates

Staphylococcus aureus and NAS were identified by α- and β-hemolysis on blood agar, Gram staining, positive catalase test, tube coagulase test, mannitol reaction, and presence of *nuc* gene (Gao et al., 2017). Species of NAS isolates was determined by partial 16S rRNA sequencing, where forward (5′-AGAGTTTGATCCTGGCTCAG-3′) and reverse (5′-GTATTACCGCGGCTGCTG-3′) primers were used to amplify a 536-bp product of the 16S rRNA gene, as described (Lange et al., 2015). The reaction mixture (25 μL) consisted of 12.5 μL of TaqMix (Transgen), 1 μL of template

DNA, 1 μL of each primer (10 μ*M*; Sunbiotech, Beijing, China), and 9.5 μL of distilled water. The PCR cycling conditions included an initial denaturation step at 95°C for 5 min, followed by 35 cycles at 95°C for 30 s, 55°C for 30 s, and 74°C for 1 min, with a final extension step at 74°C for 5 min. The PCR products were subject to sequencing after verification on a 1% agarose gel. The 16S rRNA sequences were compared with sequences deposited in the nucleotide database of the National Center for Biotechnology Information. Identification was deemed reliable if values for sequence similarities were >99% (Lange et al., 2015).

RAPD Analysis

The RAPD typing was conducted on *S. aureus* and *S. chromogenes* isolates as described for *S. aureus* (Wang et al., 2016) and NAS (Piessens et al., 2012). The RAPD-PCR primers used were 5′-GTGGATGCGA-3′ for *S. aureus* and 5′-AGTGAATTCGCGGT GAGATGCCA-3′ for NAS. A total of 50 μL of reaction mixture was prepared with 25 μL of Taqmix (Transgen), 1 μL of primer (10 μ*M*), 1 μL of template DNA, and 23 μL of distilled water. The PCR program for *S. aureus* 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 extension at 72°C for 1 min, and a final extension at 72°C for 8 min. *Staphylococcus aureus* ATCC 29213 strain was used as a quality control strain. The cycling program for NAS included 4 cycles of 94°C, 5 min; 36°C, 5 min; and 72°C, 5 min; and 30 cycles of 94°C, 1 min; 36°C, 1 min; and 72°C, 2 min, and then 72°C, 10 min. *Staphylococcus epidermidis* CMCC 26069 was used as a control strain. Isolates were clustered using InfoQuest FP software (Bio-Rad Laboratories, Hercules, CA) and groups analyzed by the Dice coefficient and the unweighted pair group method with arithmetic averages. Genetic cluster relationships were mapped.

Detection of Resistance and Biofilm Formation Genes

All *S. aureus* and NAS isolates were screened for the presence of the following genes: *blaZ*, *mecA*, *tetK*, *tetL*, *tetM*, *tetO*, *aacA-aphD*, *aadD*, *aphA3*, *ermA*, *ermB*, *ermC*, *ermT*, *msrA*, *mphC*, *lnuA*, *dfrG*, *dfrK*, *vanA*, *vanB* (ARG); *qacA*, *qacB*, and *smr* (QAC resistance genes); and *icaA* and *icaD* (biofilm formation genes). Presence of *bap* was only determined in *S. aureus* isolates. Content of the reaction mixture was the same as that used in 16S rRNA sequencing amplification mentioned above. Initial denaturation at 95°C for 5 min was followed by 30 cycles of amplification at 95°C for 45 s, annealing at specific temperatures (Table 2) for 30 s, extension at 72°C for 60 to 90 s, and a final extension step at 72°C for 10 min.

Statistical Analyses

Prevalence was obtained in cross tabulations and expressed in percentage points. Generalized linear mixed models were used to compare proportion of ARG between NAS and *S. aureus*, and between *S. chromogenes* and *S. sciuri*. Models were fit via maximum likelihood

Table 1. Origin of staphylococci used in the study

	Staphylococcus aureus		NAS	
Province	No. of isolates	No. of herds	No. of isolates	No. of herds
Anhui	2	1	1	1
Beijing	9	3	21	12
Guangdong	6	1	5	2
Hebei	11	4	23	12
Heilongjiang	34	5	17	6
Liaoning	9	2	10	3
Inner Mongolia	14	5	19	12
Shandong	2	1	1	1
Gansu	1	1	2	1
Shaanxi	4	1	3	1
Shanghai	2	1	2	1
Henan	2	1	2	1
Fujian	—	—	1	1
Shanxi	—	—	1	1
Jilin	—	—	1	1
Hubei	—	—	1	1
Jiangsu	—	—	1	1
Tianjin	—	—	1	1

with 50 quadrature points per scalar using the adaptive Gauss-Hermite quadrature. For these analyses, the logit link was used to model expected values, whereas the outcome was assumed to follow a Bernoulli distribution. Farm random effects were introduced in all analyses at the intercept level to address a hypothetical lack of independence introduced by isolates obtained from the same herds. Observations from the same province were assumed to be independent. Fisher's exact test was used for genes with <6 positive outcomes or in the presence of zero cells. For all analyses, $P < 0.05$ was considered significant. All *P*-values obtained were adjusted for multiple comparisons (Benjamini and Hochberg, 1995). Analyses were carried out in R version 3.4.2 (R Core Team, 2017), using the packages *stats* and *lme4* (Bates et al., 2015).

