J Antimicrob Chemother 2023; **78**: 1909–1920
https://doi.org/10.1093/jac/dkad182 Advance Access publication 9 June 2023

Journal of Antimicrobial Chemotherapy

In vitro and *in vivo* susceptibility to cefalexin and amoxicillin/clavulanate in canine low-level methicillin-resistant *Staphylococcus pseudintermedius*

Mattia Pirolo [1], Mareliza Menezes[1], Peter Damborg[1], Alice Wegener[2], Birgitta Duim[2], Els Broens [2], Lisbeth Rem Jessen[3], Mette Schjærff[3] and Luca Guardabassi[1]*; on behalf of the ESCMID Study Group for Veterinary Microbiology (ESGVM)

[1]*Department of Veterinary and Animal Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Frederiksberg, Denmark;* [2]*Department of Biomolecular Health Sciences, Division of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands;* [3]*Department of Veterinary Clinical Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Frederiksberg, Denmark*

*Corresponding author. E-mail: lg@sund.ku.dk

@health_amr, @Grunfio, @MattiaPirolo

Received 24 May 2023; accepted 29 May 2023

Background: Methicillin-resistant *Staphylococcus pseudintermedius* (MRSP) lineages harbouring staphylococcal cassette chromosome (SCC) *mec* types IV, V and ΨSCC*mec*57395 usually display low oxacillin MICs (0.5–2 mg/L).

Objectives: To evaluate how oxacillin MICs correlate with PBP mutations and susceptibility to β-lactams approved for veterinary use.

Methods: Associations between MICs and PBP mutations were investigated by broth microdilution, time–kill and genome sequence analyses in 117 canine MRSP strains harbouring these SCC*mec* types. Clinical outcome was retrospectively evaluated in 11 MRSP-infected dogs treated with β-lactams.

Results: Low-level MRSP was defined by an oxacillin MIC <4 mg/L. Regardless of strain genotype, all low-level MRSP isolates ($n=89$) were cefalexin susceptible, whereas no strains were amoxicillin/clavulanate susceptible according to clinical breakpoints. Exposure to 2× MIC of cefalexin resulted in complete killing within 8 h. High (≥4 mg/L) oxacillin MICs were associated with substitutions in native PBP2, PBP3, PBP4 and acquired PBP2a, one of which (V_{390}M in PBP3) was statistically significant by multivariable modelling. Eight of 11 dogs responded to systemic therapy with first-generation cephalosporins ($n=4$) or amoxicillin/clavulanate ($n=4$) alone or with concurrent topical treatment, including 6 of 7 dogs infected with low-level MRSP.

Conclusions: Oxacillin MIC variability in MRSP is influenced by mutations in multiple PBPs and correlates with cefalexin susceptibility. The expert rule recommending that strains with oxacillin MIC ≥0.5 mg/L are reported as resistant to all β-lactams should be reassessed based on these results, which are highly clinically relevant in light of the shortage of effective antimicrobials for systemic treatment of MRSP infections in veterinary medicine.

Introduction

Staphylococcus pseudintermedius is a commensal and a common opportunistic pathogen in dogs. It frequently causes canine bacterial pyoderma, otitis and post-surgical wound infections, and may also be involved in urinary tract infections, bacteraemia and osteomyelitis.[1] Systemic antimicrobial treatment has become difficult due to the emergence of methicillin-resistant *S. pseudintermedius* (MRSP), which was first reported in Europe (Germany) in 2005.[2] MRSP spreads clonally and certain MRSP lineages are predominant within a continent, as in the case of clonal complex (CC)68 in North America, CC45 in Asia, and CC71 and more recently CC258 in Europe.[3] MRSP is by definition resistant to β-lactams used as first-line agents in small animal veterinary practice, such as cefalexin and amoxicillin/clavulanate, and is often resistant to alternative antimicrobials

authorized for use in small animal veterinary practice. Consequently, MDR MRSP infections that require systemic treatment can only be managed using older antibiotics with relatively high toxicity (e.g. rifampicin or chloramphenicol) or certain antimicrobial drugs that are considered critically important in human medicine (e.g. linezolid or fosfomycin). Veterinary use of these and other drugs of high clinical importance in human medicine have been prohibited in the EU starting from February 2023,[4] thereby further limiting the availability of effective therapies for MRSP infections in veterinary medicine.

The current interpretive criteria for MRSP have been adapted from the MRSA expert rule 8.1,[5] which is based on clinical data showing that human MRSA infections respond poorly to β-lactam therapy. To adapt this rule to *S. pseudintermedius*, the oxacillin resistance breakpoint was arbitrarily reduced by 8-fold (i.e. from 4 to 0.5 mg/L) based on WT MIC distributions without any clinical evidence in support of its clinical predictive value. In a previous study,[6] we showed that low MICs of oxacillin (0.5–2 mg/L) were associated with MRSP lineages harbouring staphylococcal cassette chromosome (SCC) *mec* types IV, V and a non-typeable variant later defined as ΨSCC*mec*57395. This oxacillin phenotype was, however, not present in all strains harbouring these SCC*mec* types.

The objective of this study was to evaluate how oxacillin MICs correlate with PBP mutations and susceptibility to the two β-lactam antibiotics most commonly used in small animal veterinary practice, cefalexin and amoxicillin/clavulanate. Genome sequence analysis of an international collection of 117 MRSP strains harbouring the above-mentioned SCC*mec* types revealed the presence of PBP mutations associated with high oxacillin MICs. Regardless of strain genotype, all strains displaying low oxacillin MIC (<4 mg/L), hereafter referred to as low-level MRSP, were susceptible to cefalexin *in vitro* based on MIC and time–kill testing. In order to gain an impression of how well our *in vitro* findings predicted clinical outcome, 11 cases of MRSP infection in dogs treated empirically with β-lactams were assessed retrospectively.

Materials and methods

MRSP strains

Based on the findings of the previous study,[6] we searched our collections of genome-sequenced MRSP for strains harbouring SCC*mec* types IV, V or ΨSCC*mec*57395 collected over 17 years (2004–22). SCC*mec* types had been assigned using staphopia-sccmec v1.2.0,[7] except for ΨSCC*mec*57395, for which sequence reads were aligned against the reference sequence (GenBank accession no. HE984157.2). This search led to identification of 66, 36 and 15 strains carrying SCC*mec* types IV, V and ΨSCC*mec*57395, respectively, leading to a total of 117 strains (Dataset S1, available as Supplementary data at *JAC* Online).

All strains were oxacillin resistant according to the CLSI breakpoint for *S. pseudintermedius* (R≥0.5 mg/L)[8] and originated from different dogs in the Netherlands ($n=54$), Denmark ($n=51$), Australia ($n=4$), Canada ($n=3$), USA ($n=2$), Hong Kong ($n=2$) and Sweden ($n=1$). Among strains with a known source of isolation ($n=79$), 56 derived from skin, including pyoderma and surgical wound infections, 13 from otitis and 9 from other infections. All strains had been sequenced as part of other projects using Illumina platforms with paired-end operating mode, followed by assembly of raw sequencing reads using SPAdes v.3.13.1,[9] and quality check on QUAST v.5.0.2.[10] The sequences had been previously submitted to the NCBI Sequence Read Archive (SRA) under BioProjects PRJEB53745 and PRJNA902303.

Antimicrobial susceptibility testing

MICs of oxacillin, cefalexin and amoxicillin/clavulanate were determined by broth microdilution and interpreted according to CLSI using the clinical breakpoints for *S. pseudintermedius*,[8] namely R≥0.5 mg/L for oxacillin, ≥4 mg/L for cefalexin and ≥1 mg/L for amoxicillin/clavulanate. Each antibiotic was tested at concentrations ranging from 0.03 to 16 mg/L using *Staphylococcus aureus* ATCC 29213 as quality control strain.

