


# Specific staphylococcal cassette chromosome *mec* (SCC*mec*) types and clonal complexes are associated with low-level amoxicillin/clavulanic acid and cefalotin resistance in methicillin-resistant *Staphylococcus pseudintermedius*

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**Background:** *Staphylococcus pseudintermedius* is a common pathogen in dogs and methicillin resistance has emerged over recent decades. According to the current guidelines, *S. pseudintermedius* displaying oxacillin resistance should be reported as resistant to all  $\beta$ -lactams.

**Objectives:** To identify possible associations between  $\beta$ -lactam resistance levels and clonal complexes (CCs) and/or staphylococcal cassette chromosome *mec* (SCC*mec*) types in methicillin-resistant *S. pseudintermedius* (MRSP).

**Methods:** MICs of oxacillin, penicillin, ampicillin, amoxicillin/clavulanic acid and cefalotin were determined by broth microdilution for 86 clinical canine MRSP isolates from Denmark and the Netherlands. PCR and sequencing were used for SCC*mec* typing and MLST.

**Results:** Isolates belonged to CC71 ( $n=36$ ), CC258 ( $n=33$ ), CC45 ( $n=11$ ), CC68 ( $n=1$ ) and five singleton STs. SCC*mec*II-III was exclusively found in CC71 and SCC*mec*IV was significantly associated with CC258. SCC*mec*V and non-typeable SCC*mec* types occurred in 4 and 14 isolates, respectively. SCC*mec*IV was associated with lower MICs of oxacillin (<2 mg/L), ampicillin (<8 mg/L) and amoxicillin/clavulanic acid (<4 mg/L) and with susceptibility to cefalotin (<4 mg/L). All isolates harbouring SCC*mec*V were susceptible to cefalotin as well.

**Conclusions:** SCC*mec* types were associated with different CCs and with either high- or low-level resistance to different  $\beta$ -lactams. The finding of amoxicillin/clavulanic acid (20%) and cefalotin (70%) *in vitro* susceptibility across all CCs might have clinical implications, since amoxicillin/clavulanic acid and first-generation cephalosporins are first-choice antibiotics for treatment of *S. pseudintermedius* infections. Pharmacokinetic/pharmacodynamic and clinical outcome studies are warranted to evaluate the *in vivo* efficacy of these  $\beta$ -lactams for treatment of MRSP infections.

## Introduction

*Staphylococcus pseudintermedius* is an opportunistic pathogen in dogs. Methicillin-resistant *S. pseudintermedius* (MRSP) has emerged worldwide over the past decade.<sup>1,2</sup> Methicillin resistance is associated with *mecA* located on staphylococcal cassette

chromosome *mec* (SCC*mec*).<sup>3</sup> Various SCC*mec* types in *Staphylococcus aureus* are linked to different lineages, exhibiting different levels of oxacillin resistance.<sup>4–6</sup> In *S. pseudintermedius*, similar associations have been suggested.<sup>7,8</sup>

Screening for methicillin resistance in *S. pseudintermedius* is performed by oxacillin susceptibility testing using a considerably

lower resistance breakpoint ( $R \geq 0.5$  mg/L) than for *S. aureus* ( $R \geq 4$  mg/L).<sup>9</sup> Oxacillin-resistant isolates should be reported as resistant to all  $\beta$ -lactams according to an expert rule established for *S. aureus* to minimize major errors of antimicrobial susceptibility testing (i.e. resistant strains reported as susceptible). This rule was adapted to *S. pseudintermedius* on the basis of the oxacillin MIC distribution for *mecA*-positive isolates without being corroborated by any clinical outcome data.<sup>10</sup>

This study aimed to detect possible associations between  $\beta$ -lactam resistance levels and clonal complexes (CCs) and/or SCCmec types in MRSP. *In vitro* susceptibility to  $\beta$ -lactams commonly used in veterinary medicine was evaluated in a collection of clinical MRSP isolates that were fully characterized by SCCmec typing and MLST.

## Materials and methods

This study included 86 canine clinical MRSP (2008–14) isolates previously described in the Netherlands ( $n=50$ ) and Denmark ( $n=36$ ).<sup>2,11,12</sup> For Danish isolates, MLST was performed by PCR and Sanger sequencing and SCCmec types were determined by PCR. Dutch isolates were characterized by WGS using the batchupload pipeline from the Center for Genomic Epidemiology (Lyngby, Denmark) to assign multilocus STs and SCCmecFinder for SCCmec typing.<sup>13</sup> When no SCCmec type was detected, a local BLASTn search was performed against SCCmec elements previously identified in *S. pseudintermedius* using Geneious version 2019.0.3 (Biomatters, Auckland, New Zealand).