Table 2. PCR primers used in the study

Gene	Sequence[1] (5′ to 3′)	Annealing temperature (°C)	Amplicon size (bp)	Reference
blaZ	F: ACTTCAACACCTGCTGCTTTC R: TGACCACTTTTATCAGCAACC	57	240	Martineau et al., 2000
mecA	F: GTAGAAATGACTGAACGTCCGATAA R: CCAATTCCACATTGTTTCGGTCTAA	59	310	Spanu et al., 2004
tetK	F: GTAGCGACAATAGGTAATAGT R: GTAGTGACAATAAACCTCCTA	54	360	Strommenger et al., 2003
tetL	F: TCGTTAGCGTGCTGTCATTC R: GTATCCCACCAATGTAGCCG	60	267	Ng et al., 2001
tetM	F: AGTGGAGCGATTACAGAA R: CATATGTCCTGGCGTGTCTA	58	158	Strommenger et al., 2003
tetO	F: AACTTAGGCATTCTGGCTCAC R: TCCCACTGTTCCATATCGTCA	58	515	Ng et al., 2001
aacA-aphD	F: GAAGTACGCAGAAGAGA R: ACATGGCAAGCTCTAGGA	55	491	Choi et al., 2003
aadD	F: CAAACTGCTAAATCGGTAGAAGCC R: GGAAAGTTGACCAGACATTACGAACT	60	296	Klingenberg et al., 2004
aphA3	F: GGCTAAAATGAGAATATCACCGG R: CTTTAAAAAATCATACAGCTCGCG	58	523	Klingenberg et al., 2004
ermA	F: TATCTTATCGTTGAGAAGGGATT R: CTACACTTGGCTTAGGATGAAA	56	139	Martineau et al., 2000
ermB	F: CTATCTCATTGTTGAAGAAGGATT R: GTTTACTCTTGGTTTAGGATGAAA	55	142	Martineau et al., 2000
ermC	F: CTTGTTGATCACGATAATTTCC R: ATCTTTTAGCAAACCCGTATTC	55	190	Martineau et al., 2000
ermT	F: ATTGGTTCAGGGAAAGGTCA R: GCTTGATAAAATTGGTTTTTGGA	54	536	Fessler et al., 2010
inuA	F: GGTGGCTGGGGGGTAGATGTATTAACTGG R: GCTTCTTTTGAAATACATGGTATTTTTCGA	62	323	Lina et al., 1999
msrA	F: GGCACAATAAGAGTGTTTAAAGG R: AAGTTATATCATGAATAGATTGTCCTGTT	57	940	Lina et al., 1999
mphC	F: GAGACTACCAAGAAGACCTGACG R: CATACGCCGATTCTCCTGAT	60	722	Luthje and Schwarz, 2006
vanA	F: GGGAAAACGACAATTGC R: GTACAATGCGGCCGTTA	54	732	Simeoni et al., 2008
vanB	F: ATGGGAAGCCGATAGTC R: GATTTCGTTCCTCGACC	55	635	Simeoni et al., 2008
dfrG	F: TGCTGCGATGGATAAGAA R: TGGGCAAATACCTCATTCC	54	405	Argudin et al., 2011
dfrK	F: CAAGAGATAAGGGGTTCAGC R: ACAGATACTTCGTTCCACTC	57	229	Argudin et al., 2011
icaA	F: CCTAACTAACGAAAGGTAG R: AAGATATAGCGATAAGTGC	52	1,315	Vasudevan et al., 2003
icaD	F: AAACGTAAGAGAGGTGG R: GGCAATATGATCAAGATAC	52	381	Vasudevan et al., 2003
bap	F: CCCTATATCGAAGGTGTAGAATTGCAC R: GCTGTTGAAGTTAATACTGTACCTGC	60	971	Cucarella et al., 2004
qacA/B	F: GCTGCATTTATGACAATGTTTG R: AATCCCACCTACTAAAGCAG	55	630	Anthonisen et al., 2002
smr	ATAAGTACTGAAGTTATTGGAAGT TTCCGAAAATGTTTAACGAAACTA	54	286	Bjorland et al., 2001

[1]F = forward; R = reverse.

RESULTS

Species Identification of NAS Isolates

All PCR products generated for sequence analysis were of the expected size (i.e., 536 bp). After comparing16S rRNA sequences to sequences deposited in GenBank, 109 of 112 the NAS isolates were identified at the species level (Figure 1). The most frequent NAS species detected was *S. chromogenes* (n = 37; 33%), followed by *S. sciuri* (n = 19; 17%), *S. epidermidis* (n = 9; 8%), *S. haemolyticus* (n = 7; 6%), *S. simulans* (n = 7; 6%), *S. argenteus* (6; 5%), *S. equorum* (6; 5%), and *S. hominis* (n = 5; 4%). Three NAS isolates remained unidentified.

RAPD Profiles

The RAPD profiles of *S. aureus* and *S. chromogenes* (the predominant NAS species) resulted in DNA amplification fragments ranging in size from 200 to 2,000 bp. Genomic variability in 95 of 96 *S. aureus* isolates from 25 herds was determined using RAPD-PCR analysis (Figure 2). The remaining isolate was removed from further analysis. The 95 isolates clustered into 12 RAPD types (A–L) according to their 60% similarity. Of them, 2 prevalent *S. aureus* types (D and J) represented 56% of isolates (53/95). Type D consisted of 25 isolates collected from 14 herds in 9 Chinese provinces and type J contained 28 isolates collected from 12 herds in 5 Chinese provinces. Of the 7 herds supplying >5 isolates, only 1 herd had a single RAPD type (J), whereas the other herds had >2 types of isolates. At 80% similarity, 8 distinct RAPD types (A–H) were detected among 37 NAS *S. chromogenes* isolates (Figure 3). Two prevalent *S. chromogenes* types (A and F) represented 73% of the isolates (27/37). Type A consisted of 8 isolates from 6 herds in 5 provinces. Type F contained 19 isolates collected from 16 herds in 9 provinces. Four of 5 isolates from 1 herd belonged to a type H, whereas 3 of 4 isolates from another herd clustered to a type F.