Time–kill assays

The killing activity of cefalexin was determined in three cefalexin-susceptible strains selected on the basis of the prevalence of cefalexin susceptibility at the CC level. Briefly, single colonies from fresh Mueller–Hinton Agar (MHA, Oxoid, Roskilde, Denmark) plates were subcultured in Mueller–Hinton Broth (MHB, Oxoid). After 2 h incubation at 37°C, ∼10^6 cfu/mL of bacteria were inoculated in MHB supplemented with cefalexin concentrations corresponding to 8, 4, 2, 1, 0.5 or 0.25-fold the strain's MIC and incubated at 37°C with gentle shaking (150 rpm). Aliquots were obtained prior to cefalexin inoculation and after 2, 4, 8 and 24 h of incubation, serially diluted (1:10), and 5 μL of each dilution was spotted in triplicate on MHA plates for viable counts after 24 h of incubation at 37°C.

Genome sequence analysis

CC clustering was based on *in silico* MLST using mlst v.2.19.0 (https://github.com/tseemann/mlst) and grouping by PHYLOViZ v2.0.[11] CC clustering was refined using core-genome analysis on Prokka-annotated genomes using Roary v3.6.0,[12,13] followed by phylogenetic reconstruction using IQ-TREE v.1.5.5,[14] with the best model found by the implemented ModelFinder, and tree visualization using iTOL v6.5.8.[15]

The amino acid (aa) sequences of the four native PBPs in the selected MRSP strains were compared with those in the reference *S. pseudintermedius* strain ED99: PBP1 (GenBank accession no. ADX76886.1), PBP2 (GenBank accession no. ADX76608.1), PBP3 (GenBank accession no. ADX76509.1) and PBP4 (GenBank accession no. ADX77343.1). Reference *S. aureus* strain 85/2082 (GenBank accession no. AB037671.1) was used for detection of aa substitutions in acquired PBP2a.

We also checked for the presence of *blaZ* and *bla* accessory genes in the assembled genomes using ABRicate v1.0.1 (https://github.com/tseemann/abricate). Genomes were also screened for the presence of genes associated with the small colony variant (SCV) phenotype, namely *hemB*, *menD* and *thyA*, using *S. pseudintermedius* strain ED99 as reference. Positive hits were selected based on >95% nucleotide sequence identity and >90% coverage.

Statistical analysis

All statistical analyses were performed in R v.4.1.1. Associations between MIC values (numerical variable) and SCC*mec* types or the presence of *blaZ* were undertaken using the pairwise Wilcoxon non-parametric test. Initial associations between PBP aa substitutions and β-lactam MIC binary values were identified using chi-squared or Fisher's exact test, when appropriate. Subsequently, forward and reverse multivariable logistic regression models were constructed to include all aa substitutions that were significant at the bivariate level. Comparisons of MIC of β-lactams (numerical variable) and PBP substitutions were undertaken using the Kruskal–Wallis non-parametric test. In order to prevent type I errors, all *P* values in multiple comparisons were corrected for multiple testing using the Benjamini–Hochberg's method.

Retrospective analysis of MRSP cases treated with β-lactams

We searched the diagnostic laboratory database at the Department of Veterinary and Animal Sciences (University of Copenhagen) for canine

MRSP cases for which strains were available. Strains harbouring SCC*mec* types other than IV, V and ΨSCC*mec*57395, which were not originally included in the study, were characterized using the methods described above. Clinical medical records from MRSP cases diagnosed at the University Hospital for Companion Animals (University of Copenhagen) were thoroughly assessed by two clinicians. Clinics other than the University Hospital were contacted by e-mail or phone to enquire whether β-lactams had been empirically prescribed to these dogs prior to laboratory diagnosis of MRSP. If the dog was treated with β-lactams, information concerning age, breed, infection type, β-lactam antibiotic dosage regimen, treatment duration, adjunctive treatment (e.g. topical antiseptic shampoo, antiseptics and/or wound dressing) and clinical outcome was obtained from the clinical records. Assessment of clinical outcome was based on the clinician's report.

Results

Genotypic diversity

We detected 46 STs among the 117 selected strains, including 11 new STs (ST2182, ST2183, ST2184, ST2185, ST2186, ST2188, ST2189, ST2190, ST2191, ST2328 and ST2331) that were submitted to the PubMLST database (https://pubmlst.org). The strain collection comprised CC258 ($n=71$), CC45 ($n=19$), CC551 ($n=10$), CC68 ($n=3$) and 14 strains assigned to singleton STs (Table 1). All but five CC258 strains (93.0%) harboured SCC*mec* type IV, whereas type V was detected in all CCs, and ΨSCC*mec*57395 was only present in CC45 strains (Table 1).

Relationships between β-lactam MICs and MRSP genotypes

Amongst the 117 MRSP strains tested, 89 (76.1%) were low-level MRSP displaying oxacillin MICs below 4 mg/L. Regardless of strain genotype, all low-level MRSP were cefalexin susceptible according to the CLSI clinical breakpoint for *S. pseudintermedius* (R≥ 4 mg/L). This oxacillin phenotype included 95.5% of strains carrying SCC*mec* type IV, 66.7% of strains carrying ΨSCC*mec*57395% and 44.4% of strains carrying type V. While the vast majority (93%) of the CC258 strains and the three ST68 strains were low-level MRSP, this phenotypic trait was inconsistent in CC45 (52.6%) and absent in CC551 (Figure 1). Time–kill analyses showed bactericidal activity of cefalexin against three low-level MRSP strains, including two CC258/SCC*mec* IV strains and one ST68/SCC*mec* V strain (Figure 2). Concentrations corresponding to 2× MIC resulted in total killing of all the three strains tested after 8 h incubation. For two strains, the same effect was observed after only 4 h exposure to 8× MIC (Figure 2).

None of the MRSP strains tested were susceptible to amoxicillin/clavulanate *in vitro*. Forty-five strains (38.5%) were amoxicillin/clavulanate intermediate (MIC = 0.5 mg/L), the majority of which (95.6%) were low-level MRSP. Amoxicillin/clavulanate-intermediate strains were observed mostly among CC258 strains ($n=33$), followed by CC45 ($n=7$) and singleton STs ($n=5$). The remaining 72 strains (61.5%) were resistant according to the CLSI clinical breakpoint for *S. pseudintermedius* (R≥1 mg/L), including all CC551 ($n=10$) and ST68 ($n=3$) strains.

Among the 28 strains with high oxacillin MIC (≥4 mg/L), hereafter referred as high-level MRSP, 26 were resistant to amoxicillin/clavulanate, and 11 of them were additionally resistant to cefalexin and were assigned to CC45 ($n=9$) and CC551 ($n=2$) carrying SCC*mec* type V ($n=6$) or ΨSCC*mec*57395 ($n=5$) (Table 2). Figure 1 shows the distribution of the MICs of oxacillin, cefalexin and amoxicillin/clavulanate across the three SCC*mec* types. Statistical analysis revealed that SCC*mec* type V was significantly associated with higher MICs of oxacillin, cefalexin and

Table 1. Distribution of staphylococcal chromosomal cassette (SCC) *mec* types among STs and CCs