MICs of five  $\beta$ -lactams (oxacillin range 0.5–2 mg/L, penicillin range 0.5–8 mg/L, ampicillin range 0.25–8 mg/L, amoxicillin/clavulanic acid range 0.25–8 mg/L and cefalotin range 2–4 mg/L) were determined by broth microdilution using commercially available systems: Sensititre (Thermo Fisher Scientific, Hvidovre, Denmark) in Denmark; and MICRONAUT (MERLIN Diagnostika GmbH, Germany) in the Netherlands. Quality control (*S. aureus* ATCC 29213) was used in both laboratories and resistance was based on veterinary clinical breakpoints according to CLSI.<sup>9</sup>

Resistance was classified as high- or low-level to facilitate statistical analysis. Isolates displaying MICs of oxacillin  $\geq 2$  mg/L, of penicillin or ampicillin  $\geq 8$  mg/L and of amoxicillin/clavulanic acid  $\geq 4$  mg/L were defined as high-level resistant. Pearson's  $\chi^2$  test and Fisher's exact test (when sample size  $< 5$ ) were used to identify associations between CCs, SCCmec types and resistance levels. A *P* value for significance was determined by  $\alpha$  divided by the number of independent tests performed in a group of comparisons according to Bonferroni's method to minimize the risk of type I error.

## Results

The isolates belonged to CC71 ( $n=36$ ), CC258 ( $n=33$ ), CC45 ( $n=11$ ), CC68 ( $n=1$ ) and five singleton STs (ST268, ST269, ST284, ST286 and ST431) (Table S1, available as [Supplementary data](#) at JAC Online). Thirty-four (94%) CC71 isolates harboured SCCmecII-III, which was significantly associated with this CC ( $P < 0.001$ ) and not found in other STs. Thirty-one (94%) CC258 isolates harboured SCCmecIV, which was significantly associated with this CC ( $P < 0.001$ ). SCCmecIV was also found in three other STs (268, 269 and 286). SCCmecV was found in four different STs and not significantly associated with any CC. SCCmec was non-typeable by PCR and WGS in 14 isolates, including 10 CC45, 2 CC258 and 2 CC71. CC45 was significantly associated with harbouring a non-typeable SCCmec ( $P < 0.001$ ). No significant difference was found in the distribution of CCs and SCCmec types between Denmark and the Netherlands, although SCCmecV was found only in Danish isolates ( $n=4$ ).

As defined for MRSP, all isolates showed MICs at or above the resistance breakpoint of oxacillin ( $R \geq 0.5$  mg/L). In all SCCmecII-III isolates, oxacillin MICs were high ( $\geq 2$  mg/L), whereas 77% of other isolates had low MICs ( $< 2$  mg/L) (Figure 1). SCCmecIV was significantly associated with low-level oxacillin resistance ( $P < 0.001$ ).

All isolates displayed penicillin MICs above the clinical breakpoint ( $R > 0.25$  mg/L). There were no significant differences in penicillin MIC distribution between SCCmec types. Low-level resistance to penicillin (MIC  $< 8$  mg/L) was observed in seven isolates, all originating from Denmark ( $P = 0.001$ ).

For ampicillin, 1 isolate tested susceptible (MIC  $\leq 0.25$  mg/L), 15 isolates showed low-level resistance (MIC 0.5–4 mg/L) and 70 isolates showed high-level resistance (MIC  $> 8$  mg/L). High-level resistance was associated with SCCmecII-III ( $P < 0.001$ ) and low-level resistance with SCCmecIV ( $P = 0.007$ ). Low-level ampicillin resistance was significantly more prevalent in Denmark ( $P < 0.001$ ).

For amoxicillin/clavulanic acid, 80% of the isolates showed MICs at or above the resistance breakpoint ( $R \geq 0.5$  mg/L). High-level resistance to amoxicillin/clavulanic acid (MIC  $\geq 4$  mg/L) was significantly associated ( $P < 0.001$ ) with SCCmecII-III of which 68% had high-level resistance. Susceptibility (MIC  $\leq 0.25$  mg/L) and low-level resistance to amoxicillin/clavulanic acid (MIC  $< 4$  mg/L) were seen in 24% and 68% of SCCmecIV isolates, in 25% and 50% of SCCmecV isolates and in 21% and 57% of non-typeable SCCmec isolates, respectively. SCCmecIV was significantly associated with low-level resistance and susceptibility to amoxicillin/clavulanic acid ( $P < 0.001$ ). Low-level resistance was significantly more prevalent in the Netherlands ( $P = 0.013$ ).