Prevalence of Antimicrobial Resistance Genes

Prevalence of β-lactam resistance genes was high in *S. aureus* isolates, where 91 (95%) of *S. aureus* isolates possessed the *blaZ* gene and 15 (16%) harbored the *mecA* gene (Tables 3). Tetracycline resistance genes were prevalent in *S. aureus*, where *tetK* and *tetM* were detected in 31 and 33% of the isolates, respectively (Table 3). Other ARG in *S. aureus*, including *ermT* (erythromycin resistance gene), *inuA* (lincomycin resistance gene), and *aacA-aphD* (aminoglycoside resistance

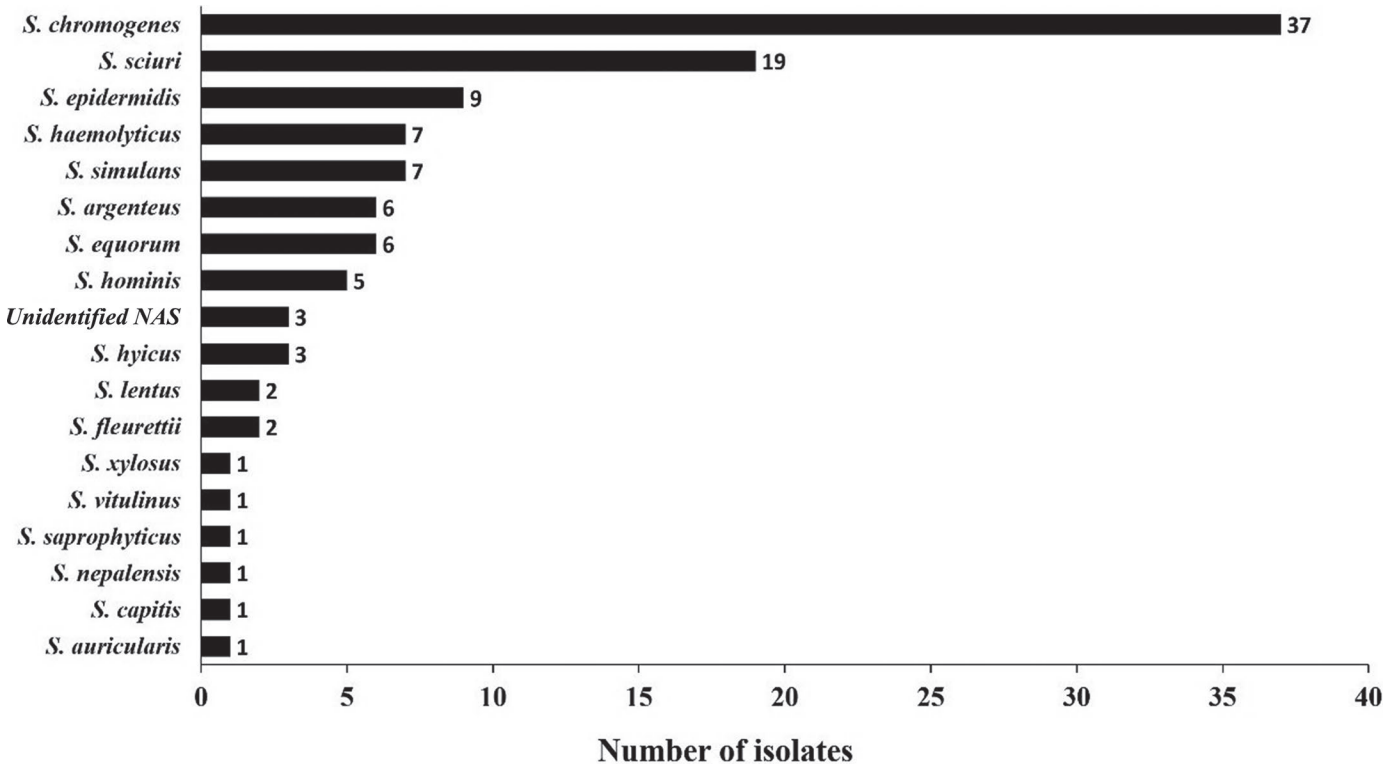

Figure 1. Species identification (partial 16S rRNA sequencing) of 112 NAS isolates from bovine mastitis in large Chinese dairy herds.