CC	ST	No. of strains	SCC*mec* IV	SCC*mec* V	ΨSCC*mec* 57395
CC258	ST258	26	26	—	—
	ST261	8	8	—	—
	ST265	5	5	—	—
	ST301	3	3	—	—
	ST2182	2	2	—	—
	ST277	2	1	1	—
	ST350	2	2	—	—
	ST498	2	2	—	—
	ST84	2	—	2	—
	ST118	1	1	—	—
	ST1403	1	1	—	—
	ST2184	1	1	—	—
	ST2185	1	1	—	—
	ST2186	1	1	—	—
	ST2331	1	1	—	—
	ST260	1	—	1	—
	ST291	1	1	—	—
	ST307	1	1	—	—
	ST312	1	1	—	—
	ST334	1	1	—	—
	ST336	1	1	—	—
	ST337	1	1	—	—
	ST342	1	1	—	—
	ST346	1	1	—	—
	ST41	1	1	—	—
	ST414	1	1	—	—
	ST430	1	1	—	—
	ST699	1	—	1	—
CC45	ST45	14	—	—	14
	ST1376	3	—	3	—
	ST659	1	—	1	—
	ST85	1	—	—	1
CC551	ST551	6	—	6	—
	ST1338	3	—	3	—
	ST2190	1	—	1	—
CC68	ST68	3	—	3	—
Singletons	ST2328	3	—	3	—
	ST1563	2	—	2	—
	ST341	2	—	2	—
	ST1849	1	—	1	—
	ST2183	1	—	1	—
	ST2188	1	—	1	—
	ST2189	1	—	1	—
	ST2191	1	—	1	—
	ST315	1	—	1	—
	ST924	1	—	1	—

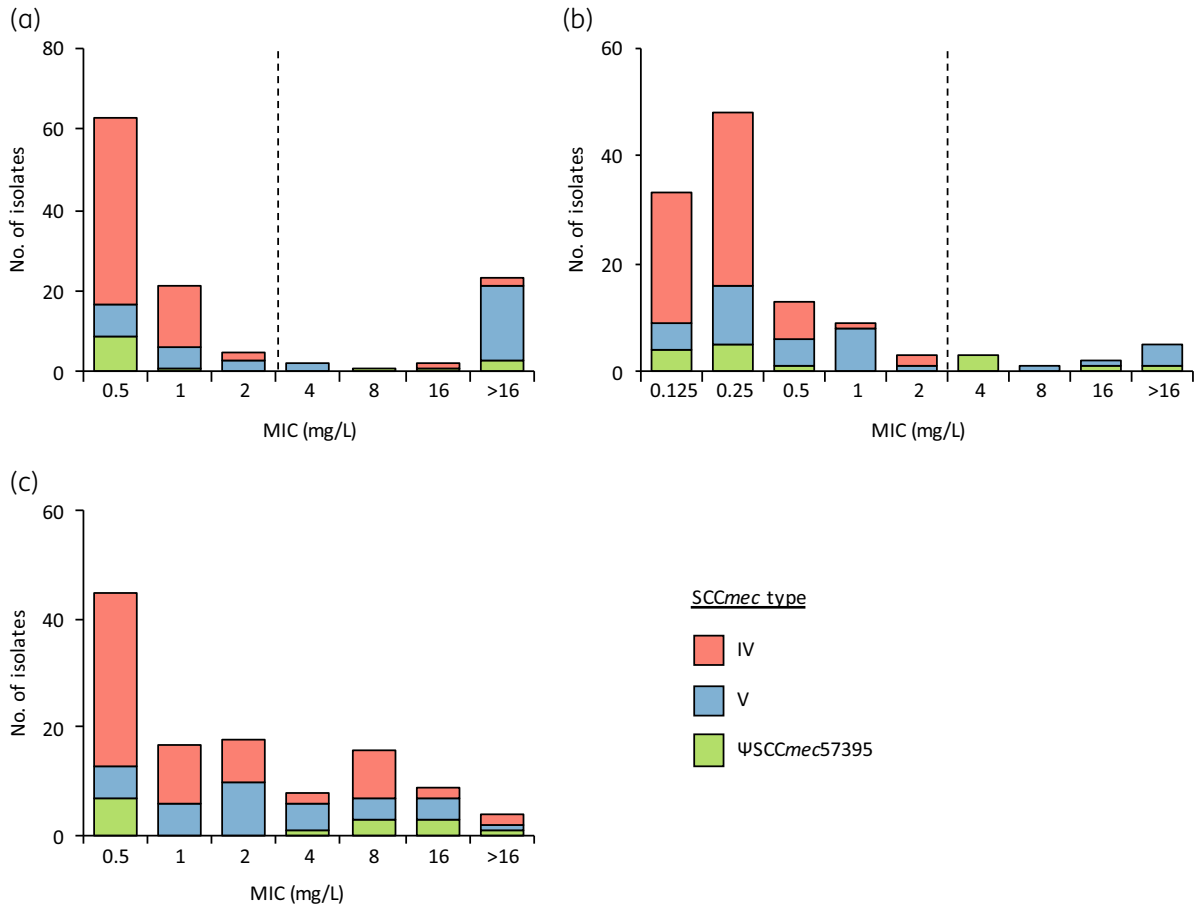

Figure 1. Distribution of SCC*mec* types and MICs of oxacillin (a), cefalexin (b) and amoxicillin clavulanate (c) in 117 genome-sequenced MRSP isolates. The dashed line in (a) indicates the oxacillin clinical breakpoint for *S. aureus*, whereas the dashed line in (b) indicates the cefalexin clinical breakpoint for *S. pseudintermedius*. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

amoxicillin/clavulanate compared with SCC*mec* type IV (adjusted *P* values < 0.001) and with higher MICs of oxacillin compared with ΨSCC*mec*57395 (adjusted *P* value = 0.049).

Relationships between β-lactam MICs, PBP mutations and MRSP genotypes

A total of 20 aa substitutions were identified across the four native PBPs in *S. pseudintermedius*, including 7, 6, 5 and 2 substitutions in PBP1, PBP2, PBP3 and PBP4, respectively. At bivariate level, no substitutions in PBP1 were associated with a change in β-lactam resistance levels, whereas single substitutions in PBP2 ($Q_{641}E$), PBP3 ($V_{390}M$) and PBP4 ($D_{326}N$) were significantly associated with high-level MRSP and cefalexin resistance (*P* values < 0.001). PBP2a protein sequence analysis led to identification of four aa substitutions, three of which ($S_{225}R$, $E_{246}G$ and $G_{489}S$) were associated with high-level MRSP. $E_{246}G$ was additionally associated with cefalexin resistance (*P* value = 0.014) (Tables S1 and S2). Multivariable modelling showed that the only aa substitution that significantly influenced the MICs of oxacillin was $V_{390}M$ in PBP3 (*P* value < 0.001). This aa substitution was associated with a significant increase in oxacillin MIC (mean 12.5 ± 6.4 mg/L for mutated strains versus 1.8 ± 3.9 mg/L for WT strains, *P* value < 0.001) and occurred in 25 strains, including 20/28 high-level MRSP (oxacillin MIC ≥4 mg/L) and 9/11 cefalexin-resistant strains. None of the substitutions were significantly associated with cefalexin and amoxicillin/clavulanate resistance by multivariable analysis. One or multiple PBP mutations were found in all the 11 cefalexin-resistant strains (Table 2) but also in 38 of the 106 cefalexin-susceptible strains.

Specific patterns were observed in the occurrence of these aa substitutions across different clonal lineages (for native PBPs) and SCC*mec* types (for PBP2a) (Figure 3). All 10 CC551 and most (7/9) CC45 strains carried the $V_{390}M$ substitution in PBP3. The $Q_{641}E$ substitution in PBP2 was exclusively present in cefalexin-resistant, high-level MRSP belonging to CC45 strains ($n = 8$). The $D_{326}N$ in PBP4 ($n = 21$) was found in all but one CC551 (9/10) and most (7/9) CC45 strains. The $E_{246}G$ substitution in PBP2a ($n = 46$) was mainly observed in SCC*mec* types V ($n = 33$) or ΨSCC*mec*57395 ($n = 12$), including 20/28 high-level MRSP and 8/11 cefalexin-resistant strains. The $S_{225}R$ ($n = 33$) and $G_{489}S$ ($n = 12$) substitutions were only observed in strains carrying SCC*mec* type V, including 18 and 10 high-level MRSP, respectively.

