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Comparative genomics of phenotypic antimicrobial resistances in methicillin-resistant *Staphylococcus pseudintermedius* of canine origin

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ABSTRACT

Staphylococcus pseudintermedius is an important pathogen in dogs. Since 2004, methicillin- resistant *S. pseudintermedius* (MRSP) isolates, often multidrug resistant, have been observed in dogs in the Netherlands. This study aims to link the observed resistance phenotypes in canine MRSP to genotypic antimicrobial resistance markers, and to study the phylogeny of MRSP by genomic comparisons.

The genomes of fifty clinical isolates of MRSP from dogs from the Netherlands were sequenced. The resistance genes were identified, and for twenty one different antimicrobials their presence and sequence were associated with the resistance phenotypes. In case of observed discrepancies, the genes were aligned with reference genes. Of the phenotypic resistances, 98.3% could be explained by the presence of an associated resistance gene or point mutation. Discrepancies were mainly resistance genes present in susceptible isolates; 43.8% (7/16) were explained by an insertion, deletion or mutation in the gene. In relation with the resistance gene presence or absence, a single-nucleotide polymorphism (SNP) based phylogeny was constructed to define the population dynamics. The resistance gene content differed according to clonal complex, from very conserved (CC45), to partly conserved (CC71) to highly diverse (CC258) resistance gene patterns.

In conclusion, this study shows that the antimicrobial genotype from whole genome sequencing is highly predictive of the resistance phenotype in MRSP. Interestingly, the observed clonal complexes of MRSP isolates were linked with resistance gene patterns

1. Introduction

Staphylococcus pseudintermedius is a major pathogen in dogs and can occasionally be found in human infection. In the last ten years, methicillin-resistant *S. pseudintermedius* (MRSP) has emerged globally, and often shows resistance to more than three classes of antimicrobials (multidrug resistant (MDR)) (Weese and van Duijkeren, 2010; Bannoehr and Guardabassi, 2012).

Resistance in MRSP covers practically all classes of antimicrobials, mainly mediated by resistance genes carried by mobile genetic elements (MGE) (Kadlec and Schwarz, 2012). Since MGEs can carry multiple resistances, MDR MRSP is thought to evolve rapidly (McCarthy et al., 2014). Only some resistances can be linked to point mutations in chromosomal genes (Descloux et al., 2008; Kadlec et al., 2011).

Multilocus sequence typing (MLST) has shown a clonal population of MRSP, and clonal complexes (CC) have been linked with different antimicrobial resistance patterns (Dos Santos et al., 2016; Duim et al., 2016). For example CC71 has been identified as the dominant clone in Europe associated with resistances to most antimicrobial classes (Perreten et al., 2010). Recently, new emerging clones showing resistances to fewer classes of antimicrobials have been reported in Finland (Grönthal et al., 2017), Denmark (Damborg et al., 2016) and the Netherlands (Duim et al., 2016).

Clonal distribution and antimicrobial resistance genes have been studied in MRSP using multi locus sequence typing (MLST), *spa* typing in combination with PCRs or microarray for specific resistance genes, but very few studies use whole genome sequencing. Whole genome sequencing (WGS) is becoming increasingly cheaper and accessible, which makes it easier to infer antimicrobial resistance from sequence data. Different online tools are available for the analysis of resistance genes in sequence data. However, most tools focus on the detection and identification of horizontally acquired resistance, in Gram-negatives

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and *Staphylococcus aureus*. (McArthur et al., 2013; Thomsen et al., 2016).

This study aims to compare genomic antimicrobial resistances with observed resistance phenotypes in canine MRSP isolates from the Netherlands. Whole genome sequencing was used on 50 MRSP isolates, resistance genes were detected using Resfinder and their presence was compared with the resistance phenotype determined by minimum inhibitory concentrations (MIC). The phylogenetic relationship of the isolates was determined and compared with observed resistance profiles.

2. Material and methods

2.1. Bacterial isolates

Fifty MRSP isolates were selected from a set of 478 MRSP isolates that had been characterized in a previous study (Duim et al., 2016). The fifty isolates were selected based on their distinctive antimicrobial resistance pattern, sequence type and year of isolation. The characteristics of the isolates are shown in Supplementary Table 1.

2.2. Antimicrobial susceptibility testing

Minimum inhibitory concentrations (MIC) were determined for a selection of twenty one antimicrobials (Table 1) by broth microdilution using the commercially available automated MICRONAUT system using the MRSA/IFSG GP 4 panel (MRSA panel), and a custom-made panel used for routine diagnostics at the Veterinary Microbiological Diagnostic Center (VMDC) of Utrecht University (VMDC panel) (MERLIN Diagnostika GmbH, Germany) (See Supplementary Table 2). Seven antimicrobials (oxacillin, clindamycin, erythromycin, fusidic acid, gentamicin, rifampicin, trimethoprim/sulfamethoxazole) were present in both panels. In cases of measurement discrepancy the highest MIC was used.

Susceptibility testing was performed as recommended by the manufacturer for inoculum preparation, broth composition and incubation conditions. *S. aureus* ATCC 29213 was used as quality control strain. Reading of the results was done automatically using a photometer (MERLIN Diagnostika GmbH, Germany). Veterinary breakpoints were used according to the Clinical and Laboratory Standards Institute (CLSI) standards (CLSI, 2015), when available. When veterinary breakpoints were missing for *S. pseudintermedius* in combination with an antimicrobial, breakpoints for other Staphylococci or human breakpoints were used (CLSI, 2017; EUCAST, 2017, 2018) (Table 1). Intermediate results were classified as resistant.

