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Identification of staphylococcal cassette chromosome *mec* in *Staphylococcus aureus* and non-*aureus* staphylococci from dairy cattle in Belgium: Comparison of multiplex PCR and whole genome sequencing

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ABSTRACT

The present study compared multiplex PCR (mPCR) and Whole Genome Sequencing (WGS) using the SCC*mec*-Finder database to identify the Staphylococcal Cassette Chromosome (SCC) *mec* in five *Staphylococcus aureus* (SA) and nine non-*aureus* staphylococci (NAS) isolated from dairy cattle. mPCR identified an SCC*mec*IV in four SA and one NAS, but could not differentiate between SCC*mec*II and IV in the fifth SA, that all harbored the *mecA* gene and were phenotypically resistant to cefoxitin. SCC*mec*Finder confirmed the presence of an SCC*mec*IVc(2B) in four SA and of the SCC*mec*IVa(2B) in the fifth SA and the one NAS. Both methods also detected one untypeable SCC*mec* in another cefoxitin-resistant NAS harboring the *mecA* gene and a pseudo SCC*mec* in one cefoxitin-sensitive NAS harboring one *mecC*-related gene. No SCC*mec* elements were identified either in one cefoxitin-sensitive NAS harboring the *mecA2* gene, or in five NAS (one resistant and four sensitive to cefoxitin) harboring the *mecA1* gene. SCC*mec*Finder could even not identify the presence of any *mecA1* gene in these five NAS, whose presence was nevertheless confirmed by ResFinder. The conclusions of this study are: (i) mPCR and WGS sequencing using SCC*mec*Finder are complementary methodologies to identify SCC*mec*; (ii) SCC*mec*Finder and ResFinder to a lesser extent cannot identify all *mec* gene allotypes; (iii) a specific classification of the SCC*mec* in NAS would be epidemiologically helpful; (iv) presence of a *mecA* gene and a complete SCC*mec* is linked to cefoxitin resistance, whereas presence of other *mec* genes and of pseudo or no SCC*mec* is not.

1. Introduction

Methicillin is a semi-synthetic penicillin active against BlaZ β-lactamase-producing *Staphylococcus aureus* (SA) that was marketed in 1959. Nevertheless, the first "Methicillin Resistant *S. aureus*" (MRSA) was reported only two years later, in 1961. For many years, MRSA were isolated from hospitalized human patients with nosocomial infections (hospital-acquired MRSA or HA-MRSA) while community-acquired MRSA (CA-MRSA) emerged in the years 1990s in humans with no history of hospitalization. On their side, animal MRSA emerged in the years 2000s in livestock and in companion animals and are generically named livestock-associated MRSA (LA-MRSA) (Lakhundi and Zhang, 2018).

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The first LA-MRSA was reported in 1972 in Belgium from a cow with mastitis (Devriese et al., 1972). Since then, MRSA have been regularly reported from cows with mastitis worldwide including in Belgium (Schnitt and Tenhagen, 2020).

The mechanism of resistance of the MRSA is the production of a β-lactam-resistant transpeptidase, or Penicillin-Binding Protein 2a (PBP2a), one enzyme involved in the bacterial cell wall formation, encoded by a de novo acquired and chromosome-located *mec* (methicillin resistance) gene. At least three different *mec* genes have been identified so far in MRSA: *mecA* with three allotypes (*mecA*, *mecA1* and