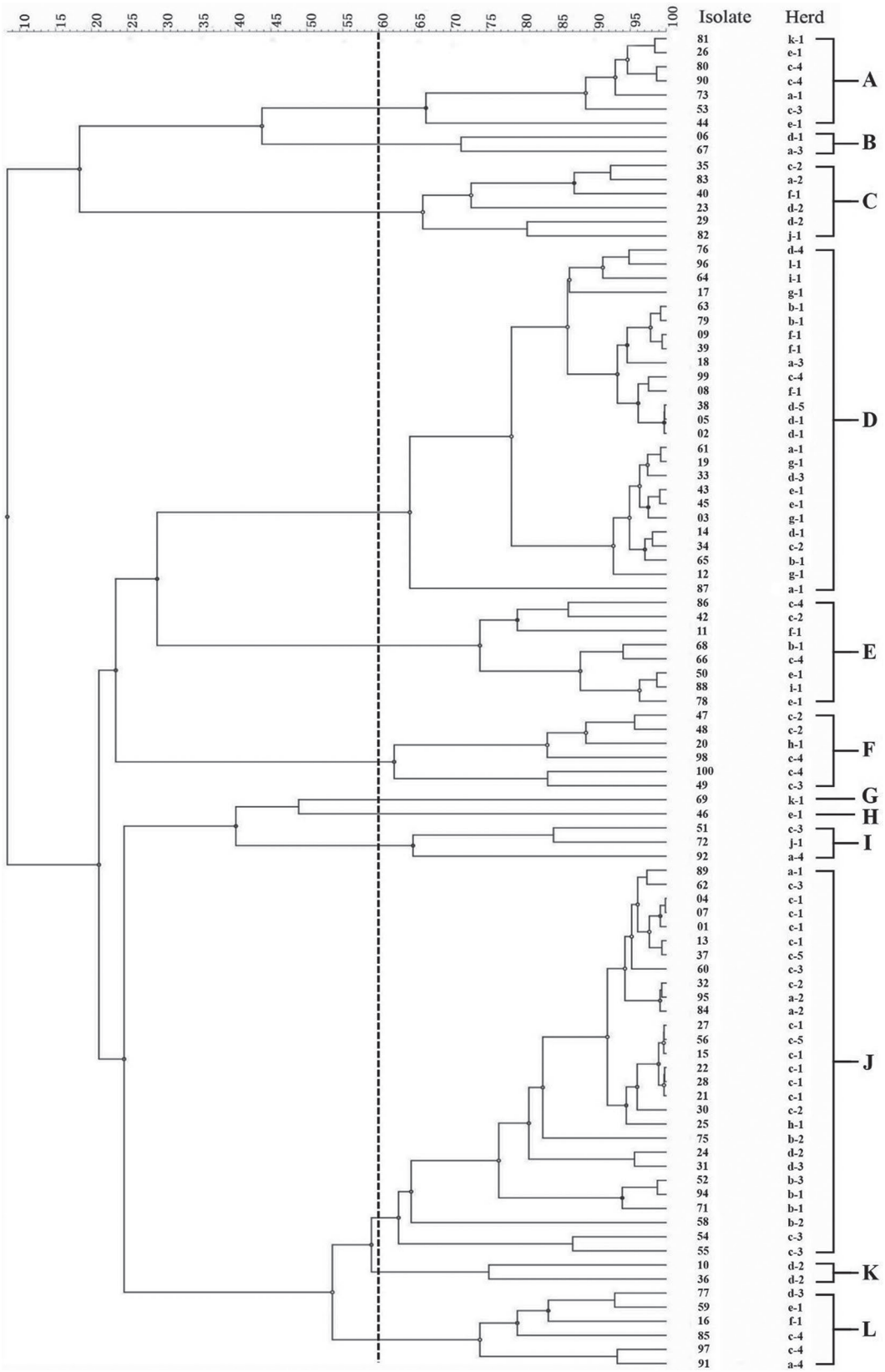

Figure 2. Dendrogram derived from random amplification of polymorphic DNA of *Staphylococcus aureus*. A 60% similarity was chosen as a discriminating threshold to define homogeneous clusters.

gene), were detected in 26, 21, and 23% of isolates, respectively (Tables 4 and 5). Four (4%) *S. aureus* isolates were *vanA* (vancomycin resistance gene) positive (Table 5). All *S. aureus* were negative for *dfrK*, *ermA*, and *vanB* (Tables 4 and 5).

For NAS, β-lactam resistance genes were highly prevalent, with the *blaZ* gene detected in all isolates and the *mecA* gene detected in 73% of isolates (Table 3). Prevalence in NAS of the tetracycline resistance genes *tetK* (79%), *tetL* (47%), and *tetM* (96%), and the MLS_B resistance genes *ermB* (20%), *inuA* (24%), *msrA* (54%), and *mphC* (63%) was high (Tables 3 and 4). In addition, *dfrG*, *aacA-aphD*, and *aphA3* were present in 25 (22%), 24 (21%), and 43 (38%) NAS isolates (Table 5). Ten (9%) isolates carried the *vanA* gene. Genes *dfrK*, *ermA*, and *vanB* were not detected in any NAS isolate (Tables 4 and 5).

The genes *mecA*, *tetK*, *tetL*, *tetM*, *dfrG*, *ermB*, *msrA*, *mphC*, and *aphA3* were detected in higher proportions of NAS isolates than in *S. aureus* ($P < 0.05$ for all comparisons), whereas *aadD* tended to be detected in high proportions ($P = 0.09$). The proportion of *ermT*-positives was higher for *S. aureus* than for NAS ($P < 0.001$). No differences were observed when comparing