2.3. Whole genome sequencing

Genomic DNA for sequencing was isolated using the molecular biology kit from ZYMO Research (Irvine, CA, USA). The genomes were sequenced by BaseClear (Leiden, The Netherlands) using a Nextera XT library with 150 bp read length, and HiSeq 2500 sequencing generating sequencing reads with an average coverage of 128x (Illumina, San Diego, USA). Sequence reads were assembled into contigs using Spades v3.1.1. (Bankevich et al., 2012). Genome sequences have been deposited in GenBank under accession numbers listed in Supplementary Table 1.

2.4. Detection of resistance genes

For detection of resistance genes in WGS data, a web-based database was used: Resfinder (version 2.1) (www.genomicepidemiology.org). For Resfinder the Batch upload pipeline (Thomsen et al., 2016) was used on all isolates with the default settings. The results of Resfinder and the phenotypic resistances were then compared. When discrepancies were observed, sequences were aligned to reference genes using custom BLAST function in Geneious v.s 9.02 (Biomatters, New Zealand) with a query centric alignment. Included reference genes of identified resistance genes from *Staphylococcus aureus* were: *ermB*, conferring resistance against macrolides and lincosamides (AB699882.1), *aac-aphD,* conferring aminoglycosides resistance (FN433596.1), *dfrG* conferring folate pathways inhibitors resistance (FN433596), *tet*(M) (CP002643), *tet*(K) (FN433596) both conferring tetracycline resistance, and *gyrA* and *grlA* (BX571857.1) conferring fluoroquinolones resistance. The sequences of the *cat*pC221, conferring resistance against chloramphenicol and *aph(3')-III* genes conferring kanamycin resistance were obtained from the Resfinder database (X02529 and M26832). When no resistance gene was detected in

Table 1
Antimicrobial ranges and breakpoints.

Antimicrobial class	Antimicrobial	Range tested	Breakpoint[a]	Document used for breakpoints
B-lactams	oxacillin	0.125-16	> = 0.5	VET01S3 (human)
	ceftarolin	0.25-2	> = 2	CLSI M100 S27 (human)
phenicols	chloramphenicol	4-32	> = 16	VET01S3 (human)
lincosamides	clindamycin	0.5-4	> = 1	VET01S3 (dogs)
macrolides	erythromycin	0.25-8	> = 1	VET01S3 (human)
inducible clindamycin resistance	erythromycin/ clindamycin	4/0.5	> = 4/0.5	(Swenson et al., 2007)
aminoglycosides	gentamicin	0.5-16	> = 8	VET01S3 (human)
	kanamycin	16-64	> = 32	CLSI M100S27(human)
folate pathway inhibitors	trimethoprim/ sulfamethoxazol	0.03125/0.59375–4/76	> = 4/76	VET01S3 (human)
tetracyclines	tetracycline	0.5-16	> = 0.5	VET01S3 (dogs)
	tigecycline	0.125-1	> 0.5	EUCAST[a]
streptogramins	quinupristin/ dalfopristin	0.5-4	> = 2	CLSI M100S27(human)
lipopeptides	daptomycin	0.5-4	> 1	EUCAST[b]
fosfomycins	fosfomycin	8-64	> 32	EUCAST[b]
steroials (fusidanes)	fusidic acid	1-2	> 1	EUCAST[b]
oxazolidinones	linezolid	1-8	> = 8	CLSI M100 S27 (human)
pseudomonic acids	mupirocin	1; 256	> 256	EUCAST, 2018[c]
glycopeptides	teicoplanin	0.125; 16	> = 16	CLSI M100 S27 (human)
	vancomycin	0.25-32	> = 8	VET01S3 (human)
fluoroquinolones	enrofloxacin	0.25-4	> = 1	VET01S3 (dogs)
	moxifloxacin	0.25-2	> = 1	CLSI M100 S27 (human)
rifampicin	rifampicin	0.0625-4	> = 2	VET01S3 (human)

[a] Breakpoints are used for non susceptibility therefore when available the intermediate breakpoint is considered.
[b] EUCAST: The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. Version 7.0, 2017.
[c] EUCAST, 2018. Version 8.0, 2018.

Resfinder and the isolate was susceptible to the antimicrobial no further genomic analysis was done.

2.5. Comparative genomics and phylogenetic analysis

The fifty MRSP genomes were compared with the genomes of 30 *S. pseudintermedius* genomes, available in the NCBI GenBank and the SRA database in December 2017 (Supplementary Table 3). For all genomes gene presence/absence was determined using Roary v3.5.6 (Page et al., 2015). Following alignment with Parsnp from the harvest toolkit, a phylogenetic tree was constructed comparing the nucleotide polymorphisms (SNPs) in the core genomes (Treangen et al., 2014). Recombinant regions in the genomes were identified using Gubbins (Croucher et al., 2015). The genes presence and absence data as well as the phenotypical resistances and susceptibility data were visualized using iTOL (Letunic and Bork, 2016).