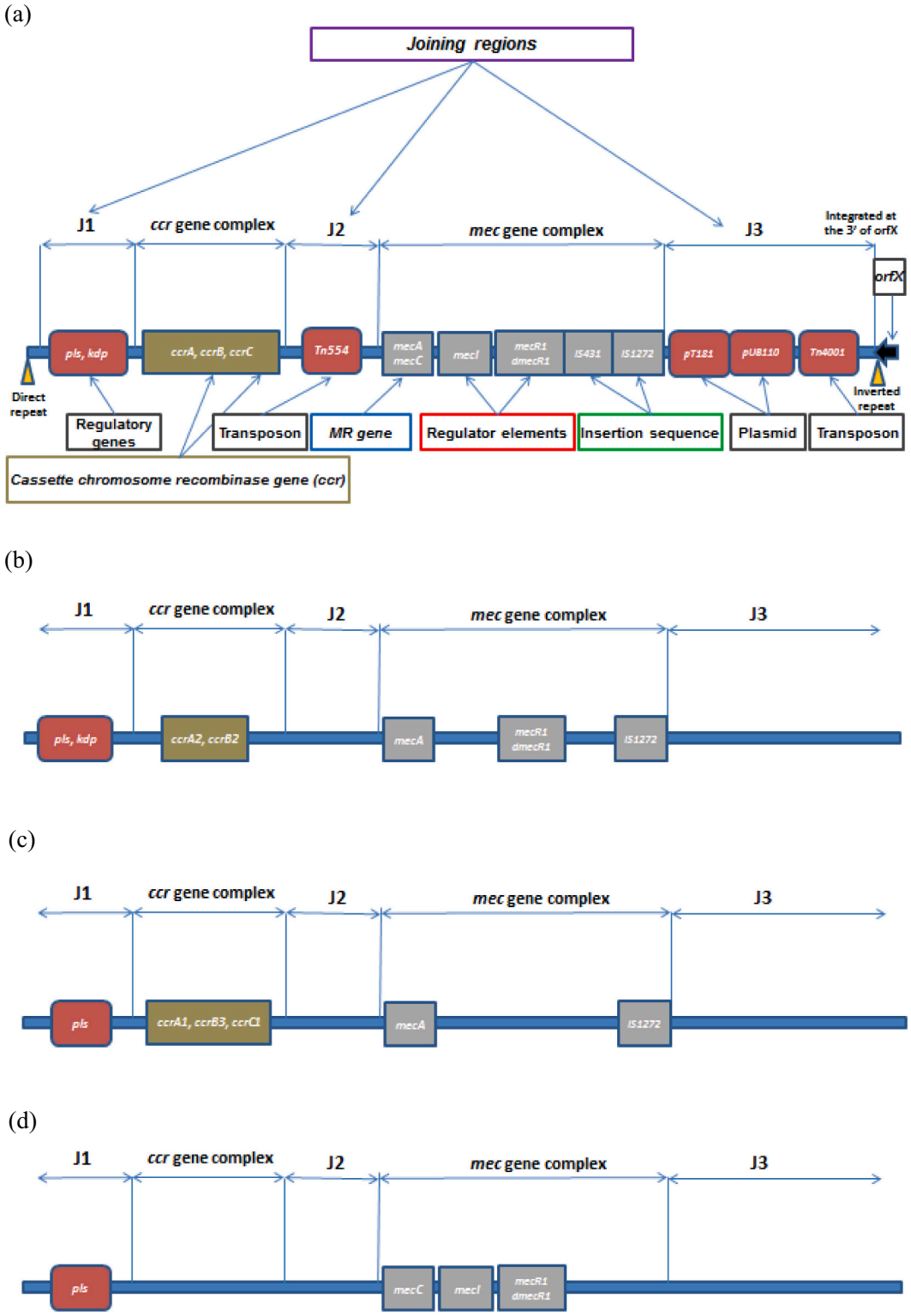

Fig. 1. Structure of SCC*mec* elements. (a) General SCC*mec* structure. (b) SCC*mec*IVc(2B) of Lg010 SA. (c) Untypeable SCC*mec* of Lg048 *S. haemolyticus*. (d) Pseudo SCC*mec* of Lg063 *S. xylosus*.

mecA2), *mecB* and *mecC* with four allotypes (*mecC*, *mecC1*, *mecC2* and *mecC3*). The *mecA* and *mecC* genes can be located on a "Staphylococcal Cassette Chromosome" (SCC*mec*) while the *mecB* gene was reported on a plasmid. The general structure of SCC*mec* (Fig. 1a) includes a *mec* gene complex, a cassette chromosome recombinase *(ccr*) gene complex, three junction (J) regions (J1, J2, J3) and flanking direct/inverted-repeat sequences (Becker et al., 2018; Lakhundi and Zhang, 2018; Liu et al., 2016; Loncaric et al., 2019; MacFadyen et al., 2019).