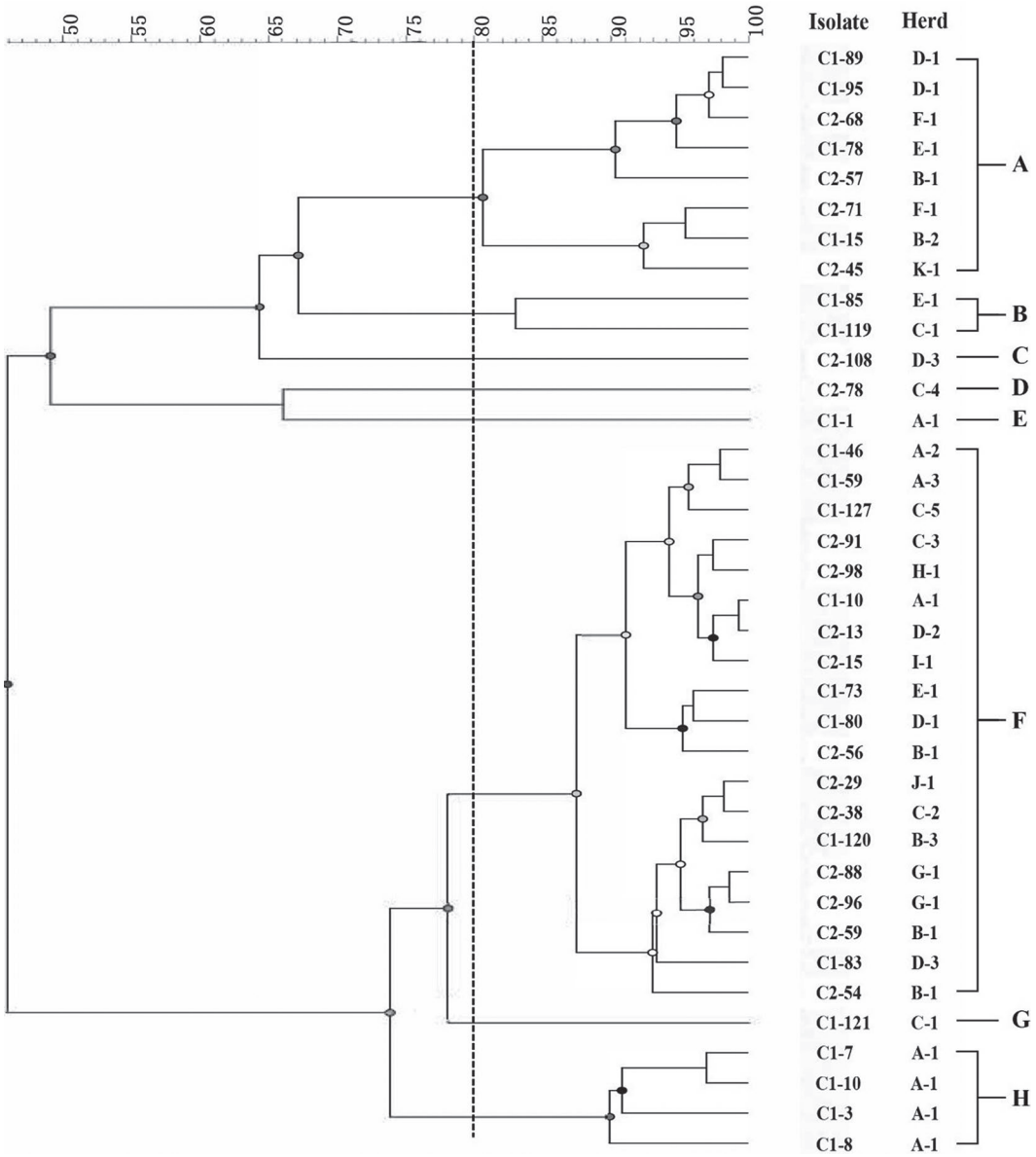

Figure 3. Dendrogram derived from the random amplification of polymorphic DNA of *Staphylococcus chromogenes*. An 80% similarity was chosen as a discriminating threshold to define homogeneous clusters.

Table 3. Prevalence of β-lactam and tetracycline resistance genes

Species	No. of isolates	No. of positive isolates (% in parentheses)					
		blaZ	*mecA*	*tetK*	*tetL*	*tetM*	*tetO*
Staphylococcus aureus	96	91 (95)	15 (16)[a]	30 (31)[a]	9 (9)[a]	32 (33)[a]	4 (4)
Total NAS	112	112 (100)	82 (73)[a]	89 (79)[a]	53 (47)[a]	108 (96)[a]	7 (6)
Staphylococcus chromogenes	37	37 (100)	26 (70)	34 (92)	17 (46)	37 (100)	2 (5)
Staphylococcus sciuri	19	19 (100)	14 (74)	16 (84)	11 (58)	18 (95)	2 (11)
Staphylococcus epidermidis	9	9 (100)	8 (89)	6 (67)	8 (89)	9 (100)	3 (33)
Staphylococcus haemolyticus	7	7 (100)	4 (57)	4 (57)	2 (29)	6 (85.7)	0
Staphylococcus simulans	7	7 (100)	5 (71)	6 (86)	2 (29)	7 (100)	0
Staphylococcus argenteus	6	6 (100)	4 (67)	3 (50)	2 (33)	5 (83)	0
Staphylococcus equorum	6	6 (100)	5 (83)	3 (50)	0	6 (100)	0
Staphylococcus hominis	5	5 (100)	5 (100)	4 (80)	3 (60)	5 (100)	0
Others	16	16 (100)	11 (69)	13 (81)	8 (50)	15 (94)	0

[a]Difference ($P < 0.05$) between *Staphylococcus aureus* and total NAS.

prevalence of ARG between *S. chromogenes* and *S. sciuri* ($P > 0.05$ for all comparisons).

Gene Patterns

Most staphylococci isolates (n = 190) had a unique gene pattern (e.g., carried a unique combination of genes). When excluding antiseptic and biofilm formation genes, the single presence of *blaZ*, followed by concomitant presence of *blaZ*, *aacA*, and *aphD*, or *blaZ*, *tetK*, and *tetM* was observed in 12 (6%), 7 (3%), and 7 (3%) isolates, respectively (Table 6), demonstrating a potential for co-selection. Notably, only 15 isolates (7%) harbored gene(s) that, if expressed, would confer resistance to a single drug class; the vast majority of isolates (146 isolates; 70%) harbored resistance elements that would confer resistance against at least 3 antimicrobial classes. Forty-four isolates (21% of total) had genetic potential to be resistant against macrolides, lincosamides, streptogramins B (MLS_B), tetracyclines, β-lactams, and aminoglycosides.

Prevalence of Antiseptic Resistance and Biofilm Formation Genes

Prevalence of antiseptic (QAC) resistance genes and biofilm formation genes (Table 7) indicated that no *S. aureus* carried the QAC resistance genes (*qacA/B* and *smr*), but that *S. aureus* carried biofilm formation genes, *icaA* (35%), *icaD* (92%), and *bap* (5%). Of the NAS isolates, 13 (12%) contained the *smr* gene, whereas 27 (24%) and 69 (62%) NAS isolates carried *icaA* and *icaD*, respectively. *Staphylococcus aureus* isolates more often carried *icaD* but less often the *smr* gene, compared with NAS ($P < 0.05$).