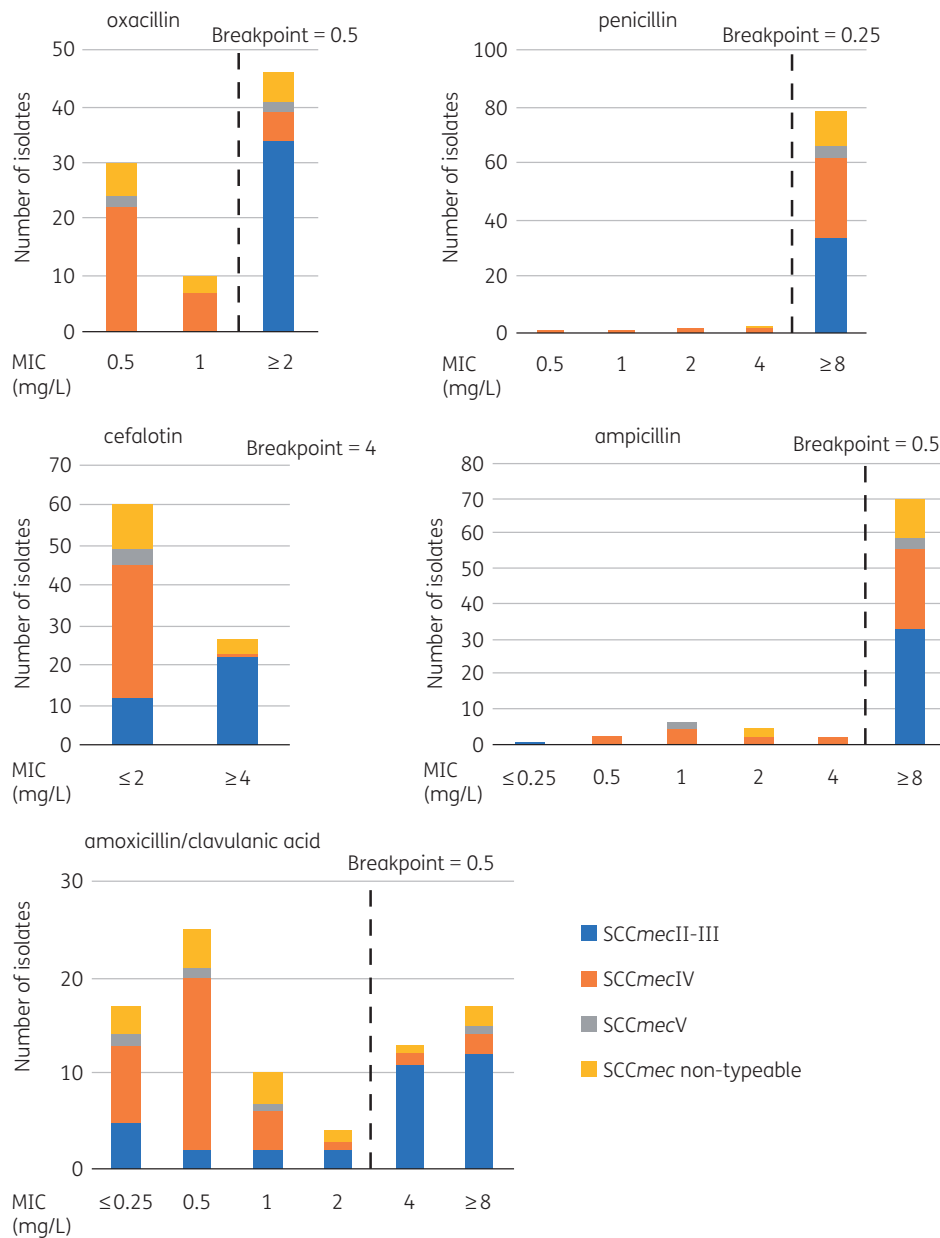
Only 30% (26/86) of the isolates showed MICs above the clinical breakpoint of cefalotin ( $R \geq 4$  mg/L). Resistance was primarily seen in SCCmecII-III isolates (65%). One isolate with SCCmecIV (3%), three isolates with non-typeable SCCmec types (21%) and none of the SCCmecV isolates displayed resistance. The associations of SCCmecII-III with resistance and SCCmecIV with susceptibility were significant ( $P < 0.001$ ).

