

Coexistence of Heavy Metal and Antibiotic Resistance within a Novel Composite Staphylococcal Cassette Chromosome in a *Staphylococcus haemolyticus* Isolate from Bovine Mastitis Milk

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The structure of a composite staphylococcal cassette chromosome (SCC) carried by a methicillin-resistant *Staphylococcus haemolyticus* (NW19A) isolated from a bovine milk sample was analyzed. The formation of the circular forms of both single SCC elements and composite SCC elements was detected in NW19A. Twenty heavy metal and antibiotic resistance-related genes coexisted in this composite SCC, suggesting that these genes might be coselected under environmental pressure. The *mec* gene complex in NW19A, designated type C3, is different from classic C1 or C2 gene complexes structurally and likely evolves differently. Furthermore, results from alignment of the SCC composite island of NW19A with 50 related sequences from different staphylococcal strains provided additional evidence to support the notion that coagulase-negative staphylococci (CoNS) are the original host of heavy metal resistance genes among staphylococci. Given that a SCC composite island could transfer freely among different staphylococcal species from different hosts, more attention should be paid to contamination with heavy metals and antibiotics in dairy farming environments, including wastewater, soil, feces, and feed.

Coreistance, one type of coselection mechanism, occurs when genes specifying resistant phenotypes are located together on the same mobile genetic element (MGE), such as plasmids, transposons, and staphylococcal cassette chromosome (SCC) elements (1). SCC elements are widely distributed in different staphylococci and highly diverse in their structural organization and genetic contents. SCC*mec* is a type of SCC element. It carries the *mec* gene complex and has broad-spectrum beta-lactam resistance encoded by the *mecA* (or *mecC*) gene. Methicillin-resistant *Staphylococcus aureus* (MRSA) strains are a leading cause of health care-associated infections and community-associated infections worldwide and can be associated with mastitis in dairy cattle (2, 3). MRSA is generated when methicillin-sensitive *Staphylococcus aureus* (MSSA) exogenously acquires an SCC*mec* element, but with an unclear mechanism (4). There is increasing, but largely indirect, evidence suggesting that coagulase-negative staphylococci (CoNS) might act as a source of SCC*mec* for MRSA. However, very little is known about genetics of SCC*mec* in CoNS (5).

In China, the average usage of veterinary antibiotics has reached approximately 6,000 tons annually (6), and numerous antibiotic-resistant bacteria have appeared (7). Abundant antibiotics are also used in dairy cattle in China because of the high incidence of mastitis and endometritis (8) and may increase the prevalence of antibiotic-resistant bacteria. In order to investigate antibiotic-resistant staphylococci from mastitis milk, in this study, a methicillin-resistant *Staphylococcus haemolyticus* (MRSH) strain from a milk sample from a Holstein cow was studied for its SCC element in terms of structural organization and genetic contents. Besides a *mecA* gene, a tetracycline resistance gene and many heavy metal resistance genes were located together in the SCCs in this strain.

MATERIALS AND METHODS

Bacterial strain and genome sequencing. Strain NW19A was isolated from a mastitis milk sample from a Holstein cow. It was identified as *S.*

haemolyticus based on 16S rRNA sequencing. Genomic DNA was extracted and the whole-genome sequencing (WGS) was performed by BGI-Shenzhen (Shenzhen, China) using the Solexa paired-end sequencing technology. A genomic DNA library was prepared with an insert size of 500 bp. WGS sequence data were assembled into contigs by using SOAPdenovo (version 1.05). Furthermore, the contigs were joined into scaffolds using paired-end information. The order of the scaffolds was determined by aligning to the genome of *S. haemolyticus* strain JCSC1435 (GenBank accession no. AP006716) using SOAPaligner (version 2.21).