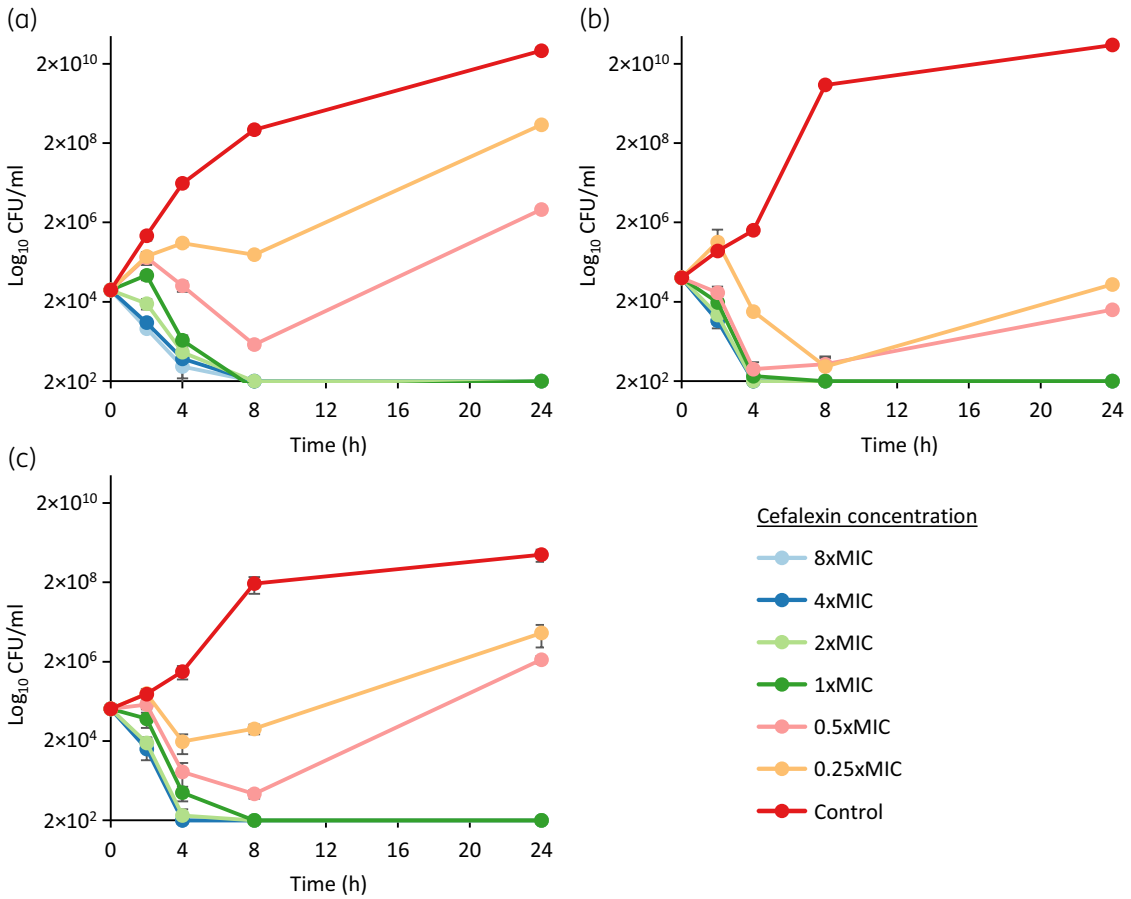

Figure 2. Cefalexin time–kill kinetics on *S. pseudintermedius*. (a) S80C7 (CC258-IV), (b) 33228 (CC258-IV) and (c) 26893 (ST68-V). All isolates exhibited cefalexin MIC of 0.25 mg/L. The limit of detection corresponds to 200 cfu/mL. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

Relationships between β-lactam MICs, blaZ and MRSP genotypes

All CC551, CC45, CC68 and singleton STs only carried the plasmid variant of *blaZ* (accession no. AJ400722), whereas in CC258, 49 (69.0%) strains carried the plasmid variant, 21 (29.6%) the chromosomal variant (accession no. AP003139) and 17 (23.9%) lacked the gene. Co-occurrence of chromosomally and plasmid-encoded *blaZ* types was detected in 16 (22.5%) CC258 strains. The presence of one or both *blaZ* types in this CC was significantly associated with higher MICs of amoxicillin/clavulanate and cefalexin compared with strains missing the gene (adjusted *P* values < 0.001). No significant association was found between presence of *bla* accessory genes (*blaI* and *blaR1*) and β-lactam resistance phenotypes.

Relationships between β-lactam MICs and treatment outcome

The retrospective search resulted in 11 clinical canine MRSP cases treated with amoxicillin/clavulanate ($n=6$), cefadroxil ($n=3$) or cefalexin ($n=2$) (Table 3). Eight of the 11 patients clinically recovered following systemic β-lactam therapy, including two dogs infected with high-level MRSP ST71 strains that were resistant to the prescribed antibiotic *in vitro* (cefalexin and amoxicillin/clavulanate in cases 6 and 7, respectively). Among the seven dogs infected with low-level MRSP, treatment with first-generation cephalosporins ($n=4$) or amoxicillin/clavulanate ($n=3$) resulted in clinical resolution in six cases. The remaining two dogs that did not respond to treatment (cases 5 and 11) were infected with high-level MRSP carrying multiple PBP mutations and displaying *in vitro* resistance to the antibiotic used (amoxicillin/clavulanate). Details related to the patients (breed and age), infection types, β-lactam treatment regimens, adjunctive treatment and phenotypic and genotypic characteristics of MRSP strains are presented in Table 3.

As a coincidental finding, two genetically distinct MRSP strains (ST924 and ST2328) were isolated from two diverse body sites (neck and groin) of the same dog (case 3). One of these strains exhibited an SCV phenotype, but no mutations in genes previously associated with SCV in *S. aureus* were identified.

Discussion

This study shows how mutations in different PBPs correlate with *in vitro* susceptibility to oxacillin, cefalexin and amoxicillin/clavulanate in MRSP, shedding light on the genetic determinants

Table 2. Origin and genotypic and phenotypic characteristics of high-level MRSP strains ($n=28$)