The *mec* gene complex comprises one *mecA* or *mecC* gene, the regulatory *mecR1* (inductor) and *mecI* (repressor) genes and the associated insertion sequences (IS). The *ccr* gene complex which includes three *ccr* genes (*ccrA, ccrB* and *ccrC* with different variants coding for the cassette chromosome recombinase) and surrounding open reading frames (ORF) is responsible for the integration and the excision of the SCC*mec* in and from the chromosome. The SCC*mec* also includes three junction (J) regions. The J1 region is located upstream of the *ccr* gene complex (L-C region) and can include several ORFs and regulatory genes (*pls* and *kdp*). The J2 region is located between the *ccr* gene complex and the *mec* gene complex (C-M region) and can include transposon Tn*554* encoding resistance to erythromycin. The J3 region is located downstream of the *mec* gene complex (M-R region) and can include different inserted genetic elements such as plasmid pT181, plasmid pUB110 and/or transposon Tn*4001* (Lakhundi and Zhang, 2018; Liu et al., 2016).

To date, 14 SCC*mec* types and several SCC*mec* subtypes have been officially recognized in MRSA based on the combinations of the *ccr* and *mec* gene complexes and on the different J regions, respectively (Lakhundi and Zhang, 2018). In addition, pseudo, composite, and hybrid SSC*mec* have also been described: pseudo SCC*mec* lack the *ccr* gene complex; composite SSC*mec* are composed of distinct genetic elements including a pseudo SCC*mec* and other SCC carrying, among others the *ccr* gene complex; hybrid SCC*mec* carry genes coding for resistance to other antibiotics or to antiseptics and/or for virulence-associated factors (Lakhundi and Zhang, 2018; Shore and Coleman, 2013).

mec genes can also be present in non-*aureus* staphylococci (NAS), including several isolates from cows with mastitis, though not all genetically positive NAS actually express resistance to methicillin. Most of the SCC*mec* described in NAS belong to one of the 14 SCC*mec* types described in MRSA, but several do not. In some NAS, these *mec* genes are even not present on any SCC*mec* (Frey et al., 2013; Garza-González et al., 2010; Hanssen and Sollid, 2007; Harrison et al., 2013; Martins and de Lourdes Cunha, 2007; McClure et al., 2021; Schnitt and Tenhagen, 2020; Shore and Coleman, 2013; Urushibara et al., 2011; Vanderhaeghen et al., 2010; Xue et al., 2015; Yu et al., 2014; Zong and Lü, 2010).

Although different phenotypic and genetic tools exist, the identification of MRSA and MRNAS is complex due to differences in expression of the *mec* genes and to the numbers of *mec* genes and SCC*mec* (sub)types (Lakhundi and Zhang, 2018; Schnitt and Tenhagen, 2020). The aim of this work was therefore to compare multiplex PCR (mPCR) and Whole Genome Sequencing (WGS) to identify the SCC*mec* present in *mec* gene-positive, methicillin-resistant or sensitive SA and NAS, isolated from bovine milk samples in Belgium.

2. Materials and methods

2.1. Isolates

A total of five SA and nine NAS (one *S. epidermidis*, one *S. haemolyticus*, five *S. sciuri*, one *S. vitulinus*, and one *S. xylosus*) were studied (Table 1). They had been previously identified phenotypically and genetically within a collection of 172 staphylococci isolated in 13 Belgian dairy farms from cows with clinical or sub-clinical mastitis (Fergestad et al., 2021a). The clinical and bacteriological diagnostic procedures and methodology have already been described (Fergestad et al., 2021a). Since the species *S. sciuri* and *S. vitulinus* were recently reassigned to the genus *Mammaliicoccus* (Madhaiyan et al., 2020), the new nomenclature is followed in this manuscript: *M. sciuri* (*S. sciuri*) and *M. vitulinus* (*S. vitulinus*).

The five SA and two NAS, *S. epidermidis*, *S. haemolyticus*, previously tested positive with the PCR for the *mecA* gene and were phenotypically resistant to cefoxitin (Table 1) while two other NAS, *M. vitulinus* and

Table 1
Resistance/susceptibility to cefoxitin, *mec* gene and SCC*mec* PCR, ResFinder and SCC*mec*Finder analysis of the WGS of 14 staphylococci isolated from dairy cattle with clinical or sub-clinical mastitis (Fergestad et al., 2021a, 2021b; this study).