3. Results

3.1. Comparison phenotypic and genotypic antimicrobial resistance

All but one of the MRSP isolates (49/50) were phenotypically classified as multidrug resistant (resistant ≥3 antimicrobial classes). The detected phenotypic resistances as well as the identified resistance genes are shown in Supplementary Table 1. For all 50 isolates, phenotypic-genotypic comparisons were made for 21 antimicrobials from 16 antimicrobial classes: 1050 comparisons in total. In 1032 (98.3%) cases the phenotypic susceptibility result could be explained by the presence or absence of a corresponding resistance gene, or a resistance associated point mutation. In 18 (1.7%) cases the phenotype result could not be explained by these factors (Table 2). The agreement and discrepancies will be discussed by antimicrobial class.

3.1.1. Bèta-lactams

All isolates tested resistant to oxacillin, an indicator for methicillin resistance in *S. pseudintermedius* (Wu et al., 2016). In all isolates the corresponding *mecA* gene was present, as was, in 49 (98%) isolates the *blaZ* gene. All isolates were susceptible to ceftarolin, a fifth generation cephalosporin antibiotic, and no resistance genes were detected for ceftarolin.

Table 2
Identified resistance genes compared with phenotypic resistances.

Antimicrobial class	Resistance gene	Antimicrobial phenotype	
		R*	S
B-lactams	*mecA*	50	0
	blaZ	49	0
	no gene	0	0
lincosamides	*lnu(B)*	3	0
	ermB	33	3
	no gene	0	14
macrolides	*ermB*	34	2
	no gene	1	13
phenicols	*cat*(pC221)	20	1
	no gene	0	29
aminoglycosides	*aac*(6)-*aph*(2)	22	9
	aph(3')-III	40	1
	no gene	0	5
FPI	*dfrG*	44	2
	no gene	0	4
tetracycline	*tet(M)*	26	0
	tet(K)	12	1
	no gene	0	12
Fluoroquinolones	S-L *gyrA* 251	29	1
	S-I *grlA* 239	30	0
	no mutations	0	19
Rifampicin	*rpoB* mutation	1	0
	no mutations	0	49

FPI; Folate pathway inhibitors, *Intermediate is considered resistant, only antimicrobial classes for which a phenotype was detected are shown.

3.1.2. Phenicols

The *cat* (Pc221) gene was detected in the genomes of all chloramphenicol resistant isolates and in one susceptible isolate (209100702102-1). Alignment of the *cat* (Pc221)gene in this isolate to the *cat* (Pc221) gene from the ResFinder database showed mutations, but these were also observed in *cat* genes from chloramphenicol resistant isolates, leaving the discrepancy unexplained.

3.1.3. Lincosamides/macrolides

For one isolate (212112902001-1) inconsistent MIC results were found for erythromycin. In the MRSA panel the MIC was 4 µg/mL and in the VMDC panel the MIC was < = 0.25 µg/mL. Since the highest MIC was used it was classified as resistant. However, the absence of an *ermB* gene in this isolate, might be explained by a phenotypic misclassification.

For one isolate (211012802302-1), inducible clindamycin resistance was detected; erythromycin tested resistant, clindamycin tested susceptible and the combination erythromycin/clindamycin tested resistant (Swenson et al., 2007).

The *ermB* gene was found in all but one resistant isolates as well as in two susceptible isolates (208081905001-1 and 213010701401-1). Alignment of the *ermB* gene of the susceptible isolates showed an additional bp (T) at position 221 in isolate 208081905001-1 and a missing bp (T) at position 154 in isolate 213010701401-1. In both cases, this resulted in inactivation of the gene by a frame shift of downstream translated amino acids, explaining the susceptibility of the two isolates. In three isolates (208082101701-1; 213032704301-1; 213101701201-2) the lincosamides resistance gene *InuB* was found in addition to *ermB*.

3.1.4. Aminoglycosides

With the ResFinder database, the *aac(6')-aph(2'')* gene, which is denoted as *aac(6')-Ie-aph(2'')-Ia* in *S. pseudintermedius* (Kadlec and Schwarz, 2012) and confers resistance to gentamicin and kanamycin, was detected in all gentamicin resistant and nine gentamicin susceptible isolates including one which was also susceptible for kanamycin. Four out of nine susceptible isolates (209022503501-1; 209031201604-5; 209040302601-1; 212042703101-1) had a bp deletion between position 1072 and 1080 resulting in a frameshift shift leading to a change of all transcribed downstream amino acids. The difference between resistant and susceptible isolates harbouring the gene could not be explained for the remaining five isolates by this alignment. The gene *aph(3')-III* conferring resistance to kanamycin, was detected in all but three kanamycin resistant isolates (those three isolates carried the *aac (6')-aph(2'')* gene) as well as in one susceptible isolate (211012802302-1). When aligned to the *aph(3')*-III from the Resfinder database no mutation was revealed. Sequence analysis further showed that the aph (3')-III was located in a highly variable region with inserted phage sequences that may have altered the gene expression. The *ant(6)-la* gene conferring resistance to streptomycin was also detected, but the relevant antimicrobial was not tested in our panel.

3.1.5. Folate pathway inhibitors

The gene *dfrG* was detected in all trimethoprim/ sulfamethoxazole resistant isolates and in two susceptible isolates. When aligned with the *dfrG* reference gene of *S. aureus*, the alignment showed 100% similarity, but no upstream promotor sequence was detected.