## Discussion

SCCmecII-III was only present in CC71, whereas SCCmecIV was associated with CC258 but also present in different STs. SCCmecV was shared by different STs. The SCCmec of the majority of CC45 isolates was non-typeable, which correlates with the previously described dissemination of novel SCCmec variants in CC45.<sup>8,14</sup> These results confirm a link between the SCCmec and CC in MRSP, as previously described.<sup>8</sup>

We found MRSP isolates susceptible to amoxicillin/clavulanic acid and cefalotin across all CCs and SCCmec types. CC71-SCCmecII-III isolates showed higher levels of resistance to oxacillin, ampicillin, amoxicillin/clavulanic acid and cefalotin, whereas SCCmecIV was associated with low-level resistance or even susceptibility to several  $\beta$ -lactams. SCCmecV and non-typeable SCCmec types were not significantly associated with either high- or low-level resistance. Especially for SCCmecV isolates, such lack of statistical association could be due to the low number of isolates representing this type.

The association of SCCmecII-III and high MICs of  $\beta$ -lactams might have contributed to the success of CC71 globally. MIC differences of  $\beta$ -lactams between different SCCmec types have been previously reported for *S. pseudintermedius*, with SCCmecII-III



**Figure 1.** MIC distributions of  $\beta$ -lactam antibiotics for different SCCmec types. MICs of oxacillin, penicillin, cefalotin, ampicillin and amoxicillin/clavulanic acid for SCCmecII-III, SCCmecIV, SCCmecV and non-typeable SCCmec types. The broken lines indicate the separation between high- and low-level resistance. This figure appears in colour in the online version of JAC and in black and white in the print version of JAC.

having higher MICs of oxacillin and other  $\beta$ -lactams than SCCmecV.<sup>7,8</sup> This study confirms those findings while expanding the range of antimicrobials tested and the diversity of CCs and SCCmec.

It is unknown how SCCmec differences influence levels of  $\beta$ -lactam resistance in MRSP and why there are differences between  $\beta$ -lactams. In *S. aureus*, a role of the *mecA* promoter on the level of PBP2a production and oxacillin resistance level has been shown,<sup>15</sup> as well as overexpression of *blaZ* resulting in low-level methicillin resistance independently of *mecA* expression in borderline oxacillin-resistant isolates.<sup>16</sup> In *S. pseudintermedius*, *mecA* (*mecI* and *mecRI*) and *blaZ* (*blaRI*

and *blaI*) regulators are known to modulate expression of *mecA*.<sup>17,18</sup> Altogether these studies suggest that the levels of  $\beta$ -lactam resistance in methicillin-resistant staphylococci are influenced by multiple loci and mechanisms. Further research on sequences, preferably from long-read sequencing, and testing of PBP production and affinity in *S. pseudintermedius* are required to understand the mechanism responsible for low-level  $\beta$ -lactam resistance in MRSP lineages.

Low-level resistance was significantly more prevalent in Denmark for penicillin and ampicillin, and in the Netherlands for amoxicillin/clavulanic acid. These findings might represent a difference in distribution of isolates between countries, but might also

be due to methodological differences between the two laboratories or minor variations in MIC affecting the classification of some isolates.

Following EUCAST and CLSI guidelines, oxacillin-resistant isolates should be reported as resistant to all  $\beta$ -lactams, although clinical evidence is lacking for *S. pseudintermedius*. The finding of cefalotin and amoxicillin/clavulanic acid susceptibility across all CCs might be clinically relevant, since amoxicillin/clavulanic acid and first-generation cephalosporins are first-line antibiotics for treatment of *S. pseudintermedius* infections. Cefalotin is highly active against staphylococci and good clinical cure rates have been reported for uncomplicated MRSA skin infections in humans.<sup>19</sup> For urinary tract infections, anti-MRSA efficacy of high doses of amoxicillin/clavulanic acid has been shown.<sup>20</sup> For canine urinary tract infections, a higher breakpoint for *Staphylococcus* spp. exists ( $R > 8$  mg/L).<sup>9</sup> Using this breakpoint, 90% of our isolates would be considered susceptible. Notably, the dosing regimens used for first-generation cephalosporins and amoxicillin/clavulanic acid in companion animals can be higher than those used for setting clinical breakpoints (12.5 mg/kg twice daily for amoxicillin/clavulanic acid and 25 mg/kg twice daily for cefalotin). It can be hypothesized that MRSP infections caused by isolates with low-level resistance to  $\beta$ -lactams can be treated by using higher doses and/or more frequent administration, thereby avoiding use of antimicrobials with adverse effects (e.g. rifampicin) or critically important antimicrobials not authorized for veterinary use (e.g. vancomycin). Pharmacokinetic/pharmacodynamic and clinical outcome studies are needed to validate this hypothesis.

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## Transparency declarations

None to declare.

## Supplementary data

Table S1 is available as [Supplementary data](#) at JAC Online.