Isolate numbers [a]	*Staphylococcus* species	Farm	Inhibition zone (R/S) [b]	*mec* gene (PCR)	*mec* gene (ResFinder) / % homology [c]	*mec* gene (SCC*mec*Finder) / % homology [d]	SCC*mec* (mPCR)	SCC*mec* (SCC*mec*Finder)	Biosample numbers
Lg_010	*S. aureus*	A	12 mm (R)	*mecA*	*mecA* / 100%	*mecA* / 100%	IV	IVc(2B)	SAMN11967865
Lg_017	*S. aureus*	A [e]	14 mm (R)	*mecA*	*mecA* / 100%	*mecA* / 100%	IV	IVc(2B)	SAMN11964773
Lg_020	*S. aureus*	A	12 mm (R)	*mecA*	*mecA* / 100%	*mecA* / 100%	IV	IVc(2B)	SAMN11964774
Lg_027	*S. aureus*	A	10 mm (R)	*mecA*	*mecA* / 100%	*mecA* / 100%	IV	IVc(2B)	SAMN11964775
Gt_111	*S. aureus*	B	6 mm (R)	*mecA*	*mecA* / 100%	*mecA* / 100%	II/IV	IVa(2B)	SAMN11964772
Lg_141	*S. epidermidis*	C	18 mm (R)	*mecA*	*mecA* / 100%	*mecA* / 100%	IV	IVa(2B)	SAMN11968195
Lg_048	*S. haemolyticus*	A	19 mm (R)	*mecA*	*mecA* / 100%	*mecA* / 100%	UT	UT	SAMN11968189
Lg_016	*M. sciuri*	A [e]	38 mm (S)	*mecA*/ Neg	*mecA1* / 99.9%	None	None	None	SAMN19114563
Lg_031	*M. sciuri*	A	23 mm (S)	*mecA*/ Neg	*mecA1* / 99.9%	None	None	None	SAMN19114570
Lg_086	*M. sciuri*	D	28 mm (S)	*mecA*/ Neg	*mecA1* / 99.9%	None	None	None	SAMN19114584
Gt_064	*M. sciuri*	E	32 mm (S)	*mecA*/ Neg	*mecA1* / 99.65%	None	None	None	SAMN19114548
Gt_123	*M. sciuri*	F	18 mm (R)	*mecA*/ Neg	*mecA1* / 99.9%	None	None	None	SAMN19114559
Lg_101	*M. vitulinus*	D	29 mm (S)	*mecA*	*mecA2* / 99.8%	*mecA* / 91%	None	None	SAMN11968159
Lg_063	*S. xylosus*	D	28 mm (S)	*mecC*	*mecC2* / 94.54% [f]	*mecC* / 93.63% [f]	Pseudo	Pseudo	SAMN19114581

[a] Lg: isolated at University of Liège (ULiège); Gt: isolated at Ghent University (UGent).
[b] to 30 microg cefoxitin disks (Neosensitabs, Rosco Diagnostica): *S. aureus*: R $\leq$ 21 mm and S $\geq$ 22 mm; NAS: R $\leq$ 24 mm and S $\geq$ 25 mm (Clinical and Laboratory Standards Institute recommendations. Performance Standards for Antimicrobial Susceptibility Testing. Zone Diameter and MIC Interpretative Standards for *Staphylococcus* spp. (M100-S23) CLSI: Annapolis Junction, MD, USA, 2013 Volume 33).
[c] Gene homology according to ResFinder with a %ID threshold of 90% and minimum length of 60%.
[d] Gene homology according to SCC*mec*Finder with a %ID threshold of 90% and minimum length of 60%.
[e] isolated from two quarters of the same cow.
[f] a BLAST search identified the *mecC* gene allotype at a 99.9% homology.

S. xylosus, were positive with the PCR for the *mecA* and *mecC* gene respectively, but phenotypically sensitive to cefoxitin. After genome sequencing, the presence of a *mecA* gene in the five SA, the *S. epidermidis* and the *S. haemolyticus* was previously confirmed using ResFinder 3.2 (Center for Genomic Epidemiology; https://cge.cbs.dtu.dk/) with a %ID threshold of 90% and minimum length of 60%, while the *M. vitulinus* harbored a *mecA2* gene and the *S. xylosus* a *mecC2* gene. In addition, the *mecA1* gene was identified in the five *M. sciuri* that were negative or inconsistently positive with the PCR for the *mecA* gene; four of them being sensitive to cefoxitin and the fifth one resistant (Table 1) (Fergestad et al., 2021a, 2021b).