3.1.6. Tetracyclines

The genes *tetM* and *tetK* were found together in one resistant isolate. The *tetM* gene was found in 26 out of 37 resistant isolates and the *tetK* gene in 12 out of 37 resistant isolates, as well as in 1 susceptible isolate (209032500801-3). In the alignment with the *tetK* gene from *S. aureus,*

the *tetK* gene of the susceptible isolate had two mutations: one bp at position 470 a transversion from C to T and the other one at position 471 a transversion from A to T, resulting in a change of amino acid from Ser to Phe. Whether this is a crucial change to explain susceptibility of the isolate is uncertain. All isolates were susceptible to tigecycline.

3.1.7. Fluoroquinolones

For fluoroquinolones enrofloxacin and moxifloxacin were tested. The phenotypic result were identical for both suggesting a common resistance mechanism. Fluoroquinolones resistance in *S. pseudintermedius* is mediated by mutations in the *gyrA* and *grlA* genes (Descloux et al., 2008). The bp mutation at position 251 results in a change in amino acid from Ser to Leu in *gyrA* as was observed in twenty nine out of 30 fluoroquinolones resistant isolates and in one susceptible isolate (213032704301-1), an unexplained discrepancy. A change in amino acid from Ser to Ile at position 239 in *grlA* was observed in all resistant isolates.

3.1.8. Rifampicin

For rifampicin, one isolate tested resistant. In this isolate, a mutation at position 1441 resulting in an amino acid change from His to Pro in the *rpoB* gene, could explain the phenotypic resistance (Kadlec et al., 2011).

3.1.9. Remaining antimicrobials

All isolates were susceptible to daptomycin, fosfomycin, fusidic acid, linezolid, teicoplanin, quinupristin/dalfopristin, and vancomycin and no resistance genes were detected for these antimicrobials.

In summary, 18 discrepancies were detected in this study: one point mutation leading to resistance in a susceptible isolate, one resistant isolate without a gene which could be explained by a misclassification, and 16 genes present in susceptible isolates from which 7 could be explained by the alteration of the corresponding gene. In total, 10 discrepancies were left unexplained.

3.2. Whole genome phylogeny

The MRSP isolates consisted of 24 different sequence types. Whole genome analysis showed clustering in clonal complexes similar to that observed with MLST, consisting of disseminated isolates in the Netherlands, belonging to CC258, CC45 and CC71 (Fig. 1). The phylogeny showed clustering of isolates with a low number of resistances at the top of the tree, followed by isolates belonging to CC45 and CC71 that are highly resistant (Fig. 1). CC45 was very conserved with a pattern of *aac(6′)-Ie-aph(2″)-Ia, ant6-la, aphIII, ermB, dfrG, tet*M, *catPc221, blaZ, and mecA,* only one isolate lacked the *tetM* gene and another one lacked the *dfrG* gene. In CC71 15 different antimicrobial resistance patterns were found. CC71 also showed a total absence of *tetM* and a high prevalence (51.7%) of *tetK*. CC258 showed less resistant patterns, with a lot of variability, mainly for the macrolides/lincosamides and chloramphenicol resistance genes. One isolate also carried the *tet*K gene which was only found in this isolate and in CC71. At the top of the tree there are diverse sequence types that contain fewer resistance genes, and belong to MRSP as well as MSSP. A majority of the discrepancies between antimicrobial genotype and phenotype (14/18) were detected in CC71 isolates.

4. Discussion

This is the first study that explores the degree to which the phenotypic resistances in MRSP can be inferred from genomic data. Discrepancies between inferred and observed resistance were observed mainly in susceptible isolates harbouring a resistance gene or point mutation linked to resistance. In 7 out of 16 cases, a gene was present in a susceptible isolate, however the gene was disrupted. In 9 cases a gene was present with no mutation in its sequence, but the isolate was still susceptible. The majority of genotype/phenotype discrepancies were detected in CC71 isolates. Discrepancies linked to the gene aac(6')-aph (2''), leading to aminoglycosides resistance, being present in susceptible isolates were detected most often. Further analysis, beyond the scope of this study, is needed to determine whether this could be due to a difference in regulation or expression, and it would be useful to analyse the promotor sequences of genes showing susceptibility despite an intact resistance gene.

The isolates examined here were selected from a previous study, with the objective of including maximum diversity of phenotypical resistance and sequence types. The fact that veterinary breakpoints and specific breakpoints for *S. pseudintermedius* are often lacking in breakpoint determination documents (CLSI, 2015; EUCAST, 2017, 2018; CLSI, 2017) might lead to an uncertainty in classification for some of the isolates. For example, in this study one isolate (212112902001-1) was considered resistant to erythromycin although its MIC was just above the breakpoint for the susceptible/ intermediate breakpoint in the MRSA panel and below it in the VMDC panel. It is possible, since the isolate showed no resistance gene, that it was misclassified.

Although the panels tested comprise a diverse number of antimicrobials not all the potentially relevant antimicrobials could be tested. As an example additional testing of amikacin or doxycycline could have brought more insight into the genotype phenotype correlation for aminoglycosides or tetracyclines respectively.