Stain ID	Country	ST	CC	SCC*mec*	MIC (mg/L)			PBP substitutions
					OXA	LEX	AMC	
26012	DK	2182	258	IV	>16	2	>16	WT
212112902001-1	NL	350	258	IV	16	0.25	1	WT
26071-1	DK	2182	258	IV	>16	2	>16	WT
Can8	CA	84	258	V	>16	1	8	WT
SP11	AU	84	258	V	>16	1	8	WT
18S02771-1	NL	1376	45	V	>16	>16	16	PBP2 $Q_{641}E$, PBP3 $V_{390}M$, PBP4 $D_{326}N$, PBP2a $S_{225}R$, PBP2a $E_{246}G$
18S02773-1	NL	1376	45	V	>16	>16	16	PBP2 $Q_{641}E$, PBP3 $V_{390}M$, PBP4 $D_{326}N$, PBP2a $S_{225}R$, PBP2a $E_{246}G$
18S02817-1	NL	1376	45	V	>16	>16	16	PBP2 $Q_{641}E$, PBP3 $V_{390}M$, PBP4 $D_{326}N$, PBP2a $S_{225}R$, PBP2a $E_{246}G$
18S02818-1	NL	659	45	V	>16	16	16	PBP2 $Q_{641}E$, PBP3 $V_{390}M$, PBP4 $D_{326}N$, PBP2a $S_{225}R$, PBP2a $E_{246}G$
209013002001-1	NL	45	45	ΨSCC*mec* 57395	>16	16	16	PBP2a $E_{246}G$
29237-1	DK	45	45	ΨSCC*mec* 57395	16	4	>16	PBP2 $Q_{641}E$, PBP3 $V_{390}M$, PBP4 $D_{326}N$
SP10	AU	45	45	ΨSCC*mec* 57395	>16	4	8	PBP2 $Q_{641}E$, PBP3 $V_{390}M$, PBP4 $D_{326}N$
SP6	AU	45	45	ΨSCC*mec* 57395	>16	4	8	PBP2 $Q_{641}E$, PBP3 $V_{390}M$, PBP4 $D_{326}N$
43025	DK	85	45	ΨSCC*mec* 57395	8	>16	4	PBP2 $Q_{641}E$, PBP2a $E_{246}G$
18S02770-1	NL	551	551	V	>16	1	4	PBP3 $V_{390}M$, PBP4 $D_{326}N$, PBP2a $S_{225}R$, PBP2a $E_{246}G$, PBP2a $G_{489}S$
18S02816-1	NL	551	551	V	>16	1	4	PBP3 $V_{390}M$, PBP4 $D_{326}N$, PBP2a $S_{225}R$, PBP2a $E_{246}G$, PBP2a $G_{489}S$
37028	DK	551	551	V	>16	1	2	PBP3 $V_{390}M$, PBP4 $D_{326}N$, PBP2a $S_{225}R$, PBP2a $E_{246}G$, PBP2a $G_{489}S$
37235	DK	551	551	V	>16	1	4	PBP3 $V_{390}M$, PBP4 $D_{326}N$, PBP2a $S_{225}R$, PBP2a $E_{246}G$, PBP2a $G_{489}S$
37711-3	DK	2190	551	V	>16	8	2	PBP3 $V_{390}M$, PBP2a $S_{225}R$, PBP2a $E_{246}G$, PBP2a $G_{489}S$
41484	DK	1338	551	V	>16	0.125	2	PBP3 $V_{390}M$, PBP4 $D_{326}N$, PBP2a $S_{225}R$, PBP2a $E_{246}G$, PBP2a $G_{489}S$
41693	DK	1338	551	V	>16	0.125	2	PBP3 $V_{390}M$, PBP4 $D_{326}N$, PBP2a $S_{225}R$, PBP2a $E_{246}G$, PBP2a $G_{489}S$
42654	DK	1338	551	V	>16	0.125	2	PBP3 $V_{390}M$, PBP4 $D_{326}N$, PBP2a $S_{225}R$, PBP2a $E_{246}G$, PBP2a $G_{489}S$
46507	DK	551	551	V	>16	>16	8	PBP3 $V_{390}M$, PBP4 $D_{326}N$, PBP2a $S_{225}R$, PBP2a $E_{246}G$, PBP2a $G_{489}S$
46726	DK	551	551	V	>16	0.5	2	PBP3 $V_{390}M$, PBP4 $D_{326}N$, PBP2a $S_{225}R$, PBP2a $E_{246}G$, PBP2a $G_{489}S$
28522	DK	2183	Singleton	V	>16	1	>16	PBP3 $V_{390}M$, PBP4 $D_{326}N$, PBP2a $S_{225}R$, PBP2a $E_{246}G$
18S02748-1	NL	341	Singleton	V	4	0.25	0.5	PBP2a $S_{225}R$, PBP2a $E_{246}G$
41258	DK	2191	Singleton	V	>16	2	2	PBP3 $V_{390}M$, PBP4 $D_{326}N$, PBP2a $S_{225}R$, PBP2a $E_{246}G$
45498	DK	1563	Singleton	V	4	1	0.5	PBP3 $V_{390}M$, PBP2a $S_{225}R$, PBP2a $E_{246}G$

DK, Denmark; NL, the Netherlands; CA, Canada; AU, Australia; AMC, amoxicillin/clavulanate; LEX, cefalexin; OXA, oxacillin.

responsible for MIC variability. The study reveals a PBP3 point mutation ($V_{390}M$) statistically associated with higher oxacillin MICs by multivariate analysis, as well as five additional mutations in native PBP2, PBP4 or acquired PBP2a that deserve further investigation, as they were significantly associated with high oxacillin and cefalexin MICs at univariate level. The high oxacillin MICs in CC551 and the variability of oxacillin and cefalexin MICs in CC45 were largely attributable to the presence of these PBP mutations (Figure 3). However, 5 of the 28 high-level MRSP (17.8%) contained WT PBPs and 26 of the 89 low-level MRSP (29.2%) harboured PBP mutations, indicating that oxacillin MICs are also influenced by other factors that are not linked to PBPs. Cefalexin and amoxicillin/clavulanate MICs in CC258 were additionally influenced by the presence of one or both plasmid- and chromosomally encoded *blaZ*. Collectively, these results indicate that β-lactam MIC variability in MRSP is influenced by

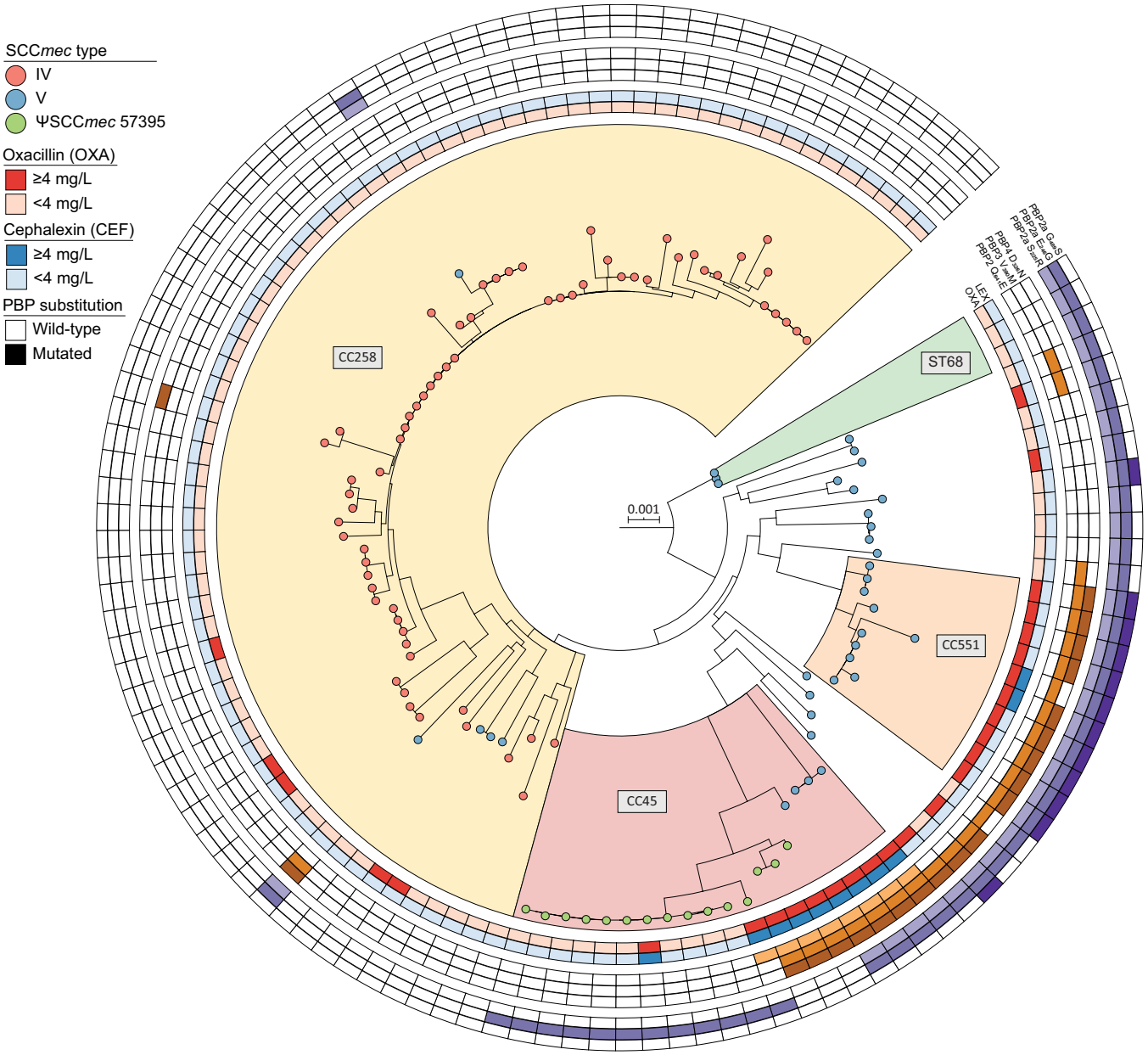

Figure 3. Core-genome phylogeny based on 117 MRSP isolates. The tree was arbitrary rooted on CC68 isolates. The scale bar indicates the expected number of substitutions per site. Coloured filled circles correspond to SCC*mec* types. Inner circles indicate oxacillin and cefalexin MIC above the epidemiological cut-off (ECOFF) values for *S. aureus* and *S. pseudintermedius*, respectively. Middle and outer circles indicate the presence or absence of substitution in native PBPs and acquired PBP2a, respectively. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

additive effects of mutations in multiple PBP target sites, *blaZ*-mediated enzymatic drug inactivation and other unknown mechanisms not mediated by *mecA*.