Four SA, the *S. haemolyticus* and two *M. sciuri* came from the same farm (A) (Table 1), with one SA and one *M. sciuri* isolated from two quarters of the same cow. Another *M. sciuri*, the *M. vitulinus* and the *S. xylosus* also came from one same farm (D).

2.2. Multiplex PCR

The multiplex PCR (mPCR) scheme to identify the SCC*mec* types I to XI was based on five multiplex PCR (PCR1a, PCR1b, PCR 2, PCR3 and PCR4) targeting the *ccr* and *mec* gene complexes (Table 2). The primer sequences and the PCR amplification conditions (Suppl. Table) were adapted from Argudín and collaborators as previously described (Argudín et al., 2016; Ngassam Tchamba et al., 2021), using the multiplex PCR kit® (QIAGEN, The Netherlands) on DNA extracted by boiling from one colony after overnight growth on Luria-Bertani (LB) agar (VWR Chemical, Belgium). All amplified DNA fragments were analyzed by electrophoresis in 1.5% (*w/v*) agarose gel after staining with Midori green Advance DNA Stain (Nippon Genetics, Germany).

2.3. Whole genome sequencing

The Whole Genome Sequencing (WGS) procedure has already been described (Fergestad et al., 2021b). All genome sequences were compared with the SCC*mec*Finder 1.2 software and database (Center for Genomic Epidemiology; https://cge.cbs.dtu.dk/) for the identification of SCC*mec* types and subtypes with a %ID threshold of 90% and minimum length of 60%. Furthermore, a BLAST search was performed to identify the *mecC* gene allotype in the *S. xylosus* (Camacho et al., 2009; Clausen et al., 2018; Kaya et al., 2018; Oliveira and de Lencastre, 2011). All genomic data related to this project, including raw reads, are available via the NCBI BioProject PRJNA609060 (Table 1) (Fergestad et al., 2021b) (https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA609060).

3. Results

3.1. Multiplex PCR

The SCC*mec* type IV (Table 1) was identified with the mPCR in the four SA from the same farm and in the *S. epidermidis* from another farm, but the mPCR could not distinguish SCC*mec* types II and IV in the fifth SA. Conversely, the PCR amplification profile of the *S. haemolyticus* did not match any recognized SCC*mec* type. In addition, amplification of the *mec* gene complex, but not of the *ccr* gene complex was obtained for the *S. xylosus*, while only the *mecA2* gene was amplified in the *M. vitulinus* and no amplification of the *mec* and *ccr* gene complexes was obtained for the five *M. sciuri*.

3.2. WGS

Using SCC*mec*Finder database with a %ID threshold of 90% and minimum length of 60%, the WGS confirmed the presence of an SCC*mec* type IV in the five SA and in the *S. epidermidis*. The SCC*mec* subtype IVc (2B) was identified in the four SA from the same farm and subtype IVa (2B) in the fifth SA (Fig. 1b) and in the *S. epidermidis* (Table 1).

Table 2
Resistance/susceptibility to cefoxitin and SCC*mec* mPCR results of the different staphylococcal isolates (see also Suppl. Table).

Isolates [a]	Cef [b]	PCR1 (a, b)						PCR2			PCR3		PCR4			SCC*mec* types
		mecA	type 1 *ccr*	type 2 *ccr*	type 3 *ccr*	type 4 *ccr*	type 5 *ccr*	type 7 *ccr*	class A *mec*	class B *mec*	class C2 *mec*	*mecA*	class C1 *mec*	*mecC*	class E *mec*	type 8 *ccr*	
Lg_010 (*S. aureus*)	R	+		+						+		+					IV
Lg_017 (*S. aureus*)	R	+		+						+		+					IV
Lg_020 (*S. aureus*)	R	+		+						+		+					IV
Lg_027 (*S. aureus*)	R	+		+						+		+					IV
Gt_111 (*S. aureus*)	R	+		+					+	+		+					II /IV
Lg_141 (*S. epidermidis*)	R	+		+						+		+					IV
Lg_048 (*S. haemolyticus*)	R	+					+	+		+		+					UT [c]
Lg_101 (*M. vitulinus*)	S	+										+					No other amplification

[a] Lg: isolated at University of Liège (ULiège); Gt: isolated at Ghent University (UGent). No amplification (even for the *mec* genes) was obtained with the five *M. sciuri* (*mecA1*) and the *S. xylosus* (*mec C2*) (see also Table 1).
[b] Cef = cefoxitin: R = Resistant; S = sensitive (see also Table 1).
[c] UT = Untypeable.