Resfinder, already evaluated for Gram-negative bacteria and *S. aureus* (Zankari et al., 2012) was able to identify genes in *S. pseudintermedius* with high accuracy. It uses a Blast method; the default identity is 90% over at least 60% of the reference gene. It can sometimes find inactive genes containing a mutation, deletion or insertion, which still meet these criteria (Zankari et al., 2012). Resfinder does not seem to miss resistance genes as all phenotypical resistances (except the above mentioned misclassified isolate) were explained by the presence of a gene or mutation. Resfinder can only find genes conferring resistance, that are currently present in the Resfinder database, and will not identify unknown genes or point mutations that could confer resistance. Furthermore, Resfinder was unable to detect point mutations in chromosomal genes leading to antimicrobial resistance. Thus, manual searches were still needed for resistances involving point mutations. The genes present in Resfinder that are known to confer resistance to fosfomycins, fusidic acid, oxazolidones and vancomycin can be identified. For these antimicrobial classes no resistance was detected in this study, and other studies have reported low resistances to these antimicrobial classes in *S. pseudintermedius* (Kadlec and Schwarz, 2012). Currently it is unknown whether those genes that are present in Resfinder confer resistances in *S. pseudintermedius*. For instance, the *fosB* gene associated with fosfomycin resistance in staphylococci, is not homologous to the *fosB* gene found in Resfinder (Zakour et al., 2011). This gene is commonly present in *S. pseudintermedius* but was not associated with fosfomycin resistance in a previous study, indicating that characterization of the gene associated with fosfomycin is needed (DiCicco et al., 2014). Also, the *fusC* gene, for fusidic acid resistance in *S. pseudintermedius,* has been described in single study and was not present in the Resfinder database (O'Neill et al., 2007). Resistance to tigecycline has until now not been reported in *S. pseudintermedius*.

Other databases, such as the Comprehensive Antimicrobial Resistance Database (CARD) (McArthur et al., 2013), could be used to identify point mutations. In this study the genes identified with Resfinder were also found with CARD identity searches, but the chromosomal point mutations in the fluoroquinolone resistance regions were not identified (data not shown). The CARD analysis identified multiple genes with very low identity that were therefore considered as false positives. Four supplementary genes were found with high identity *mecR*1 and *mecI* (regulators of the *mecA* operon), *ant*9-Ia (which confers resistance to aminoglycosides), and *sat*4 (which confers resistance to streptothricin which was not tested in our panel with antimicrobials) (data not shown).