Assemblage and identification of SCC elements in NW19A. To map SCC elements, every scaffold of NW19A was subjected to a BLAST search against marker genes, including *orfX*, the *mec* gene complex, and the *ccr* gene complex. Positive scaffolds were aligned against SCC*mec* V(5C2&5)c of *S. aureus* P126 (GenBank accession no. KF593809) to confirm their genetic organization. Gaps were closed by PCR followed by sequencing. Gene prediction in NW19A was performed using Glimmer 3.0. The nucleotide sequences of predicted genes and their deduced amino acid sequences were subsequently compared mainly against the nucleotide collection (nr/nt) database and the nonredundant protein database provided by NCBI, separately. A second set of PCRs was also performed for investigating possible SCC*mec* excision, with extracted genomic DNA from

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TABLE 1 Results of susceptibility testing against heavy-metal compounds and antibiotics of NW19A

Strain (species)	MIC ^a (mM) of metal				MIC ^b (μg/ml) of antimicrobial agent									
	CuSO ₄	Cd(NO ₃) ₂	ZnSO ₄	NaAsO ₂	GEN	PEN	OXA	FOX	TET	LIN	CIP	TEC	TMP	SMZ
NW19A (<i>S. haemolyticus</i>)	8	0.4	4	8	>256	16	>256	32	>256	>256	32	8	0.5	>256
RN4220 (<i>S. aureus</i>)	4	0.015	4	0.015	1	1	0.5	2	1	1	2	2	1	>256
BB02312 (<i>S. haemolyticus</i>)	2	0.25	1	0.25	2	16	1	4	2	128	4	4	2	>256

^a All MIC determinations were performed in agar dilution assays, and a tentative breakpoint for heavy metal resistance was defined as a MIC of >2 mM.

^b GEN, gentamicin; PEN, penicillin G; OXA, oxacillin; FOX, cefoxitin; TET, tetracycline; LIN, lincomycin; CIP, ciprofloxacin; TEC, teicoplanin; TMP, trimethoprim; SMZ, sulfamethoxazole.

stationary-phase cultures as templates. Primer sequences are listed in Table S1 in the supplemental material.

MIC determinations. MICs for the antimicrobial agents listed in Table 1 were determined for strain NW19A using an agar dilution method recommended by the CLSI (9). MICs of heavy metal ions were measured as previously described (10). Bacterial suspensions of McFarland 0.5 standard were prepared for the tests. Mueller-Hinton agar plates with different final concentrations of heavy metal ions were prepared [CuSO₄ · 5H₂O for copper, Cd(NO₃)₂ · 4H₂O for cadmium, NaAsO₂ for arsenic, and ZnSO₄ · 7H₂O for zinc]. The strains *S. aureus* RN4220 and *S. haemolyticus* BB02312 served as controls for MIC determination of both antibiotics and heavy metal ions.

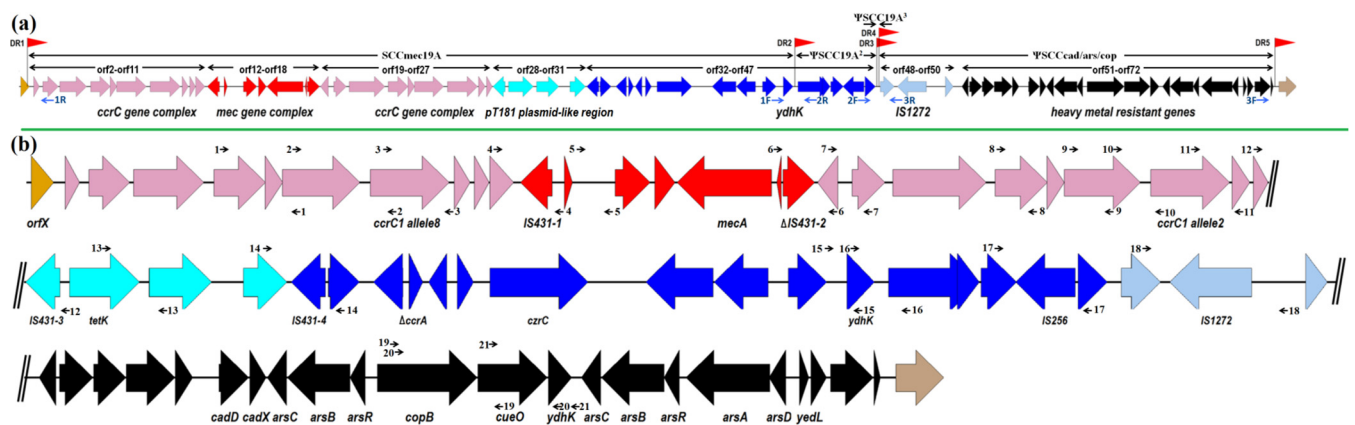
Bioinformatics analyses. In order to determine the role of NW19A in the emergence and evolution of MRSA, the NCBI nucleotide BLAST software was used for genome alignments in the nr/nt database with default parameters (accessed on 11 September 2014). The query sequence was the identified nucleotide sequence of CI_{19A} (the SCC composite island of NW19A) with a length of 69.7 kb. The top 50 matched sequences with both a maximum score greater than 10,000 and a total score greater than 40,000 were chosen for detailed analyses. The comparison of different SCC elements was performed using BLASTN with the software Mauve (11), ACT (12), and Easyfig (13). The direct repeat unit (DRU) was analyzed as suggested elsewhere (14).