DISCUSSION

In the present study, a reliable DNA-based method (Lange et al., 2015) was used to characterize species in 109 of 112 NAS isolates from CM. Our results confirmed the importance of *S. chromogenes* as the most frequent NAS species isolated from CM in Chinese dairy herds,

Table 4. Prevalence of macrolide, streptogramin B, and lincomycin resistance genes

Species	No. of isolates	No. of positive isolates (% in parentheses)						
		ermA	*ermB*	*ermC*	*ermT*	*inuA*	*msrA*	*mphC*
Staphylococcus aureus	96	0	7 (7)[a]	14 (15)	25 (26)[a]	20 (21)	10 (10)[a]	7 (7)[a]
Total NAS	112	0	22 (20)[a]	10 (9)	3 (3)[a]	27 (24)	60 (54)[a]	70 (63)[a]
Staphylococcus chromogenes	37	0	7 (19)	3 (8)	0	4 (11)	20 (54)	19 (51)
Staphylococcus sciuri	19	0	5 (26)	4 (21)	0	6 (32)	10 (53)	16 (84)
Staphylococcus epidermidis	9	0	2 (22)	2 (22)	0	2 (22)	4 (44)	5 (56)
Staphylococcus haemolyticus	7	0	2 (29)	0	0	3 (43)	2 (29)	3 (43)
Staphylococcus simulans	7	0	1 (14)	1 (14)	2 (3)	1 (14)	4 (57)	3 (43)
Staphylococcus argenteus	6	0	1 (17)	0	0	0	2 (33)	3 (50)
Staphylococcus equorum	6	0	1 (17)	0	0	0	3 (50)	2 (33)
Staphylococcus hominis	5	0	0	0	0	5 (100)	5 (100)	5 (100)
Others	16	0	3 (19)	0	1 (6)	6 (38)	10 (63)	14 (88)

[a]Difference ($P < 0.05$) between *Staphylococcus aureus* and total NAS.

Table 5. Prevalence of carrying aminoglycoside, vancomycin, and sulfanilamide resistance genes

Species	No. of isolates	No. of positive isolates (% in parentheses)						
		aacA-aphD	*aadD*	*aphA3*	*vanA*	*vanB*	*dfrG*	*dfrK*
Staphylococcus aureus	96	22 (23)	1 (1)	7 (7)[a]	4 (4)	0	5 (5)[a]	0
Total NAS	112	24 (21)	12 (11)	43 (38)[a]	10 (9)	0	25 (22)[a]	0
Staphylococcus chromogenes	37	7 (19)	2 (5)	11 (30)	3 (8)	0	10 (27)	0
Staphylococcus sciuri	19	5 (26)	0	8 (42)	0	0	5 (26)	0
Staphylococcus epidermidis	9	4 (44)	1 (11)	4 (44)	0	0	5 (56)	0
Staphylococcus haemolyticus	7	1 (14)	0	3 (43)	0	0	1 (14)	0
Staphylococcus simulans	7	2 (29)	0	1 (14)	1 (14)	0	0	0
Staphylococcus argenteus	6	2 (33)	1 (17)	2 (33)	0	0	0	0
Staphylococcus equorum	6	2 (33)	0	2 (33)	0	0	0	0
Staphylococcus hominis	5	0	5 (100)	4 (80)	4 (80)	0	3 (60)	0
Others	16	1 (6)	3 (19)	8 (50)	2 (13)	0	1 (6)	0

[a]Difference ($P < 0.05$) between *Staphylococcus aureus* and total NAS.

similar to studies carried out in the United States, the Netherlands, and Canada (Gillespie et al., 2009; Sampimon et al., 2011; Condas et al., 2017), although other species have been also frequently detected in clinical and subclinical mastitis milk (Thorberg et al., 2009; Frey et al., 2013). *Staphylococcus sciuri* was the second most common NAS (17%), a percentage similar to a previous report in a Canadian study by Condas et al. (2017). In contrast, we rarely isolated *Staphylococcus xylosus* (<1%), a NAS species more common in milk of cows with subclinical mastitis or low SCC (Thorberg et al., 2009; Frey et al., 2013). Similar to our results, *S. xylosus* was less frequently isolated in CM cases in another report (Condas et al., 2017). It has been reported that cow factors contribute to NAS distribution (Thorberg et al., 2009; Condas et al., 2017). For instance, *S. xylosus* was frequently isolated from quarters with low SCC, whereas *S. sciuri* was commonly present in CM cases (Condas et al., 2017). In addition, *S. chromogenes* IMI was most common in heifers, whereas multiparous cows more often than first lactation heifers had *S. epidermidis* IMI (Thorberg et al., 2009; Condas et al., 2017). In the present study, a considerable proportion of CM was caused by NAS species, which does not agree with the traditional statement regarding NAS as minor pathogens (Taponen and Pyörälä, 2009). Though we could determine the overall prevalence of NAS in CM, more details on animals (lactation, milking days, and severity of disease) were not obtained in the study. Therefore, we can only hypothesize that the mammary immunity of cows in many Chinese herds may be poorer, so that NAS (opportunistic pathogens) easily proliferate within the udder, causing severe inflammation with clinical signs. The main reason behind this hypothesis is that as a result of high genetics and improved nutrition, milk yield of cows in Chinese herds has rapidly increased, but important mastitis management practices such as milking management are still not optimal, resulting in insufficient mammary resistance to pathogens. Conversely, we could hypothesize that NAS isolates from Chinese herds on average are more virulent. Thus, in China, udder health management and its association with pathogen-specific CM incidence need to be studied. Additionally, virulence of NAS isolates in Chinese dairy herds needs to be characterized, as well as the association with clinical severity. It is important to emphasize, though, that this study was not designed to estimate the prevalence of mastitis-causing pathogens. Therefore, the apparent high prevalence of

Table 6. Gene patterns of staphylococci isolates

Gene pattern[1]	No. of isolates	%
blaZ	12	5.8
blaZ aacA aphD	7	3.4
blaZ tetK tetM	7	3.4
blaZ mecA tetK tetL tetM	4	1.9
blaZ mecA tetK tetM aphA3 msrA mphC dfrG	4	1.9
blaZ ermC ermT	3	1.4
blaZ ermT	3	1.4
blaZ tetM	3	1.4
*blaZ Inu*A	2	1.0
blaZ tetK tetM aphA3 inuA mphC	2	1.0
blaZ tetK tetM inuA	2	1.0
blaZ tetM msrA mphC	2	1.0
blaZ mecA aacA_aphD inuA	2	1.0
blaZ mecA tetK ermT	2	1.0
blaZ mecA tetK tetL tetM ermB msrA dfrG	2	1.0
blaZ mecA tetK tetL tetM tetO aphA3 msrA mphC	2	1.0
blaZ mecA tetK tetM	2	1.0
blaZ mecA tetK tetM aphA3 inuA msrA mphC	2	1.0
blaZ mecA tetK tetM aphA3 mphC	2	1.0
blaZ mecA tetK tetM ermB msrA mphC	2	1.0
blaZ mecA tetK tetM mphC	2	1.0
blaZ mecA tetK tetM msrA	2	1.0
blaZ mecA tetK tetM msrA mphC	2	1.0
Unique patterns	135	64.9
Total	208	100

[1]Excluding antiseptic resistance and biofilm formation genes.