Although we did not study the altered functions of the mutated PBPs, we hypothesize that these mutations may lower their binding affinity for different β-lactams and thereby increase transpeptidase protein activity (gain of function). A recent single study on PBP-binding affinities in *S. pseudintermedius* showed that cefalexin had high affinity for PBP2 in a methicillin-susceptible strain,[16] whereas neither cefalexin nor oxacillin had selective affinity for specific PBPs in an MRSP strain. Conversely, studies on *S. aureus* (MSSA) suggest a high affinity of cefalexin for PBP3 and of oxacillin for PBP2 and PBP3.[17] It is difficult to study the effects of the newly identified aa substitutions on affinity to different β-lactams in MRSP, since no crystal structures of PBPs are available for *S. pseudintermedius*. However, numerous studies have investigated the effects of aa substitutions in native PBP and PBP2a of MRSA.[18–23] The PBP2 of *S. aureus* (727 aa) is composed of a transglycosylase (positions 82–257) and transpeptidase domain (positions 361–631) and shares 71.2% of

Table 3. Clinical cases of MRSP-infected dogs treated with a β-lactam antibiotic

Case number	Breed	Age	ST (CC)	SCC*mec*	PBP substitutions	OXA	LEX	AMC	Type of infection	Systemic treatment	Adjunctive treatment	Clinical response	Comorbidities
1	Bullterrier	2y	ST265 (CC258)	IV	WT	1	0.25 (S)	0.5 (I)	Superficial pyoderma	AMC 14.2 mg/kg twice as day for 50 days	None	Clinical resolution	Demodicosis, atopic dermatitis
2	Dogue de Bordeaux	9y	ST2186 (CC258)	IV	WT	0.5	0.125 (S)	2 (R)	Folliculitis (superficial pyoderma)	LEX 25 mg/kg for 2 weeks (only 18 mg/kg for the first 10 days)	Chlorhexidine shampoo, 4%	Clinical resolution	None reported
3 (First strain - neck)	Pug	8y	ST2328 (singleton)	V	$S_{225}R$ and $E_{246}G$ in PBP2a	0.5	0.25 (S)	0.5 (I)	Folliculitis, (superficial pyoderma) and furunculosis (deep pyoderma)	Cefadroxil 26 mg/kg twice a day for 16 days	Chlorhexidine shampoo, 3% was used as a sole treatment 2 weeks prior to systemic antimicrobial treatment without clinical response	Clinical resolution	Atopic dermatitis, adverse food reaction
3 (Second strain - groin)[a]	Pug	8y	ST924 (singleton)	V	$V_{390}M$ in PBP3, $D_{326}N$ PBP4, and $S_{225}R$, $E_{246}G$ and $G_{489}S$ in PBP2a	1	0.5 (S)	4 (R)					
4	Dogue de Bordeaux	7m	ST258 (CC258)	IV	WT	0.5	0.5 (S)	4 (R)	Deep pyoderma	Cefadroxil 15 mg/kg twice a day for 14 days followed by doxycycline 10 mg/kg once a day on/off for 6 months	None	No clinical resolution with β-lactam. A partial clinical response after 6 months of doxycycline. Euthanasia due to comorbidity.	Generalized demodicosis
5	Doberman	1y	ST1338 (CC551)	V	$V_{390}M$ in PBP3, $D_{326}N$ PBP4, and $S_{225}R$, $E_{246}G$ and $G_{489}S$ in PBP2a	>16	0.125 (S)	2 (R)	Cut wound infection	AMC 13.5 mg/kg twice a day for 10 days	Chlorhexidine 0.05% and manuka honey	No clinical resolution upon systemic antimicrobial treatment. Clinical cure after 3 weeks of secondary healing and topical treatment.	None reported
6	Great Dane	11m	ST71 (CC71)	II-III	$D_{326}N$ in PBP4 and $E_{246}G$ in PBP2a	>16	>16 (R)	16 (R)	Ulcerative deep pyoderma	LEX 24.8 mg/kg twice a day for 21 days	Chlorhexidine shampoo, 3% started 10 days into cefalexin therapy	Clinical resolution	Necrotizing stomatogingivitis, lymphadenitis
7	Mixed breed	6.75y	ST71 (CC71)	II-III	$D_{326}N$ in PBP4 and $E_{246}G$ in PBP2a	>16	8 (R)	16 (R)	Post surgical wound infection	AMC 13.5 mg/kg twice a day for 8 days	Wet-to-dry bandage	Clinical resolution	Mast cell tumour leg
8	Saint Bernard	5.92y	ST2035 (singleton)	II-III	$V_{390}M$ in PBP3	2	1 (S)	0.5 (S)[b]	Cystitis	AMC 17.6 mg/kg twice a day for 5 days	None	Clinical resolution of MRSP, but followed by *E. coli* cystitis. Euthanasia due to comorbidities.	Pyelonephritis, hypoadrenocorticism, chronic kidney disease, melena

Continued

Table 3. *Continued*

Case number	Breed	Age	ST (CC)	SCC*mec*	PBP substitutions	OXA	LEX	AMC	Type of infection	Systemic treatment	Adjunctive treatment	Clinical response	Comorbidities
9	Coton de Tulear	7m	ST258 (CC258)	IV	WT	0.5	0.25 (S)	2 (R)	Juvenile cellulitis (deep pyoderma)	AMC 13.6 mg/kg twice a day for 28 days	Fusidic acid and wipes containing 0.05% Ophytrium and chlorhexidine shampoo, 3%. Prednisolone 0.6 mg/kg twice a day 30 days followed by tapering over 2 weeks.	Clinical resolution	None reported
10	Swiss Shepherd Dog	8y	ST1403 (CC258)	IV	$D_{326}N$ in PBP4	1	0.5 (S)	8 (R)	Superficial pyoderma	Cefadroxil 13 mg/kg twice a day for 14 days.	0.033% hypochloric acid spray and wipes containing 0.05% Ophytrium and 3% chlorhexidine. Cyclosporine 5 mg/kg once a day.	Clinical resolution	Perianal fistulas
11	Great Dane	1y	ST71 (CC71)	II-III	$D_{326}N$ in PBP4, and $E_{246}G$ PBP2a	>16	>16 (R)	16 (R)	Cut wound infection	AMC 14.3 mg/kg twice a day for 7 days	None	No clinical resolution on antimicrobial treatment. Clinical cure after surgical wound debridement.	Wound necrosis

y, years; m, months; S, susceptible; I, intermediate; R, resistant; AMC, amoxicillin/clavulanate; LEX, cefalexin.
[a]This strain presented an SCV phenotype and MICs of β-lactams were determined after 48 h of incubation.
[b]This MIC value was categorized according to the CLSI breakpoint for urinary tract infection (R≥16 mg/L).

homology with the PBP2 of *S. pseudintermedius* (740 aa). Based on the position of the equivalent aa in *S. aureus* (Q645), the $Q_{641}E$ substitution in PBP2 of *S. pseudintermedius* is predicted to fall outside the transpeptidase domain. Conversely, the PBP3 $V_{390}M$ and PBP4 $D_{326}N$ substitutions in *S. pseudintermedius* are predicted to fall inside the transpeptidase and DUF1958 domains, respectively, based on modelling of PBP3 (positions 351–656, 73.3% homology) and PBP4 (position 317–382, 60.0% homology) in *S. aureus*.