RESULTS

General features of *S. haemolyticus* NW19A and the SCC composite island in it. Strain NW19A was resistant to a range of antimicrobial drugs (Table 1). The MICs of copper, cadmium, arsenic, and zinc ions were 8 mM, 0.4 mM, 8 mM, and 4 mM, respectively. The WGS sequence data of 280 Mb from NW19A,

giving approximately 100-fold genome coverage, were generated and assembled into 161 contigs. Subsequently, the contigs were joined into 77 scaffolds, with coverage of 89.99% against the human-origin strain *S. haemolyticus* JCSC1435. Among the 77 assembled scaffolds, 14 were identified as components of SCC elements. Twenty-one PCRs were performed to close the gaps between/in these scaffolds. Finally, the SCC composite island of NW19A was determined and designated CI_{19A}, with a length of 69.7 kb (GenBank accession no. [KM369884](#)). It consisted of one intact SCC element designated SCCmec19A (42.9 kb) and three pseudo-SCC elements designated ΨSCC19A²-ΨSCC19A³-ΨSCCcad/ars/cop, from left to right (Fig. 1a). Among the 71 predicted open reading frames (ORFs) in CI_{19A}, 19 ORFs were resistance-associated genes (2 for antibiotics and 17 for heavy metals) and 12 ORFs were related to DNA transfer (6 insertion sequences and 6 recombination-related genes) (see Table S2 in the supplemental material). CI_{19A} contained 4 integration site sequences (ISS) in addition to the one in *orfX*. Only one SCC retained the cassette chromosome recombinase (*ccr*) gene essential for SCC movement. Other SCCs were thus considered remnants of SCCs and designated ΨSCCs.

PCR were performed to detect putative extrachromosomal circular intermediates of each element situated between direct repeats (DRs). Strongly positive PCR amplicons were detected not only for the primer sets 1R+1F, 2R+2F, and 3R+3F but also for the primers sets 1R+2F, 1R+3F, and 2R+3F (data not shown). These results confirmed the formation of the circular forms in NW19A of both



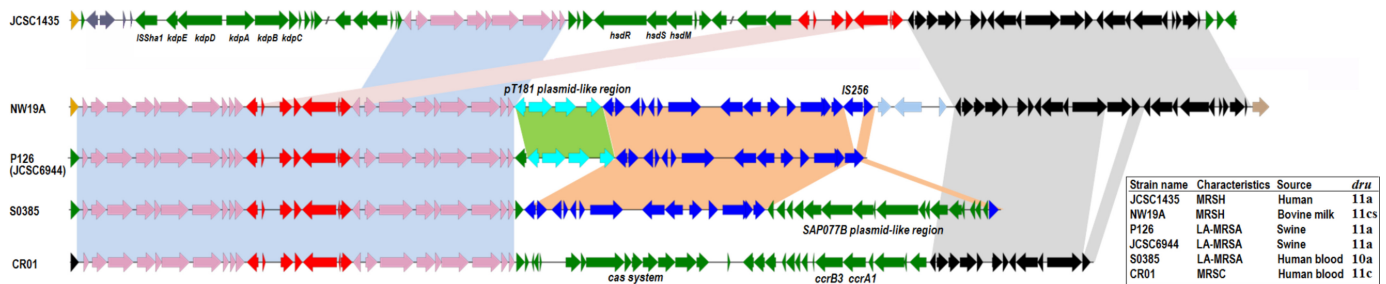


FIG 2 Comparative structure analyses of CI_{19A} against sequences of *S. haemolyticus* JCSC1435, *S. aureus* P126, *S. aureus* JCSC6944, *S. aureus* S0385, and *Staphylococcus capitis* CR01. Homologous gene clusters in different strains are shaded in different colors.

single SCC elements (SCCmec19A, $\Psi SCC19A^2$, and $\Psi SCCcad/ars/cop$) and composite SCC elements (CI_{19A} , SCCmec19A- $\Psi SCC19A^2$, and $\Psi SCC19A^2$ - $\Psi SCC19A^3$ - $\Psi SCCcad/ars/cop$).