Conversely, no SCC*mec* type could be identified in the *S. haemolyticus* though the *ccrA1, ccrB3, ccrC1* and *mecA* genes and the *IS1272* were detected (Fig. 1c). The WGS analysis using SCC*mec*Finder also confirmed the mPCR results for the *S. xylosus* with presence of only the *mec* gene complex (Fig. 1d) and for the five *M. sciuri* and the *M. vitulinus* with the absence of any SCC*mec* (Table 1).

Actually, the SCC*mec*Finder database could not detect even the presence of any *mec* gene in these five *M. sciuri* (Table 1). Moreover, one *mecA* gene with 91% homology was detected in the *M. vitulinus* and one *mecC* gene with 93.63%, homology in the *S. xylosus* (Table 1). Therefore, a new ResFinder analysis was carried out and the previous results were confirmed (Table 1): presence of a *mecA1* gene in five *M. sciuri*, of a *mecA2* gene in the *M. vitulinus* and of a *mecC2* gene in the *S. xylosus*. Nevertheless, the *mecC2* gene homology was low, 94.56% (Table 1). Therefore, a BLAST search was performed and a 99.9% homology was found with the *mecC* gene of *S. xylosus* AD10b.

4. Discussion

Detection of methicillin resistance in staphylococci is performed by phenotypic assays, such as the disk diffusion method with cefoxitin discs (EUCAST, www.eucast.org), while the presence of *mec* genes and SCC*mec* cassettes is identified by genetic assays, like (m)PCR and WGS (Argudín et al., 2016; Kaya et al., 2018; Schnitt and Tenhagen, 2020; Uehara, 2022). Although the mPCR made no erroneous identification in this study, the WGS was more precise for the identification of the SCC*mec* types and subtypes using SCC*mec*Finder (Table 1). Nevertheless, SCC*mec*Finder is more limited regarding the identification of the *mec* genes compared to ResFinder and to PCR, since it does not include all *mecA* and *mecC* allotypes.

For instance, the presence of the *mecA1* gene in the five *M. sciuri* is detected by ResFinder but not by SCC*mec*Finder. Another discrepancy is related to the identification of the *mecC* gene in the *S. xylosus*. According to SCC*mec*Finder it is one of the *mecC1*, *mecC2* or *mecC3* allotypes, while ResFinder identifies the *mecC2* gene. However, the homology to the *mecC2* gene was below the level of 95% (Table 1). Moreover, a BLAST search identifies a 99.9% homology with the *mecC* gene of the AD10b strain (Loncaric et al., 2019), which is nevertheless in contradiction to SCC*mec*Finder result. The authors have no clear explanation for such contradictory results. Actually, mPCR performs as well as WGS in identifying *mec* genes providing the primers are accurate (Hajia, 2017) considering that the *mec* gene sequence can be slightly different within each allotype (Frey et al., 2013; Lakhundi and Zhang, 2018; Loncaric et al., 2019; Miragaia, 2018; Shore and Coleman, 2013). Primer lack of perfect accuracy (not shown) was indeed the cause of the *mecA* PCR inconsistent results on the five *M. sciuri* (Table 1).

As far as identification of the *mec* genes and SCC*mec* is concerned, the five SA and the *S. epidermidis* harbor a classic *mecA* gene and a classic SCC*mec* type IV, similarly to previous results obtained in Belgium, though types III and V have also been identified in SA and NAS from dairy cattle (Bardiau et al., 2013; Vanderhaeghen et al., 2010, 2013). Apart from the four SA isolated in farm A, the results of this study, however do not support the observation of others (Bardiau et al., 2013; Frey et al., 2013; Vanderhaeghen et al., 2010, 2013) that the *mec* genes and SCC*mec* are more homogeneous between isolates from the same herd, suggesting different origins of contamination in herds A and D (Table 1).