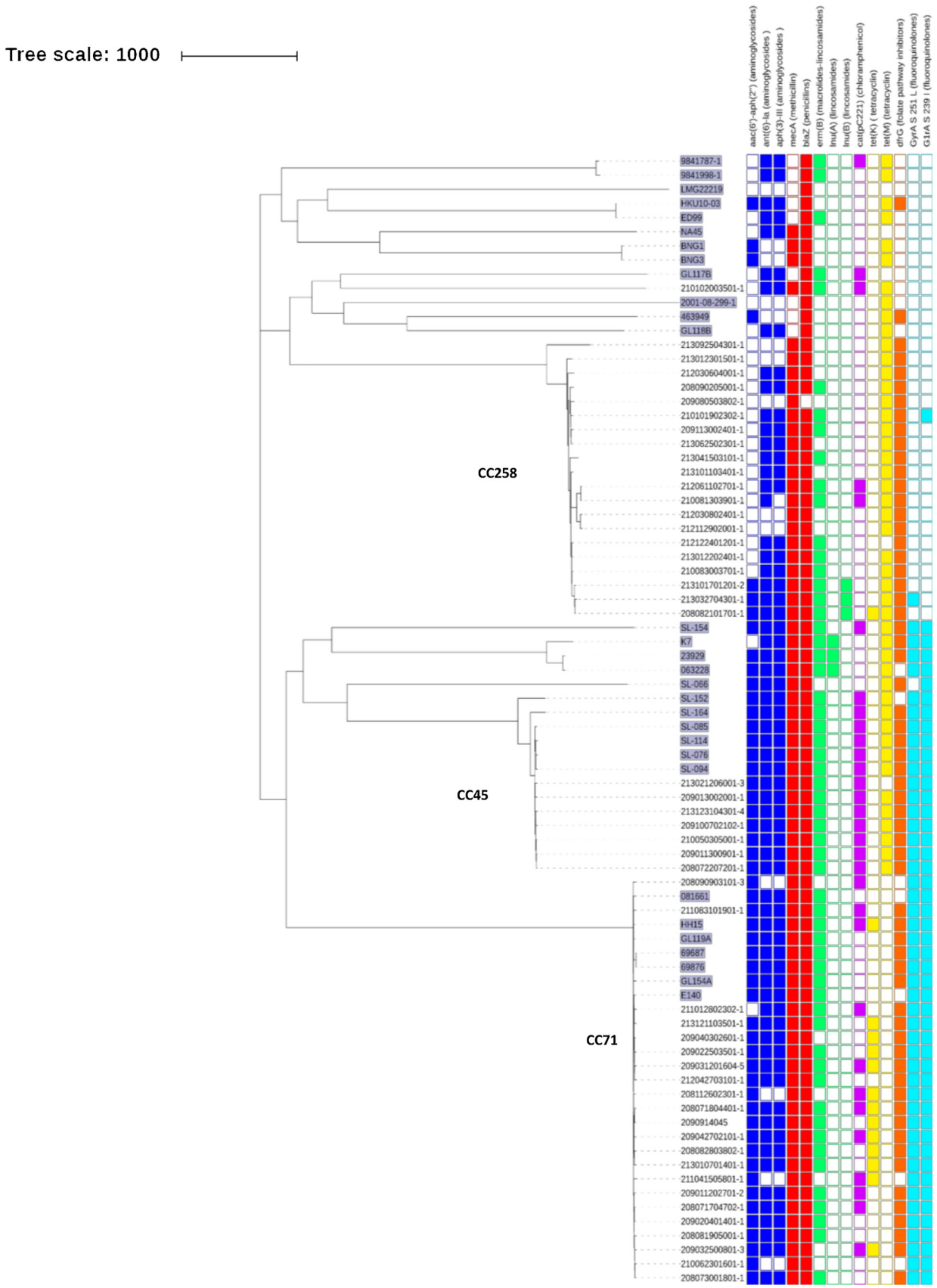

Fig. 1. Genome phylogeny based on SNP in the core genome of 50 dog isolates and 30 reference isolates. The coloured boxes display the antimicrobial resistance gene presence (full box) and absence (empty box). The reference strains are shown highlighted.

In *S. pseudintermedius* resistance to fluoroquinolones is known to be mediated by the *gyrA* 251 Ser/Leu and the *grlA* 239 Ser/Ile mutations (Descloux et al., 2008). In this study all but one resistant isolates harboured both mutations, in one resistant isolate only the *grlA* 239 Ser/Ile mutation was present with a MIC similar to the one of other resistant isolates, and in one susceptible isolate the *gyrA* 251 S/L mutation was present without an increase in the MIC. This contradicts reports identifying *gyrA* 251 S/L as the main mutation involved in fluoroquinolone resistance (Loiacono et al., 2017). Since only one susceptible isolate showed the mutation it is difficult to conclude that one mutation is always sufficient for resistance. This point has been raised in other studies (Descloux et al., 2008; Gómez-Sanz et al., 2011; Onuma et al., 2011). In our study the effect of MIC increase by a combination of mutations could have been missed due to the limited concentration range tested for fluoroquinolones.

Resistance to rifampicin is known to be caused by different mutations in the *rpoB* gene. In this study resistance to rifampicin was seen in only one isolate and coincided with a mutation in *rpoB*. As the position of this mutation His to Pro at amino acid position 481, differs from the one previously described in *S. pseudintermedius* (Kadlec et al., 2011), it is difficult to ascertain that this mutation is associated with resistance, however resistance conferring mutations 481 His→Tyr and 481 His→Asn have been described in *S. aureus* (Wichelhaus et al., 2002; Tang et al., 2016), making this highly likely.

The patterns of antimicrobial resistances were linked to the clonal distribution confirming the results of previous studies using MLST (Dos Santos et al., 2016; Duim et al., 2016). CC71 isolates exhibited diverse resistance patterns and carried only the *tetK* and not the *tetM* gene for tetracycline resistance. This has also been shown for MRSP strains in Europe and America (Perreten et al., 2010). CC45 on the other hand, had very conserved resistance gene patterns and CC258 showed fewer resistances and more diverse resistance patterns. This suggests that the distribution of resistance genes is correlated with clonal spread, but also indicates that horizontal gene transfer could play a role as previously suspected in this species. (Perreten et al., 2010; McCarthy et al., 2014; Dos Santos et al., 2016). The diversity in CC71 resistance patterns, suggests that individual strains of this CC may apply different mechanisms for incorporation and maintenance of antimicrobial resistance genes i.g. different restriction recombination systems. This could also explain why the dynamics in the population of CC258 isolates is different from that of CC45 and CC71 isolates. We speculate that antimicrobial resistance was the primary driver of success of CC71 and CC45 isolates among dogs, and that CC258 was later introduced with more susceptible isolates. The dynamics and the success of MRSP with dissemination of antimicrobial resistances, must be addressed by analysing a wider population, including methicillin susceptible *S. pseudintermedius*.

5. Conclusions

Whole genome sequencing combined with Resfinder and manual searches predicts most resistances accurately; only a small proportion of phenotypically susceptible isolates would be misclassified as resistant due to the presence of inactive or disrupted resistance genes. Phylogenetic analysis indicates that clonal spread of MRSP is linked with diversity in resistance patterns.

Conflict of interest

None to declare.

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Appendix A. Supplementary data

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References

Bankevich, A., Nurk, S., Antipov, D., Gurevich, A.A., Dvorkin, M., Kulikov, A.S., Lesin, V.M., Nikolenko, S.I., Pham, S., Prjibelski, A.D., Pyshkin, A.V., Sirotkin, A.V., Vyahhi, N., Tesler, G., Alekseyev, M.A., Pevzner, P.A., 2012. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. J. Comput. Biol. 19, 455–477. https://doi.org/10.1089/cmb.2012.0021.

Bannoehr, J., Guardabassi, L., 2012. *Staphylococcus pseudintermedius* in the dog: taxonomy, diagnostics, ecology, epidemiology and pathogenicity. Vet. Dermatol. 23https://doi.org/10.1111/j.1365-3164.2012.01046.x. 253-e52.

CLSI, 2015. Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated From Animals. 3rd Ed. CLSI Supplement VET01S. Clinical and Laboratory Standards Institute, Wayne, PA.

CLSI, 2017. Performance Standards for Antimicrobial Susceptibility Testing. 27th Ed. CLSI Supplement M100. Clinical and Laboratory Standards Institute, Wayne, PA.

Croucher, N.J., Page, A.J., Connor, T.R., Delaney, A.J., Keane, J.A., Bentley, S.D., Parkhill, J., Harris, S.R., 2015. Rapid phylogenetic analysis of large samples of recombinant bacterial whole genome sequences using Gubbins. Nucleic Acids Res. 43, e15. https://doi.org/10.1093/nar/gku1196.