SCCmec19A was identified immediately downstream of *orfX* and bracketed by two DRs (Fig. 1a). SCCmec19A showed 99.1% DNA sequence identity to SCCmec type V (5C2&5)c of *S. aureus* P126 (GenBank accession no. KF593809) and was classified as the same type. It was a composite of a *ccrC1* allele 8 gene complex (*orf5* to *orf11*), a type V SCCmec carrying a class C2-like *mec* gene complex (*orf12* to *orf18*), and a *ccrC1* allele 2 gene complex (*orf22* to *orf27*). The *mec* gene complex was composed of *IS431-1*, $\Delta mvaS$, *ugpQ*, *maoC*, *mecA*, $\Delta mecR1$, and $\Delta IS431-2$ genes in the order. Unlike the class C2 *mec* gene complex, the *IS431-2* was truncated in NW19A. A pT181-like fragment was bracketed by two insertion sequences (*IS431-3* and *IS431-4*) and contained a gene encoding a tetracycline resistance protein (*tetK*) (Fig. 1b). Furthermore, a region of 10 ORFs (*orf33* to *orf42*) located downstream of the pT181-like fragment. The first three genes were related to chromosome recombination (see Table S2 in the supplemental material). The last seven genes might be associated with heavy metal resistance (*orf36* to *orf38*), putative metallo β -lactamase resistance (*orf39*), and anion transposition (*orf40* to *orf42*) (see Table S2 in the supplemental material).

The $\Psi SCC19A^2$ was 4.5 kb long and harbored an *IS256* (*orf46*), which divided a gene into two nonfunctional fragments (*orf45* and *orf47*) (Fig. 1b and 2). $\Psi SCC19A^3$ was 0.1 kb long and did not contain any ORF. $\Psi SCCcad/ars/cop$ was 22.2 kb long, with 25 ORFs (*orf48* to *orf72*) harboring resistance genes for cadmium, arsenic, and copper (Fig. 1b; also, see Table S2 in the supplemental material). Two *ars* operons were found in $\Psi SCCcad/ars/cop$ as *arsC-arsB-arsR* and *arsC-arsB-arsR-arsA-arsD*, separately.

Comparative analyses of SCCs in NW19A with those in related bacteria. Fifty sequences from different staphylococcal strains with the highest similarities with CI_{19A} were chosen for detailed analyses (see Table S3 in the supplemental material). It is noteworthy that 74% of the sequences investigated (37 out of 50) contained defensive systems such as a type I or II restriction-modification system or a CRISPR-associated system (CAS) (see Table S3 in the supplemental material). However, none of the defensive system existed in CI_{19A} (see Table S3 in the supplemental material). Among the 50 strains investigated, the five showing highest scores with CI_{19A} were P126, JCSC6944, S0385, CR01, and JCSC1435.

CI_{19A} shared the region of *orf2* to *orf27* with P126, JCSC6944, S0385, and CR01. It also shared the regions of *orf12* to *orf18* and *orf19* to *orf27* with strain JCSC1435, but with different arrange-

ment of positions (Fig. 2). Interestingly, the *IS431-2* gene was intact in JCSC1435 instead of a truncated version in P126, JCSC6944, S0385, and CR01 as well as NW19A. In addition, strains JCSC1435, JCSC6944, and P126 shared DRU type 11a, suggesting that the acquisition of *mec* gene complexes among them was evolutionarily related (Fig. 2).

The region from *orf32* to *orf47* in CI_{19A} showed high sequence identities with P126, JCSC6944, and S0385. CI_{19A} , P126, and JCSC6944 contained an integrated pT181-like plasmid (*orf28* to *orf31*), which was absent in the genome of S0385 (Fig. 2). Interestingly, a separate plasmid, pS0385-1, that was nearly identical to *orf28* to *orf31* was found in S0385 (GenBank accession no. AM990993). Furthermore, similar interruption of *IS256* in CI_{19A} was observed in the genome of S0385, where 17 genes were inserted (Fig. 2). These 17 genes showed 98% sequence identity with the ORFs in *S. aureus* plasmid SAP077B (GenBank accession no. GQ900429). These results suggest the existence of many insertion events in CI_{19A} mediated by plasmids and close evolutionary relationships in the region of *orf28* to *orf47* among these four strains.