## References

- Pires dos Santos T, Damborg P, Moodley A *et al.* Systematic review on global epidemiology of methicillin-resistant *Staphylococcus pseudintermedius*: inference of population structure from multilocus sequence typing data. *Front Microbiol* 2016; **7**: 1599.
- Duim B, Verstappen KM, Broens EM *et al.* Changes in the population of methicillin-resistant *Staphylococcus pseudintermedius* and dissemination of antimicrobial-resistant phenotypes in the Netherlands. *J Clin Microbiol* 2016; **54**: 283–8.
- International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements (IWG-SCC). Classification of staphylococcal cassette chromosome *mec* (SCCmec): guidelines for reporting novel SCCmec elements. *Antimicrob Agents Chemother* 2009; **53**: 4961–7.
- Ho C-M, Lin C-Y, Ho M-W *et al.* Methicillin-resistant *Staphylococcus aureus* isolates with SCCmec type V and *spa* types t437 or t1081 associated to discordant susceptibility results between oxacillin and ceftiofur, Central Taiwan. *Diagn Microbiol Infect Dis* 2016; **86**: 405–11.
- Chen F-J, Huang I-W, Wang C-H *et al.* *mecA*-positive *Staphylococcus aureus* with low-level oxacillin MIC in Taiwan. *J Clin Microbiol* 2012; **50**: 1679–83.
- Conceição T, Coelho C, de Lencastre H *et al.* Frequent occurrence of oxacillin-susceptible *mecA*-positive *Staphylococcus aureus* (OS-MRSA) strains in two African countries. *J Antimicrob Chemother* 2015; **70**: 3200–4.
- Kasai T, Saegusa S, Shirai M *et al.* New categories designated as healthcare-associated and community-associated methicillin-resistant *Staphylococcus pseudintermedius* in dogs. *Microbiol Immunol* 2016; **60**: 540–51.
- Worthing KA, Schwendener S, Perreten V *et al.* Characterization of staphylococcal cassette chromosome *mec* elements from methicillin-resistant *Staphylococcus pseudintermedius* infections in Australian animals. *mSphere* 2018; **3**: e00491-18.
- CLSI. *Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated From Animals—Fourth Edition*: VET08. 2018.
- CLSI. *Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated From Animals—Fifth Edition*: VET01. 2018.
- Wegener A, Broens EM, Zomer A *et al.* Comparative genomics of phenotypic antimicrobial resistances in methicillin-resistant *Staphylococcus pseudintermedius* of canine origin. *Vet Microbiol* 2018; **225**: 125–31.
- Damborg P, Moodley A, Aalbak B *et al.* High genotypic diversity among methicillin-resistant *Staphylococcus pseudintermedius* isolated from canine infections in Denmark. *BMC Vet Res* 2016; **12**: 131.
- Kaya H, Hasman H, Larsen J *et al.* SCCmecFinder, a web-based tool for typing of staphylococcal cassette chromosome *mec* in *Staphylococcus aureus* using whole-genome sequence data. *mSphere* 2018; **3**: e00612-17.
- Duim B, Verstappen K, Kalupahana RS *et al.* Methicillin-resistant *Staphylococcus pseudintermedius* among dogs in the description of novel SCCmec variants. *Vet Microbiol* 2018; **213**: 136–41.
- Harrison EM, Ba X, Coll F *et al.* Genomic identification of cryptic susceptibility to penicillins and  $\beta$ -lactamase inhibitors in methicillin-resistant *Staphylococcus aureus*. *Nat Microbiol* 2019; **4**: 1680–91.
- Hryniewicz MM, Garbacz K. Borderline oxacillin-resistant *Staphylococcus aureus* (BORSA) – a more common problem than expected? *J Med Microbiol* 2017; **66**: 1367–73.
- Liu P, Xue H, Wu Z *et al.* Effect of *bla* regulators on the susceptible phenotype and phenotypic conversion for oxacillin-susceptible *mecA*-positive staphylococcal isolates. *J Antimicrob Chemother* 2016; **71**: 2105–12.
- Black CC, Eberlein LC, Solyman SM *et al.* The role of *mecA* and *blaZ* regulatory elements in *mecA* expression by regional clones of methicillin-resistant *Staphylococcus pseudintermedius*. *Vet Microbiol* 2011; **151**: 345–53.
- Chen AE, Carroll KC, Diener-West M *et al.* Randomized controlled trial of cephalexin versus clindamycin for uncomplicated pediatric skin infections. *Pediatrics* 2011; **127**: e573–80.
- Guignard B, Entenza JM, Moreillon P.  $\beta$ -Lactams against methicillin-resistant *Staphylococcus aureus*. *Curr Opin Pharmacol* 2005; **5**: 479–89.