Damborg, P., Moodley, A., Aalbæk, B., Ventrella, G., dos Santos, T.P., Guardabassi, L., 2016. High genotypic diversity among methicillin-resistant *Staphylococcus pseudintermedius* isolated from canine infections in Denmark. BMC Vet. Res. 12. https://doi.org/10.1186/s12917-016-0756-y.

Descloux, S., Rossano, A., Perreten, V., 2008. Characterization of new staphylococcal cassette chromosome mec (SCCmec) and topoisomerase genes in fluoroquinolone- and methicillin-resistant *Staphylococcus pseudintermedius*. J. Clin. Microbiol. 46, 1818–1823. https://doi.org/10.1128/JCM.02255-07.

DiCicco, M., Weese, S., Neethirajan, S., Rousseau, J., Singh, A., 2014. Fosfomycin susceptibility of canine methicillin-resistant *Staphylococcus pseudintermedius* isolates. Res. Vet. Sci. https://doi.org/10.1016/j.rvsc.2014.02.004.

Dos Santos, T.P., Damborg, P., Moodley, A., Guardabassi, L., 2016. Systematic review on global epidemiology of methicillin-resistant *Staphylococcus pseudintermedius*: inference of population structure from multilocus sequence typing data. Front. Microbiol. https://doi.org/10.3389/fmicb.2016.01599.

Duim, B., Verstappen, K.M., Broens, E.M., Laarhoven, L.M., Van Duijkeren, E., Hordijk, J., De Heus, P., Spaninks, M., Timmerman, A.J., Wagenaar, J.A., 2016. Changes in the population of methicillin-resistant *Staphylococcus pseudintermedius* and dissemination of antimicrobial-resistant phenotypes in the Netherlands. J. Clin. Microbiol. 54, 283–288. https://doi.org/10.1128/JCM.01288-15.

Gómez-Sanz, E., Torres, C., Lozano, C., Saenz, Y., Zarazaga, M., 2011. Detection and characterization of methicillin-resistant *Staphylococcus pseudintermedius* in healthy dogs in La Rioja, Spain. Comp. Immunol. Microbiol. Infect. Dis. 34, 447–453. https://doi.org/10.1016/j.cimid.2011.08.002.

Grönthal, T., Eklund, M., Thomson, K., Piiparinen, H., Sironen, T., Rantala, M., 2017. Antimicrobial resistance in *Staphylococcus pseudintermedius* and the molecular epidemiology of methicillin-resistant S. *pseudintermedius* in small animals in Finland. J. Antimicrob. Chemother. 72, 1021–1030. https://doi.org/10.1093/jac/dkw559.

Kadlec, K., Schwarz, S., 2012. Antimicrobial resistance of Staphylococcus pseudintermedius. Vet. Dermatol. 23, 19–25. https://doi.org/10.1111/j.1365-3164.2012.01056.x.

Kadlec, K., van Duijkeren, E., Wagenaar, J.A., Schwarz, S., 2011. Molecular basis of rifampicin resistance in methicillin-resistant *Staphylococcus pseudintermedius* isolates from dogs. J. Antimicrob. Chemother. 66, 1236–1242. https://doi.org/10.1093/jac/dkr118.

Letunic, I., Bork, P., 2016. Interactive tree of life (iTOL) v3: an online tool for the display and annotation of phylogenetic and other trees. Nucleic Acids Res. 44, W242–W245. https://doi.org/10.1093/nar/gkw290.

Loiacono, M., Martino, P.A., Albonico, F., Dell'Orco, F., Ferretti, M., Zanzani, S., Mortarino, M., 2017. High-resolution melting analysis of gyrA codon 84 and grlA codon 80 mutations conferring resistance to fluoroquinolones in *Staphylococcus pseudintermedius* isolates from canine clinical samples. J. Vet. Diagn. Invest. 29, 711–715. https://doi.org/10.1177/1040638717712330.

McArthur, A.G., Waglechner, N., Nizam, F., Yan, A., Azad, M.A., Baylay, A.J., Bhullar, K., Canova, M.J., De Pascale, G., Ejim, L., Kalan, L., King, A.M., Koteva, K., Morar, M., Mulvey, M.R., O'Brien, J.S., Pawlowski, A.C., Piddock, L.J.V., Spanogiannopoulos, P., Sutherland, A.D., Tang, I., Taylor, P.L., Thaker, M., Wang, W., Yan, M., Yu, T., Wright, G.D., 2013. The comprehensive antibiotic resistance database. Antimicrob. Agents Chemother. 57, 3348–3357. https://doi.org/10.1128/AAC.00419-13.

McCarthy, A.J., Harrison, E.M., Stanczak-Mrozek, K., Leggett, B., Waller, A., Holmes, M.A., Lloyd, D.H., Lindsay, J.A., Loeffler, A., 2014. Genomic insights into the rapid emergence and evolution of MDR in *Staphylococcus pseudintermedius*. J. Antimicrob.