The *orf28* to *orf47* region was absent in CR01 and JCSC1435. Most genes in this region were related to antibiotic resistance (*orf29* and *orf39*), heavy metal resistance (*orf36* to *orf38*), or chromosome transfer (*orf28*, *orf30*, *orf32*, *orf33*, *orf34*, *orf35*, and *orf46*). The corresponding region in CR01 and JCSC1435 was composed mainly of the CAS system and type I restriction-modification system, respectively.

The region encompassing *orf51* to *orf72* in CI_{19A} was rich in heavy metal resistance genes, conferring resistance to cadmium, arsenic, and copper. JCSC1435 shared this region with CI_{19A} , but its *copB* gene (*orf61*) was truncated; CR01 contains a part of this region. P126 and S0385 did not contain $\Psi SCC19A^3$ - $\Psi SCCcad/ars/cop$ as in NW19A, suggesting that at least two more recombination events occurred in NW19A in comparison with them (Fig. 2).

DISCUSSION

Compared to a single SCCmec, composite SCC elements contain many more resistance genes (15, 16). A novel composite SCC from *S. haemolyticus* NW19A has been revealed in this study. It is characterized not only by containing a large proportion of heavy-metal resistance genes and antibiotic resistance genes but also by including a surprising number of ISs and recombinases. Several circular intermediates of SCC elements were detected in this study, implying that CI_{19A} could be excised as a whole or in part. It has been reported that soil, water, crops, animal feed, and feces on dairy farms in China were contaminated with heavy metals (17). Hence, the coexistence of both types of determinants in the same

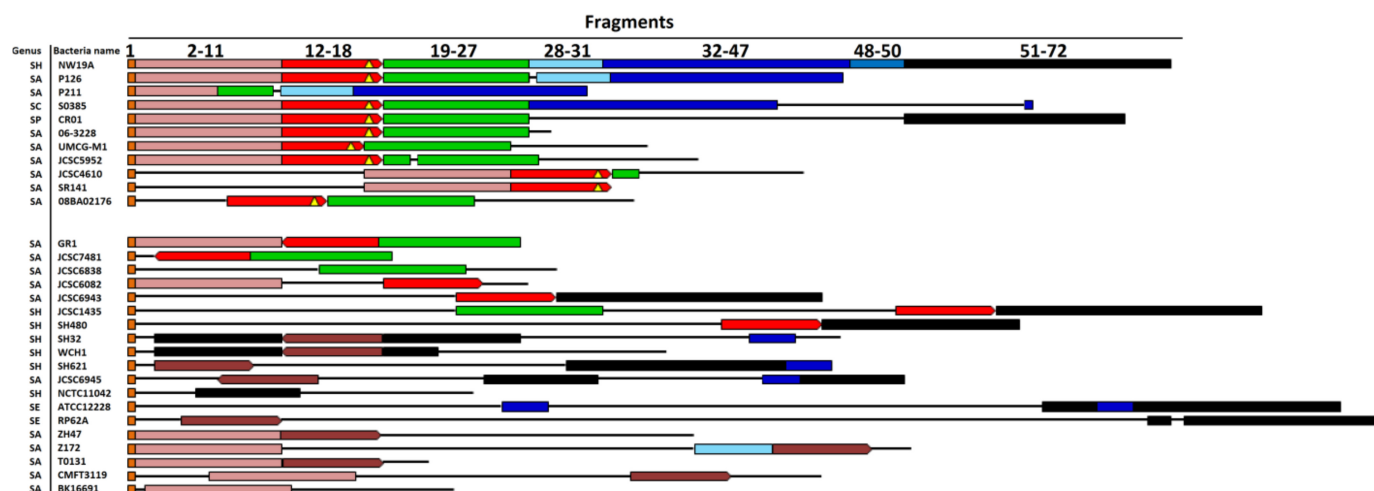


FIG 3 Comparative analysis of CI_{19A} with the 50 bacteria investigated. Various fragments are differentiated by different colors. The red and brown boxes with arrows represent *mec* gene complexes and their directions. The red boxes with and without a yellow triangle represent type C3 and C2 *mec* gene complexes, respectively. The brown boxes represent other *mec* complex types. The thin black line indicates sequences with no identities among different SCC elements. SH, SA, SC, SP, and SE represent *S. haemolyticus*, *S. aureus*, *S. capitis*, *Staphylococcus pseudintermedius*, and *Staphylococcus epidermidis*, respectively. More detailed comparisons and descriptions of strains are given in Table S4 in the supplemental material.

genetic element of bacteria may allow antibiotic resistance to be selected upon heavy metal selective pressure in the contaminated environment.