The presence of one untypeable SCC*mec* in the *S. haemolyticus* is not surprising, since new and untypeable SCC*mec*, including pseudo, composite and hybrid SCC*mec* are regularly described in NAS including *S. haemolyticus* (McClure et al., 2021; Shore and Coleman, 2013; Xue et al., 2015; Yu et al., 2014; Zong and Lü, 2010). In that respect, a specific classification of SCC*mec* present in NAS would be epidemiologically interesting, since they are increasingly involved in infections in humans and animals, including in dairy cattle (De Visscher et al., 2017; Fergestad et al., 2021a; Garza-González et al., 2010; Hanssen and Sollid, 2007; Ito et al., 2009; Ngassam Tchamba et al., 2019; Urushibara et al., 2011; Vanderhaeghen et al., 2013), providing they follow the requirements for defining new SSC*mec* (Uehara, 2022; Yamada et al., 2017). The other NAS harbor a pseudo SCC*mec* lacking the *ccr* gene complex (*S. xylosus*) or no SCC*mec* at all (the five *M. sciuri* and *M. vitulinus*), as already described by others (Shore and Coleman, 2013). Interestingly RAST annotation of these genomes identified no other genes related to the SCC*mec* in these different NAS (not shown).

Nevertheless, the genetic assays have a limitation for practitioners and clinicians. Indeed the detection of a *mec* gene by PCR or WGS does not systematically means resistance to β-lactams especially among NAS (Becker et al., 2018; Fergestad et al., 2020; Frey et al., 2013), as illustrated by the susceptibility to cefoxitin of four out of the five *M. sciuri*, the *M. vitulinus* and the *S. xylosus* (Table 1). Moreover, the levels of resistance to cefoxitin of the staphylococci harboring the *mecA* gene and a full SCC*mec* (five SA, *S. epidermidis* and *S. haemolyticus*) are different according to the results of the disk diffusion assay (Table 1). The reasons of these differences in the resistance levels to cefoxitin are several like: (i) the presence of mutations in the *mec* gene complex (Frey et al., 2013; Harrison et al., 2013); (ii) the presence of a *blaZ* gene conferring resistance to penicillin, like in four SA and in the *S. haemolyticus* (Fergestad et al., 2021b), since their respective regulatory genes are similar (Miragaia, 2018; Shore and Coleman, 2013; Song et al., 1987); (iii) the general genetic background of each staphylococcus isolate (Miragaia, 2018); (iv) the existence of another resistance mechanism, like mutations in the PBP-encoding genes, or cell-walls with intrinsic β-lactam resistance, and others (Cai et al., 2021; Miragaia, 2018; Pantosti et al., 2007).

5. Conclusions

As a general conclusion, the use of a phenotypic or a genetic assay to detect methicillin resistance and/or the *mec* genes in staphylococci depends on the actual purpose of the study:

(i) the phenotypic assay to detect clinical resistance to cefoxitin is more appropriate for practitioners and clinicians;

(ii) the (m)PCR to detect the presence of *mec* genes and SCC*mec* cassettes are appropriate for investigation in hospitals or farms facing internal outbreaks;

(iii) the WGS allowing not only resistance gene identification, but also Multi-Locus Sequence Typing, virulotyping and clonal relationships is more appropriate for large scale epidemiological studies in relation with public health questions, although it is much dependent on the completeness of the data banks.

Author contributions

Conceptualization: C.N.T., J.M., D.T.; Formal analysis: C.N.T., M.F., D.T.; Funding acquisition: S.D.V., Y.W., T.L'A., Y.B., J.M.; Investigation: C.N.T., M.F., A.D.V.; Methodology: C.N.T., F.T., M.F., A.D.V., Y.B., M.A.A.; Project administration: J.M.; Software: C.N.T., F.T., M.A.A., D.T.; Supervision: S.D.V., Y.W., J.M., D.T.; Writing-original draft, C.N.T., J.M. and D.T.; Writing-review & editing, F.T., M.F., Y.W., and M.A.A. All authors approved the final manuscript.

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Declaration of Competing Interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

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