PBP2a mutations increasing β-lactam resistance levels in MRSA have been previously reported in the allosteric regulatory site (residues 27–326) or in the penicillin-binding pocket (i.e. active site) of the transpeptidase region (residues 327–668) of this protein.[18,19,24,25] We identified three aa substitutions in PBP2a ($S_{225}R$, $E_{246}G$ and $G_{489}S$) that were associated with increased oxacillin MICs in MRSP, and one of them ($E_{246}G$) was additionally associated with cefalexin resistance. Mutations in the allosteric site $S_{225}R$ and $E_{246}G$ were previously identified in MRSA resistant to ceftobiprole and ceftaroline without interfering with susceptibility to these to fifth-generation cephalosporins.[19,24,25] The $E_{246}G$ mutation has also been associated with growth advantage in the presence of penicillin in *S. aureus*, which is impaired in the presence of clavulanate as a probable case of collateral sensitivity.[21] To the best of our knowledge, the $G_{489}S$ substitution in the transpeptidase domain has not been reported in MRSA.

It has previously been argued that the MRSA expert rule, which defines all oxacillin-resistant strains as resistant to all β-lactams, was applied to MRSP without sufficient microbiological and clinical evidence, and that β-lactam resistance might be overreported in MRSP because the oxacillin breakpoint assigned to this species on the basis of MIC distribution ($R \geq 0.5$ mg/L) is much lower than for MRSA (≥4 mg/L).[26,27] Indeed, two previous studies showed that *in vitro* cross-resistance to cephalosporins (i.e. cefazolin, cefoxitin and cefalotin) was inconsistent in strains with oxacillin MIC < 4 mg/L.[6,27] The present study provides further microbiological evidence that this lack of cross-resistance in low-level MRSP is consistent for cefalexin regardless of strain genotype, and that low-level MRSP are rapidly killed by cefalexin concentrations approximating the strain's MIC. The cefalexin-susceptible phenotype was observed in all strains belonging to the emerging lineage CC258/SCC*mec* IV as well as in other widespread MRSP lineages such as CC45, CC551 and ST68.[3]

Our *in vitro* results were confirmed by a retrospective clinical study showing that 8 of 11 MRSP patients, including 7 of the 8 dogs infected with low-level MRSP, were successfully treated with β-lactam treatment with or without adjunctive topical antiseptic treatment. The only case of low-level MRSP infection not responding to β-lactam treatment (case 4, Table 3), was a case of deep pyoderma with concurrent generalized demodicosis, which likely contributed to treatment failure due the underlying immunodeficiencies associated with this parasitic disease.[28] For at least two dogs, clinical resolution could be attributed to systemic β-lactam therapy alone since they did not receive concurrent topical treatment (cases 1 and 8). Three additional dogs responding to β-lactam therapy (cases 3, 6 and 9) were affected by deep pyoderma, for which topical treatment alone is generally regarded as inadequate.[29,30] Clinical cure was even achieved in two patients infected with high-level MRSP ST71 strains that were resistant to the β-lactam antibiotic chosen for treatment *in vitro* (cefalexin and amoxicillin/clavulanate in cases 6 and 7, respectively). For these patients, cure may have been enhanced by adjunctive treatment, especially in case 7 (Table 3), where surgical site infection was managed locally using a wet-to-dry bandage, which does not in itself contain an antimicrobial component, but leads to mechanical debridement due to moistened gauze left to dry on the wound.

Of note, two genetically distinct MRSP strains were isolated from different body sites in the same patient (case 3). It has been previously shown that multiple *S. pseudintermedius* strains with distinct antimicrobial resistance profiles may occur in the same lesion or in different lesions from the same dog.[31] However, in this case both strains were susceptible to the antibiotic used for therapy (cefadroxil). One of the two strains showed an SCV phenotype but it did not carry any of the mutations in genes previously associated with the SCV in *S. aureus*, i.e. *hemB*, *menD* and *thyA*.[32] Thus, we hypothesize that this phenotype could be attributed to transcriptomic changes.[33]

Despite its merits, the study has some evident limitations. First of all, the sample size of the retrospective clinical study was relatively small, which reflects the challenge of recruiting cases of MRSP-infected dogs treated with β-lactams, since β-lactam treatment of MRSP patients is currently considered unethical based on the MRSP expert rule. Thus, we could only rely on cases of dogs that had been treated empirically with β-lactams prior to receiving the laboratory diagnostic report indicating MRSP isolation. Secondly, the clinical study was affected by several confounders that could influence treatment outcome, including concomitant topical treatment with antiseptics in five out of the eight cases resulting in clinical cure, and the lack of a predetermined definition of clinical resolution, which could vary between MRSP cases due to subjectivity of the clinician's assessment.

In conclusion, this study provides new knowledge about the relationships between PBP mutations in MRSP and susceptibility to the most frequently used β-lactam antibiotics in small animal veterinary practice. Despite the limitations of our retrospective clinical study, cefalexin and amoxicillin/clavulanate appear to be viable therapeutic options for managing MRSP infections that require systemic treatment and are caused by low-level MRSP strains displaying low oxacillin MIC or susceptibility to the β-lactam of choice according to clinical breakpoint. Effective management of these infections with β-lactams would reduce the use of critically important antimicrobials such as fluoroquinolones, which often are the only approved veterinary drugs to which MRSP strains are susceptible,[3] as well as antimicrobials that are not licensed for use in animals, including older antibiotics with relatively high toxicity (e.g. chloramphenicol, rifampicin and aminoglycosides) or newer drugs (e.g. linezolid, vancomycin and fosfomycin) that should be reserved for human use according to the new EU legislation.[4] In light of these important animal and public health implications, we conclude that the current MRSP expert rule should be reassessed by the competent international committees for susceptibility testing of animal pathogens using large clinical studies integrated with strain phenotypic and genotypic data. Ideally, such studies should be performed prospectively to gather detailed, accurate and extended information on treatment outcome.

Acknowledgements

We would like to acknowledge Lene Boysen (Dyrlæge Boysen), Anders Jensen (Anicura Københavns Dyrehospital) and Lise Hedegaard (Bygholm Dyrehospital) for providing clinical data on canine MRSP cases.

Funding

The work was funded by Agria and SKK Research Fund for Companion Animals (project number N2019-0021).

Transparency declarations

All authors have no conflict of interest to declare.

Supplementary data

Dataset S1 and Tables S1 and S2 are available as Supplementary data at *JAC* Online.

References

1 Bannoehr J, Guardabassi L. *Staphylococcus pseudintermedius* in the dog: taxonomy, diagnostics, ecology, epidemiology and pathogenicity. *Vet Dermatol* 2012; **23**: 253–66, e51–2. https://doi.org/10.1111/j.1365-3164.2012.01046.x

2 Loeffler A, Linek M, Moodley A *et al*. First report of multiresistant, *mecA*-positive *Staphylococcus intermedius* in Europe: 12 cases from a veterinary dermatology referral clinic in Germany. *Vet Dermatol* 2007; **18**: 412–21. https://doi.org/10.1111/j.1365-3164.2007.00635.x

3 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. https://doi.org/10.3389/fmicb.2016.01599

4 EUR-Lex. Commission Implementing Regulation (EU) 2022/1255 of 19 July 2022 designating antimicrobials or groups of antimicrobials reserved for treatment of certain infections in humans, in accordance with Regulation (EU) 2019/6 of the European Parliament and of the Council (Text with EEA relevance). 2022. https://eur-lex.europa.eu/eli/reg_impl/2022/1255/oj.

