Genomic dynamics of antimicrobial resistance in canine and human derived *Staphylococcus pseudintermedius*

ALICE WEGENER

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Genomic dynamics of antimicrobial resistance in canine and human derived *Staphylococcus pseudintermedius*

Genomische dynamiek van antibioticumresistentie in *Staphylococcus pseudintermedius* bij hond en mens

(met een samenvatting in het Nederlands)

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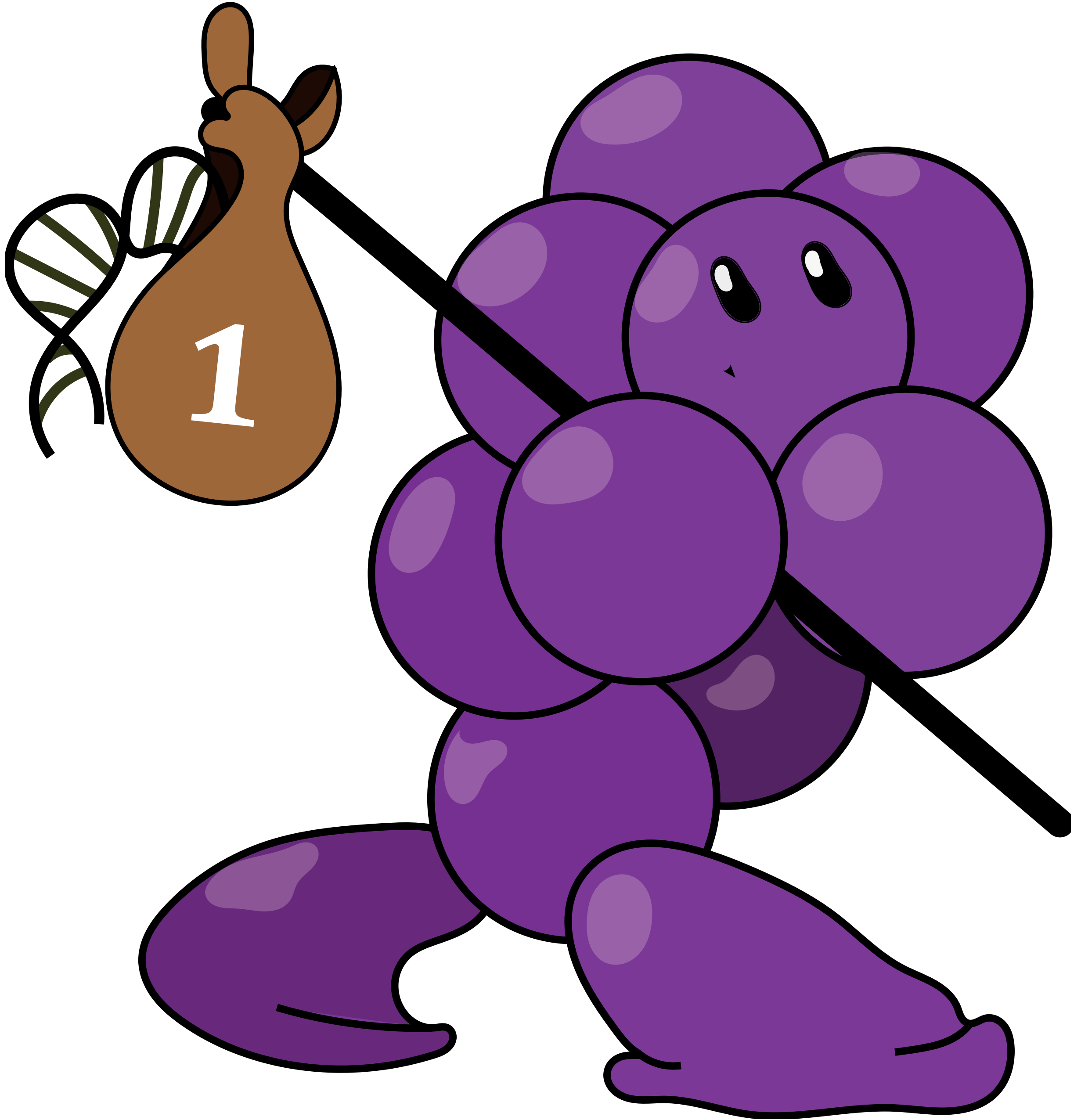
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CHAPTER 1

General introduction

Population structure and evolution of antimicrobial resistance in *Staphylococcus pseudintermedius*

Staphylococci

Staphylococcus is a genus of Gram-positive bacteria of clinical importance for humans as well as animals. The taxonomy of this genus has undergone significant changes in the last decades and currently comprises 75 species and 30 subspecies, while new species are still regularly being described (1, 2). Staphylococci are commensals colonising skin and mucous membranes of humans and a wide variety of animals without causing health problems. However, some staphylococcal species are also known as important opportunistic pathogens, causing infections if host defences are weakened. Infections in humans and animals can occur in the community (sporadic cases or outbreaks), or as nosocomial infections in health care settings.