The gene *czrC* (orf38) is related to zinc and cadmium resistance (18); both CopB (orf61) and CueO (orf62) can transport copper from the cytoplasm to the extracellular area, decreasing the damage of bacteria by copper (19); CadD (orf56) is a putative cadmium transporter, which is regulated by CadX (orf57) (19). These proteins conferred zinc, copper, and cadmium resistance to NW19A, which was determined by heavy metal resistance assay. Two groups of arsenic operons are present in NW19A, giving the strain strong resistance to arsenic (MIC = 8 mM), since the proteins ArsA (orf67) and ArsD (orf68) could greatly enhance the arsenic efflux capacity of the ArsB pump (20).

In this study, bioinformatics analyses were performed in order to reveal potential sequences for formation of CI_{19A} . As shown in Fig. 3 and in Table S4 in the supplemental material, CI_{19A} can be divided into 3 constitutive regions (orf2 to orf27, orf28 to orf50, and orf51 to orf72). The first region is comprised of three independent units: the *ccrC1* allele 8 gene complex, the *mec* gene complex, and the *ccrC1* allele 2 gene complex. The second region might be formed by three recombination events: entry of orf28 to orf31 by the pT181-like plasmid, entry of orf32 to orf47 mediated by CcrAB recombinases, and entry of orf48 to orf50 probably by IS1272. The third region possesses many heavy metal resistance genes. Eleven investigated strains as well as NW19A contain all or parts of this region. It is noteworthy that 10 out of the 12 strains are CoNS (Fig. 3; also, see Table S4 in the supplemental material), suggesting that this heavy metal multiresistant region may originate from CoNS. In summary, we hypothesize that the formation of CI_{19A} might be conducted with the help of *S. aureus* P126, JCSC6944, or S0385 for the first two constitutive regions and the help of *S. haemolyticus* JCSC1435 for the third constitutive region.

There were several types of *mec* gene complexes in the strains investigated. A large proportion of strains contain a truncated IS431-2 (Fig. 3). IS431-2 has been proposed to be involved in the

formation of the *mec* gene complex (21). Therefore, a truncated IS431-2 could eliminate the mobility of the *mec* gene complex. Furthermore, the IS-mediated *mecA* deletion occurs at much higher rates than the *ccr*-mediated complete excision of SCC*mec* (4, 22), so a truncated IS431-2 probably could decrease the frequency of losing the *mec* gene complex. We confirmed this hypothesis by the finding that the Δ IS431-contained class C2 *mec* gene complexes were always interlocked with surrounding sequences (Fig. 3), suggesting the presence of a new evolutionary tendency of the *mec* gene complex. There are two distinct class C *mec* gene complexes (C1 and C2) differentiated by the orientation of their IS431-2. They have likely evolved independently (23). Based on this, we suggest classifying this *mec* gene complex as a class C3 complex.

It seems that the chromosome of NW19A is very plastic and might have encountered various types of environmental pressure. The CAS and restriction-modification system enable bacteria to degenerate exogenous DNA and protect the stability of chromosome, so the lack of these systems in NW19A may facilitate acquisition of exogenous DNA and consequently speed up the evolutionary process of this bacterium. The emergence of livestock-associated MRSA infection constitutes an additional obstacle to control MRSA in humans (24, 25) and to treat mastitis in dairy cattle. A study has provided evidence for a horizontal transfer of a type V SCC*mec* from MRSH to a MSSA to create a new clone of MRSA (26). Considering the fact that abundant heavy metal-resistant genes and antibiotic resistance genes coexist in a mobile SCC element and could be coselected, we suggest reducing the use of antibiotics and heavy metals and improving animal management systems to limit the selection of resistant bacteria and the emergence of MRSA in dairy cattle.

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We have no conflicts of interests to declare.

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