Table 7. Prevalence of antiseptic resistance and biofilm formation genes

Species	No. of isolates	No. of positive isolates (% in parentheses)				
		qacA/B	*smr*	*icaA*	*icaD*	*bap*
Staphylococcus aureus	96	0	0[a]	34 (35)	88 (92)[a]	5 (5)
Total NAS	112	0	13 (12)[a]	27 (24)	69 (62)[a]	—
Staphylococcus chromogenes	37	0	2 (5)	4 (11)	20 (54)	—
Staphylococcus sciuri	19	0	2 (11)	1 (5)	8 (42)	—
Staphylococcus epidermidis	9	0	0	5 (56)	7 (78)	—
Staphylococcus haemolyticus	7	0	0	2 (29)	6 (86)	—
Staphylococcus simulans	7	0	0	1 (14)	5 (71)	—
Staphylococcus argenteus	6	0	1 (17)	5 (83)	6 (100)	—
Staphylococcus equorum	6	0	0	6 (100)	5 (83)	—
Staphylococcus hominis	5	0	5 (100)	1 (20)	3 (60)	—
Others	16	0	3 (19)	2 (13)	9 (56)	—

[a]Difference ($P < 0.05$) between *Staphylococcus aureus* and total NAS.

pathogens not frequently isolated from CM (e.g., NAS) in other studies should not be overinterpreted.

We characterized *S. aureus* by RAPD-PCR with 60% similarity threshold (Wang et al., 2016) and identified 2 predominant RAPD types representing 56% of trans-regional isolates. Similar results were reported in another Chinese study, with 2 predominant RAPD types of 6 representing 71% *S. aureus* isolates (Wang et al., 2016). Therefore, particular *S. aureus* strains might transmit more from cow to cow. Another explanation for the higher incidence of certain strains in CM would be that these strains are more successful in establishing chronic IMI if they are not removed from herds, resulting in flare-ups as CM cases. In regards to NAS, because RAPD fingerprints of *S. chromogenes* isolates were homogeneous (Piessens et al., 2012), the similarity threshold was increased to 80% to improve discriminative power. Predominant types were observed among *S. chromogenes* in NAS isolates, with 2 predominant among 8 RAPD types. Though a study reported conserved genetic patterns of *S. chromogenes* (Shimizu et al., 1997; Piessens et al., 2012), diversity of *S. chromogenes* was conversely reported in dairy herds from United States using the PFGE method (Gillespie et al., 2009). Thus, although NAS are commonly considered environmental pathogens, NAS species may exhibit discriminatory diversity (Piessens et al., 2012) and appearance of predominant types in *S. chromogenes* may be due to udder adaption. Cow-to-cow transmission probably remains important in dissemination of *S. chromogenes*.

Knowledge of distribution of ARG among pathogenic and non-pathogenic udder microbes is key to understand evolution of multi-drug resistant agents in dairy cattle. All *Staphylococcus* isolates carried at least 1 ARG and nearly all isolates (98%) carried the *blaZ* gene, indicative of penicillin resistance (Wendlandt et al., 2013). Likewise, *mecA* gene (resistance to β-lactam antibiotics; Wendlandt et al., 2013) was identified in >15 and 70% of *S. aureus* and NAS isolates, respectively. Prevalence of *mecA* among *Staphylococcus* isolates in this study was higher on these Chinese dairy farms than in CM isolates from other studies (Frey et al., 2013; Ruegg et al., 2015). This high prevalence of *blaZ* and *mecA* and potential resistance to β-lactam antibiotics in Chinese bovine *Staphylococcus* isolates highlighted a decisive point in the use of these antimicrobials, commonly used in mastitis treatment, in the dairy industry in China and other countries.

Of tetracycline resistance genes, *tetK* and *tetM* genes were more frequently detected than *tetL* and *tetO*. In bovine staphylococci, *tetK* gene codes for membrane associated efflux proteins, whereas *tetM* gene products represent ribosome protective proteins which mediate resistance to all tetracyclines, including minocycline (Wendlandt et al., 2013). High prevalence of *tetK* and *tetM* demonstrated that 2 mechanisms may contribute to tetracycline resistance in these Chinese staphylococci. The *tetO* gene was similar to other reports, but rarely detected in these staphylococci (Schwarz et al., 1998; Liu et al., 2017).

Among MLS_B resistance genes, this study focused on *erm*, *msrA*, *mphC*, and *lnuA* classes as key genes in resistance to macrolides. In particular, *erm* codes for methylases conferring combined resistance to macrolides, lincosamides, and streptogramin B, *msrA* gene for a transporter protein conferring resistance to macrolides and streptogramin B, *mphC* for a macrolide phosphotransferase conferring only resistance to macrolides, and *lnuA* for nucleotidyltransferase conferring only resistance to lincosamides (Wendlandt et al., 2013). The *ermA/B/C* genes were not commonly detected in either *S. aureus* or NAS (<20%), which was different from other Chinese studies (Li et al., 2015; Wang et al., 2015). Thus, few staphylococci may have combined resistance to macrolides, lincosamides, and

streptogramin B. However, predominance of *ermT* in *S. aureus* among MLS_B resistance genes was a concern because this is a plasmid-born gene with an additional resistance component (Fessler et al., 2010). In addition, more than half of NAS carried *msrA* and *mphC* and could develop further resistance to macrolides and streptogramin B.