Staphylococci are traditionally separated in two groups based on the production of coagulase, an enzyme coagulating plasma. Coagulase-positive staphylococci are considered the major pathogenic species, whereas coagulase-negative species are traditionally considered relatively minor pathogens. However nowadays, coagulase negative staphylococcal species represent one of the major nosocomial pathogens in human hospitals (3). Another clinically relevant property of staphylococci is their ability to acquire resistance to several antimicrobials. The most significant acquired antimicrobial resistance from staphylococci is methicillin resistance encoded by the *mecA* gene, which is carried on a distinct mobile genetic element, the Staphylococcal Chromosomal Cassette *mec* (SCC*mec*) (4).

The most clinically relevant staphylococci in animals are coagulase-positive staphylococci associated with specific infections in animal hosts, e.g. *Staphylococcus aureus* causing mastitis in ruminants, *S. hyicus* causing dermatitis in pigs and *S. pseudintermedius* causing pyoderma in dogs. This thesis focuses on population structure and gene content of the latter species that was described in 2005.

Staphylococcus pseudintermedius

In 1976 the species *Staphylococcus intermedius* has been described, based on a study of 50 isolates originating from pigeons, dogs, mink and horses (5). This study showed

isolates with properties homologous to those of *S. aureus* (coagulase production and heat stable nuclease) and those of *S. epidermidis* (serine in peptidoglycan), therefore the name *S. intermedius* was chosen. At that time phenotypic variation in the *S. intermedius* isolates was already noted and over the years more diversity was reported among isolates assigned to *S. intermedius* (5–7). The use of molecular techniques led in 2005 to the description of the *Staphylococcus intermediu*s group (SIG) containing three species, *S. intermedius*, *S. delphini* (8) and *S. pseudintermedius* (9). More recently two new species, *S. cornubiensis* (10) and *S. ursi* (11) have been added to this group.

It has been hypothesised that all species within the SIG group are associated with specific hosts, although the ecology of the SIG members remains diverse. *S. intermedius* has been associated with feral pigeons, *S. delphini* with domestic pigeons, mink and horses, *S. pseudintermedius* mainly with dogs but occasionally with cats and humans, and *S. ursi* with black bears (7, 11, 12). The description of *S. cornubiensis* was based on an isolate recovered from a human skin infection, which was genetically similar to a previously isolated canine isolate, therefore the origin and host species are unclear (10).

Distinction between different members of the SIG-group using biochemical methods is rather difficult. Molecular methods such as PCR-RFLP of the *Mbo*I restriction of the *pta* gene can differentiate *S. pseudintermedius* from other SIG members and other pathogenic staphylococci (13). The introduction of MALDI-TOF analysis for the identification of bacterial species in routine diagnostics allows a more accurate and faster identification of the individual species within the SIG group (14, 15), although the more recently discovered species (*S. cornubiensis* and *S. ursi)* are currently not included in the standard database.

After the description of *S. pseudintermedius* it was revealed that this species, and not *S. intermedius,* was the common canine pathogen. It has therefore been suggested that *S. intermedius* in dogs described before 2005, should be considered as *S. pseudintermedius,* unless shown by genomic comparison to belong to another species (12).

S. pseudintermedius is both frequently identified as a commensal, and as a major opportunistic pathogen in dogs, causing mainly skin and soft tissue infection (SSTI) (16). *S. pseudintermedius* infections in dogs without predisposition such as allergies, can generally be treated easily using antimicrobials. Infections with multidrug-resistant (MDR) isolates, including methicillin-resistant *S. pseudintermedius* (MRSP), are of clinical concern as treatment options are limited. In dogs *S. pseudintermedius*

can colonize different body sites (e.g. skin, ear, nose, mouth, perineum), and genetic heterogeneity among isolates from one dog is common (16–19). *S. pseudintermedius* can be transmitted within households from dog to dog, and between dogs and other household members, including humans (20–22). *S. pseudintermedius* has been found in other animals such as cats, horses, and humans, although they are not considered its natural hosts. Host adaptation of *S. pseudintermedius* to dogs has been suggested, because isolates from dogs adhered better to corneocytes of dogs than to those of humans, and the extracellular matrix adherence proteins SpsL and SpsD were host-species dependent; these proteins are hypothesised to be responsible of this host specificity (23, 24).