5 Leclercq R, Cantón R, Brown DFJ *et al*. EUCAST expert rules in antimicrobial susceptibility testing. *Clin Microbiol Infect* 2013; **19**: 141–60. https://doi.org/10.1111/j.1469-0691.2011.03703.x

6 Wegener A, Damborg P, Guardabassi L *et al*. 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*. *J Antimicrob Chemother* 2020; **75**: 508–11. https://doi.org/10.1093/jac/dkz509

7 Petit RAP, Read TD. *Staphylococcus aureus* viewed from the perspective of 40,000+ genomes. *PeerJ* 2018; **6**: e5261. https://doi.org/10.7717/peerj.5261

8 CLSI. *Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals—5th Edition: VET01S*. 2020.

9 Prjibelski A, Antipov D, Meleshko D *et al*. Using SPAdes de novo assembler. *Curr Protoc Bioinformatics* 2020; **70**: e102. https://doi.org/10.1002/cpbi.102

10 Gurevich A, Saveliev V, Vyahhi N *et al*. QUAST: quality assessment tool for genome assemblies. *Bioinformatics* 2013; **29**: 1072–5. https://doi.org/10.1093/bioinformatics/btt086

11 Nascimento M, Sousa A, Ramirez M *et al*. PHYLOViZ 2.0: providing scalable data integration and visualization for multiple phylogenetic inference methods. *Bioinformatics* 2017; **33**: 128–9. https://doi.org/10.1093/bioinformatics/btw582

12 Seemann T. Prokka: rapid prokaryotic genome annotation. *Bioinformatics* 2014; **30**: 2068–9. https://doi.org/10.1093/bioinformatics/btu153

13 Page AJ, Cummins CA, Hunt M *et al*. Roary: rapid large-scale prokaryote pan genome analysis. *Bioinformatics* 2015; **31**: 3691–3. https://doi.org/10.1093/bioinformatics/btv421

14 Nguyen L-T, Schmidt HA, von Haeseler A *et al*. IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. *Mol Biol Evol* 2015; **32**: 268–74. https://doi.org/10.1093/molbev/msu300

15 Letunic I, Bork P. Interactive tree of life (iTOL) v4: recent updates and new developments. *Nucleic Acids Res* 2019; **47**: W256–9. https://doi.org/10.1093/nar/gkz239

16 Gagetti P, Rosato RR, Rosato AE. *Staphylococcus pseudintermedius*'s PBP4 is directly associated with the dissociated oxacillin and cefoxitin phenotype. *Antibiotics (Basel)* 2021; **10**: 1299. https://doi.org/10.3390/antibiotics10111299

17 Ferrer-González E, Kaul M, Parhi AK *et al*. β-Lactam antibiotics with a high affinity for PBP2 act synergistically with the FtsZ-targeting agent TXA707 against methicillin-resistant *Staphylococcus aureus*. *Antimicrob Agents Chemother* 2017; **61**: e00863-17. https://doi.org/10.1128/AAC.00863-17

18 Long SW, Olsen RJ, Mehta SC *et al*. PBP2a mutations causing high-level ceftaroline resistance in clinical methicillin-resistant *Staphylococcus aureus* isolates. *Antimicrob Agents Chemother* 2014; **58**: 6668–74. https://doi.org/10.1128/AAC.03622-14

19 Alm RA, McLaughlin RE, Kos VN *et al*. Analysis of *Staphylococcus aureus* clinical isolates with reduced susceptibility to ceftaroline: an epidemiological and structural perspective. *J Antimicrob Chemother* 2014; **69**: 2065–75. https://doi.org/10.1093/jac/dku114

20 Chan LC, Gilbert A, Basuino L *et al*. PBP4 mediates high-level resistance to new-generation cephalosporins in *Staphylococcus aureus*. *Antimicrob Agents Chemother* 2016; **60**: 3934–41. https://doi.org/10.1128/AAC.00358-16

21 Harrison EM, Ba X, Coll F *et al*. Genomic identification of cryptic susceptibility to penicillins and β-lactamase inhibitors in methicillin-resistant *Staphylococcus aureus*. *Nat Microbiol* 2019; **4**: 1680–91. https://doi.org/10.1038/s41564-019-0471-0

22 Fergestad ME, Stamsås GA, Morales Angeles D *et al*. Penicillin-binding protein PBP2a provides variable levels of protection toward different β-lactams in *Staphylococcus aureus* RN4220. *Microbiol Open* 2020; **9**: e1057. https://doi.org/10.1002/mbo3.1057

23 Ali T, Basit A, Karim AM *et al*. Mutation-based antibiotic resistance mechanism in methicillin-resistant *Staphylococcus aureus* clinical isolates. *Pharmaceuticals* 2021; **14**: 420. https://doi.org/10.3390/ph14050420

24 Mendes RE, Tsakris A, Sader HS *et al*. Characterization of methicillin-resistant *Staphylococcus aureus* displaying increased MICs of ceftaroline. *J Antimicrob Chemother* 2012; **67**: 1321–4. https://doi.org/10.1093/jac/dks069

25 Schaumburg F, Peters G, Alabi A *et al*. Missense mutations of PBP2a are associated with reduced susceptibility to ceftaroline and ceftobiprole in African MRSA. *J Antimicrob Chemother* 2016; **71**: 41–4. https://doi.org/10.1093/jac/dkv325

26 Guardabassi L, Damborg P, Stamm I *et al*. Diagnostic microbiology in veterinary dermatology: present and future. *Vet Dermatol* 2017; **28**: 146–e30. https://doi.org/10.1111/vde.12414

27 Adiguzel MC, Schaefer K, Rodriguez T *et al*. Prevalence, mechanism, genetic diversity, and cross-resistance patterns of methicillin-resistant *Staphylococcus* isolated from companion animal clinical samples submitted to a veterinary diagnostic laboratory in the midwestern United States. *Antibiotics (Basel)* 2022; **11**: 609. https://doi.org/10.3390/antibiotics11050609

28 Ferrer L, Ravera I, Silbermayr K. Immunology and pathogenesis of canine demodicosis. *Vet Dermatol* 2014; **25**: 427–e65. https://doi.org/10.1111/vde.12136

29 Summers JF, Brodbelt DC, Forsythe PJ *et al*. The effectiveness of systemic antimicrobial treatment in canine superficial and deep pyoderma: a systemic review. *Vet Dermatol* 2012; **23**: 305–29. https://doi.org/10.1111/j.1365-3164.2012.01050.x

30 Beco L, Guaguère E, Lorente Méndez C *et al*. Suggested guidelines for using systemic antimicrobials in bacterial skin infections: part 2 — antimicrobial choice, treatment regimens and compliance. *Vet Rec* 2013; **172**: 156–60. https://doi.org/10.1136/vr.101070

31 Pinchbeck LR, Cole LK, Hillier A *et al*. Pulsed-field gel electrophoresis patterns and antimicrobial susceptibility phenotypes for coagulase-positive staphylococcal isolates from pustules and carriage sites in dogs with superficial bacterial folliculitis. *Am J Vet Res* 2007; **68**: 535–42. https://doi.org/10.2460/ajvr.68.5.535

32 Kriegeskorte A, Grubmüller S, Huber C *et al*. *Staphylococcus aureus* small colony variants show common metabolic features in central metabolism irrespective of the underlying auxotrophism. *Front Cell Infect Microbiol* 2014; **4**: 141. https://doi.org/10.3389/fcimb.2014.00141

33 Loss G, Simões PM, Valour F *et al*. *Staphylococcus aureus* small colony variants (SCVs): news from a chronic prosthetic joint infection. *Front Cell Infect Microbiol* 2019; **9**: 363. https://doi.org/10.3389/fcimb.2019.00363