Chemother. 70, 997–1007. https://doi.org/10.1093/jac/dku496.
O'Neill, A.J., McLaws, F., Kahlmeter, G., Henriksen, A.S., Chopra, I., 2007. Genetic basis of resistance to fusidic acid in Staphylococci. Antimicrob. Agents Chemother. 51, 1737–1740. https://doi.org/10.1128/AAC.01542-06.
Onuma, K., Tanabe, T., Sato, H., 2011. Antimicrobial resistance of *Staphylococcus pseudintermedius* isolates from healthy dogs and dogs affected with pyoderma in Japan. Vet. Dermatol. 23https://doi.org/10.1111/j.1365-3164.2011.00995.x. 17-e5.
Page, A.J., Cummins, C.A., Hunt, M., Wong, V.K., Reuter, S., Holden, M.T.G., Fookes, M., Falush, D., Keane, J.A., Parkhill, J., 2015. Roary: rapid large-scale prokaryote pan genome analysis. Bioinformatics 31, 3691–3693. https://doi.org/10.1093/bioinformatics/btv421.
Perreten, V., Kadlec, K., Schwarz, S., Andersson, U.G., Finn, M., Greko, C., Moodley, A., Kania, S.A., Frank, L.A., Bemis, D.A., Franco, A., Iurescia, M., Battisti, A., Duim, B., Wagenaar, J.A., van Duijkeren, E., Weese, J.S., Fitzgerald, J.R., Rossano, A., Guardabassi, L., 2010. Clonal spread of methicillin-resistant *Staphylococcus pseudintermedius* in Europe and North America: an international multicentre study. J. Antimicrob. Chemother. 65, 1145–1154. https://doi.org/10.1093/jac/dkq078.
Swenson, J.M., Brasso, W.B., Ferraro, M.J., Hardy, D.J., Knapp, C.C., McDougal, L.K., Reller, L.B., Sader, H.S., Shortridge, D., Skov, R., Weinstein, M.P., Zimmer, B.L., Patel, J.B., 2007. Detection of inducible clindamycin resistance in Staphylococci by Broth microdilution using erythromycin-clindamycin combination wells. J. Clin. Microbiol. 45. https://doi.org/10.1128/JCM.01501-07.
Tang, H.-J., Lai, C.-C., Hsueh, P.-R., Chen, C.-C., Wu, K.-Y., Lin, Y.-C., Zhang, C.-C., Weng, T.-C., Chiu, Y.-H., Toh, H.-S., Chiang, S.-R., Yu, W.-L., Ko, W.-C., Chuang, Y.-C., 2016. RNA polymerase B subunit gene mutations in biofilm-embedded methicillin-resistant *Staphylococcus aureus* following rifampin treatment. J. Microbiol. Immunol. Infect. 49, 394–401. https://doi.org/10.1016/j.jmii.2015.06.006.
The European Committee on Antimicrobial Susceptibility Testing, 2017. Breakpoint Tables for Interpretation of MICs and Zone Diameters. Version 7.0. http://www.eucast.org.
The European Committee on Antimicrobial Susceptibility Testing, 2018. Breakpoint Tables for Interpretation of MICs and Zone Diameters. Version 8.0. http://www.eucast.org.
Thomsen, M.C.F., Ahrenfeldt, J., Cisneros, J.L.B., Jurtz, V., Larsen, M.V., Hasman, H., Aarestrup, F.M., Lund, O., 2016. A bacterial analysis platform: an integrated system for analysing bacterial whole genome sequencing data for clinical diagnostics and surveillance. PLoS One 11. https://doi.org/10.1371/journal.pone.0157718.
Treangen, T.J., Ondov, B.D., Koren, S., Phillippy, A.M., 2014. The harvest suite for rapid core-genome alignment and visualization of thousands of intraspecific microbial genomes. Genome Biol. 15, 524. https://doi.org/10.1186/s13059-014-0524-x.
Weese, J.S., van Duijkeren, E., 2010. Methicillin-resistant *Staphylococcus aureus* and *Staphylococcus pseudintermedius* in veterinary medicine. Vet. Microbiol. 140, 418–429. https://doi.org/10.1016/j.vetmic.2009.01.039.
Wichelhaus, T.A., Böddinghaus, B., Besier, S., Schäfer, V., Brade, V., Ludwig, A., 2002. Biological cost of rifampin resistance from the perspective of *Staphylococcus aureus*. Antimicrob. Agents Chemother. 46, 3381–3385. https://doi.org/10.1128/AAC.46.11.3381-3385.2002.
Wu, M.T., Burnham, C.-A.A.D., Westblade, L.F., Bard, J.D., Lawhon, S.D., Wallace, M.A., Stanley, T., Burd, E., Hindler, J., Humphries, R.M., 2016. Evaluation of oxacillin and cefoxitin disk and MIC breakpoints for prediction of methicillin resistance in human and veterinary isolates of *Staphylococcus intermedius* group. J. Clin. Microbiol. 54, 535–542. https://doi.org/10.1128/JCM.02864-15.
Zakour, N.L.B., Bannoehr, J., van den Broek, A.H.M., Thoday, K.L., Fitzgerald, J.R., 2011. Complete genome sequence of the canine pathogen *Staphylococcus pseudintermedius*. J. Bacteriol. https://doi.org/10.1128/JB.00137-11.
Zankari, E., Hasman, H., Cosentino, S., Vestergaard, M., Rasmussen, S., Lund, O., Aarestrup, F.M., Larsen, M.V., 2012. Identification of acquired antimicrobial resistance genes. J. Antimicrob. Chemother. 67, 2640–2644. https://doi.org/10.1093/jac/dks261.