S. pseudintermedius is infrequently reported in humans, although infections might be underdiagnosed, due to phenotypic misidentification as *S. aureus* (15, 25–27). Since the implementation of MALDI-TOF MS in routine diagnostics, the number of reported *S. pseudintermedius* cases in humans has increased (15, 25–27). Human infections are often attributed to zoonotic transmission between dogs and humans (28, 29) confirmed by the similarity of isolates from dogs and humans within the same household and the occupational risk of people working with dogs, e.g. veterinarians (20, 21). In a few human cases, *S. pseudintermedius* isolates from humans and dogs were distinct (29, 30). This might be explained by the genetic heterogeneity among isolates colonising one dog (16–19), while in comparative studies usually only one isolate per individual is analysed (29, 30). Human-to-human transmission of *S. pseudintermedius* is limited. This has only been reported once for a cluster of infections caused by MRSP in a tertiary hospital (31).

In humans, *S. pseudintermedius* is often found as part of a polymicrobial infection with other staphylococci, although it can also be the only pathogen (28, 29). In humans it is, like in dogs, associated with skin and soft tissue infections (SSTI), and is mainly found in elderly patients with comorbidities (28, 32). In the largest study on *S. pseudintermedius* in humans (24 cases) the median age was 61 years, the most common comorbidity was diabetes mellitus (29.2%), followed by peripheral vascular disease (20.8%), and cardiovascular disease (16.7%) (28). Despite increasing reports, the occurrence of *S. pseudintermedius* in humans is very limited (0.05% of SSTI) compared to that of *S. aureus* (30% of SSTI) (28, 30). In humans *S. pseudintermedius* infections are most often mild and easily treated, but invasive infections have been observed, not resulting in mortality (28, 29).

The epidemiology of *S. pseudintermedius* in human infections is poorly studied and

information on the genetic variation or host adaptation of *S. pseudintermedius* is limited. Wider population studies comparing dog and human isolates on a genomic level and detection of host associated characteristics on a global level are lacking.

S. pseudintermedius population structure

Most studies on the distribution of *S. pseudintermedius* are biased towards analysis of methicillin-resistant isolates. Several molecular typing methods such as Multi-Locus Sequence Typing (MLST) (33), protein A repeats (*spa*) typing (34) and direct repeat units (dru) typing (35), have been used to study the population structure of *S. pseudintermedius.* More recently Whole Genome Sequencing (WGS) was used for this purpose. Methicillin-susceptible *S. pseudintermedius* (MSSP) isolates showed high diversity with nearly every isolate belonging to a different sequence type (36, 37). A totally different picture is shown for MRSP which forms a clonal population. Clonal Complex (CC) 71 has been identified as the dominant clone in Europe since 2004 (38). More recently, new emerging clones have been reported in Finland (39), Denmark (19) and the Netherlands (40). On other continents specific epidemic MRSP clones have also been found: ST496 in Australia (41), CC45 in Asia (36), and ST68 in North America (38). Resistance gene patterns in MRSP seem to be associated with clonal complexes, with CC71 and CC45 being associated with resistance to most antimicrobial classes, and CC258 showing less resistances (16, 36, 38, 40, 42). More studies using WGS are needed to elucidate the diversity, epidemiological characteristics, and gene content of both MSSP and MRSP isolates.

Antimicrobial resistance in *S. pseudintermedius*

In the past decades, methicillin-resistant *S. pseudintermedius* (MRSP) has emerged globally. In a study in the USA 30% of the tested *S. pseudintermedius* isolates in 2007 was MRSP positive, and in another study in 2005 15.6 % of the isolates were positive for MRSP (43). In Europe the first MRSP isolate was detected in 2007 (44). Besides resistance to β-lactam antimicrobials encoded by the *mecA* gene carried on a mobile genetic element (SCC*mec*), MRSP often shows resistance to several other antimicrobial classes. Many more studies have focused on antimicrobial resistances in MRSP isolates compared to just a few studies on MSSP isolates. Overall, is it assumed that MRSP isolates are resistant to more antimicrobial classes (on top of β-lactams) than MSSP isolates. Table 1 provides an overview of the reported

antimicrobial resistances in MRSP isolates from different continents (Europe, North America and Asia) (38), and in MSSP isolates obtained in a large European study (37).

Table 1. An overview of antimicrobial resistances in MRSP and MSSP (37, 38).

	MRSP (38) (n=103)	**MSSP (37) (n=95)**
Geographical origin	Europe, North America and Asia	Europe
Absence of resistance	0%	17.9%
β-lactam resistance	100%	76.8%
Aminoglycosides	71.3% gentamicin 5.2% amikacin 20% streptomycin 16.8% kanamycin	3.2% gentamicin
Macrolides/ Lincosamides	93.5% erythromycin 92.7% clindamycin	17.9%
Tetracyclins	70.7%	34.7%
Chloramphenicol	43.1%	12.6%
Trimethoprim	76.6%	2.1%
Fluoroquinolones	73.8%	0%