Among aminoglycoside resistance genes, *aacA-aphD* in *S. aureus* and *aphA3* predominated in NAS. Gene *aacA-aphD* is widely distributed in staphylococci of animal origin, including *S. aureus* from bovine mastitis (Fessler et al., 2010), whereas *aphA3* was mainly present in canine and feline NAS (Wendlandt et al., 2013). In terms of antimicrobial resistance, *aacA-aphD* codes for a bifunctional enzyme and confers resistance to gentamicin, kanamycin, tobramycin, and amikacin, whereas *aphA3* codes for a phosphotransferase mediating resistance to kanamycin, neomycin, and amikacin (Wendlandt et al., 2013). Thus, different distribution of *aacA-aphD* and *aphA3* genes between *S. aureus* and NAS from CM may lead to different resistant phenotypes to aminoglycosides.

Our finding of vancomycin resistance genes in *S. aureus* (*vanA*, 4%) and NAS (*vanA*, 9%) isolated from CM in China was alarming for public health, as vancomycin is a main antimicrobial agent to treat serious infections caused by methicillin-resistant staphylococci in human medicine. Proportions of NAS carrying ARG were higher than in *S. aureus* (*mecA*, *tetK/L/M*, *dfrG*, *ermB*, *msrA*, *mphC*, *aadD*, and *aphA3*). As NAS easily develop multi-resistance compared with *S. aureus* (Taponen and Pyörälä, 2009), bovine NAS carrying multidrug resistance genes in Chinese dairy farms represent a reservoir of ARG and a potential threat to human health.

The majority of isolates carried a combination of resistance genes that could confer resistance to 2 or more distinct drug classes. Due to the alarming number and combination of genes observed, we hypothesize that resistance genes in staphylococci isolates of bovine origin in China are being carried out in plasmids for at least 2 distinct reasons. First, the location of a resistance gene on a mobile genetic element results in an efficient dissemination across different pathogens such as NAS and *S. aureus* (Wendlandt et al., 2015). Second, staphylococci resistance plasmids that contain multidrug resistance genes such as *erm* genes frequently carry 1 or more additional resistance elements (Wendlandt et al., 2015). Irrespective of their location, our findings imply that resistance genes in staphylococci isolated from bovine mastitis in China can be co-selected and potentially persist in the absence of selective pressure introduced by a particular antimicrobial class. Therefore, a plan to delay emergence of AMR on the same population would require prudent use of several antimicrobial classes, increasing the complexity of such a strategy.

Evaluation of potential resistance of staphylococci isolates to QAC has been helpful for developing effective disinfecting strategies. Two genes (*qacA/B* and *smr*) responsible for efflux-mediated resistance to QAC were detected in the present study. Unlike widespread distribution of QAC-resistant strains in Norwegian herds (Bjorland et al., 2005), *qacA/B* was not detected in any isolate, whereas *smr* was less frequently detected than reported (Bjorland et al., 2005). Low prevalence of QAC-resistant genes provides more choices of disinfectants to control mastitis staphylococci in Chinese herds.

Biofilm-forming ability of staphylococci has increasingly been accepted as another contributor to antimicrobial resistance. Of 3 genes related to biofilm formation, *icaD* was most common in both *S. aureus* and NAS, whereas *bap* was detected in few *S. aureus*. This distribution of biofilm genes was similar to other Chinese studies on *S. aureus* isolated from subclinical mastitis (He et al., 2014) and in *S. aureus* from other countries (Aslantas and Demir, 2016; Felipe et al., 2017). Despite being the most prevalent gene, *icaD* was detected less frequently in NAS (62%) than from *S. aureus* (92%) isolates. Because overexpression of *icaD* improved internalization of *S. aureus* to mammary epithelial cells (Pereyra et al., 2016), higher carriage of *icaD* could make *S. aureus* more pathogenic in the udder than NAS.

In the present study, a high degree of relatedness of isolates was present within herds (similar RAPD types within herds for *S. aureus* and *S. chromogenes*), as would be expected for contagious mastitis pathogens. Although *P*-values were obtained in herd-conditional models, our point estimates can be regarded as vulnerable to clustering of isolates obtained from the same herds (or provinces) and should, therefore, be interpreted with caution.

CONCLUSIONS

Among 112 NAS isolated from cows with CM, *S. chromogenes* was the most common species, followed by *S. sciuri* and *S. epidermidis*. Two predominant RAPD types of *S. aureus* (comprising 56% of isolates) as well as 2 prevalent types of *S. chromogenes* (containing 73% of isolates) were detected. The *blaZ* was the most prevalent ARG in *S. aureus* isolates, followed by *tetM*, *tetK*, *ermT*, and *aacA-aphD*. The *blaZ*, *mecA*, *tetK*, *tetM*, *mphC*, and *msrA* were confirmed as the most frequent ARG in NAS. Overall, *mecA*, *tetK*, *tetL*, *tetM*, *dfrG*, *ermB*, *msrA*, *mphC*, and *aphA3* were more frequently

detected in NAS than in *S. aureus*. The *icaA* and *icaD* (biofilm formation genes) were frequently detected in *S. aureus* and also, but to a lesser degree, in NAS isolates. Prevalent RAPD types of *S. aureus* and *S. chromogenes* isolates demonstrated that specific strains are more udder-adapted and probably disseminate within herds. High carriage rates of multi-ARG in NAS suggest potential resistance to those antimicrobials.

ACKNOWLEDGMENTS

This research was financially supported by the National Natural Science Foundation of China (No. 31772813, No. 31572587), High-end Foreign Experts Recruitment Program (No. GDT20171100013), National Key R&D Project (No. 2016YFD0501203), National Undergraduate Research and Innovation Project (No. 201710019107), and Young Teacher Innovation Project of China Agricultural University (Beijing; 2018QC026). The authors thank John Kastelic (University of Calgary, Calgary, AB, Canada) for editing the manuscript.

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