Antimicrobial resistance can be assessed phenotypically or by screening for resistance genes. Phenotypic resistance is performed by determining the minimum inhibitory concentration (MIC). Its interpretation requires clinical breakpoints specific to the antimicrobial and bacterial species, that can vary depending on the host and infection site. Two main organisations have established standard breakpoints: CLSI and EUCAST (45, 46). Veterinary breakpoints have been established by CLSI (47), but for *S. pseudintermedius* breakpoints for only a limited number of antimicrobial classes exist. When veterinary breakpoints for *S. pseudintermedius* are lacking, there is no general accepted rule and a variety of interpretive criteria have been used in the past complicating comparison of results between studies. It seems to be appropriate to use veterinary or human breakpoints for *S. aureus*, coagulase-negative staphylococci or *Staphylococcus* spp. (47). However, there is an important discrepancy in the screening for methicillin resistance in *S. pseudintermedius* compared to other staphylococci. The cefoxitin disk test that is recommended for other staphylococci is less predictive

for the presence of methicillin resistance in *S. pseudintermedius.* Instead, oxacillin susceptibility testing, using a considerably lower resistance breakpoint than for *S. aureus* should be used in *S. pseudintermedius* (48). If methicillin resistance is detected, MRSP isolates are considered resistant to all β-lactams according to an expert rule established for MRSA. This rule was adapted to MRSP without being corroborated by clinical data (49). In contrast to this rule differences have been observed in MICs of different β-lactams, in MRSA and MRSP clinical data. In MRSA, various SCC*mec* types are linked to different levels of oxacillin resistance (50–52). In *S. pseudintermedius*, similar associations have been suggested (53, 54). These observations need further analysis and validation in *S. pseudintermedius.*

Antimicrobial resistance can also be inferred from whole genome sequences by detecting resistance genes or mutations leading to resistances. To this end different online tools are available, such as ResFinder or CARD (55, 56). However, most tools focus on the detection and identification of horizontally acquired resistance, in Gram-negative bacteria and *Staphylococcus aureus* (55, 56). These tools rely on databases of known resistance genes and therefore do not allow the detection of new resistance genes. Furthermore, the presence of a resistance gene might not always result in phenotypical resistance, as regulation mechanisms might hinder its expression. Equally the presence of a resistance gene does not give information about the resistance level. This shows that the accuracy of antimicrobial resistance genes prediction and its correspondence to phenotypical resistance needs to be confirmed for individual antimicrobials and bacterial species. In *S. pseudintermedius* the association between antimicrobial genotype and phenotype had not been confirmed, prior to this thesis.

Several genes, also present in *S. aureus*, are known to be responsible for antimicrobial resistance in *S. pseudintermedius*: *mecA* and *blaZ* for β-lactam resistance; *erm*A, *erm*B, *erm*C, *msr*A and *Inu*A for macrolides and lincosamides resistance, *cat*$_{\text{pC221}}$ for chloramphenicol resistance, *aac(6')-aph(2"), ant6-la, aphIII-3' and sat4* for aminoglycosides resistance; *tet*(M), *tet(*K*) tet(*O*), tet(*L*)* for tetracycline resistance; *dfr*G for trimethoprim resistance and *fus*C for fusidic acid resistance, although the latter is very rare (43, 57). Resistances to fluoroquinolones and the very rare rifampicin resistance are both linked to point mutations (43, 58).

The finding of several resistance genes that have been identified in other staphylococcal species before shows that *S. pseudintermedius* can acquire genetic

material from other bacterial species. This genetic material is often carried by mobile genetic elements (MGEs) including transposons, plasmids, as well as integrated bacteriophages. MGEs play an important role in bacterial adaptation, as they can carry antimicrobial and heavy metal resistance genes, virulence genes and bacteriocins. In *S. aureus* the MGE content can vary within the same clonal complex (CC) by frequent acquisition and loss events (59). It is currently unknown whether this is the case in *S. pseudintermedius*.

The most studied MGE is the staphylococcal chromosomal cassette *mec* (SCC*mec)* carrying the *mecA* gene that confers methicillin resistance. It is characterised by a *ccr* gene complex, mec complex (*mecA* or *mecC* gene with the specific regulator genes *mecI, mecRI* and *mecR2*) and joining regions, which can contain different genes and transposons (60). The SCC*mec* cassette generally integrates in the *orfx* region of the chromosome. It was hypothesised that SCC*mec* developed in the *S. sciuri* group, where it acquired its composition and localisation through several recombination events (61). Different SCC*mec* types have been described and classified according to their type of *ccr* and *mec* gene complex. The joining regions are used to characterise the subtypes (62). Classification of these elements are made difficult by their variability as well as by the presence of composite SCC*mec* elements originating from the recombination of two SCC*mec* types. The current classification is mainly based on the SCC*mec* types present in *S. aureus* (62).

While MRSP carries SCC*mec* types common with *S. aureus* (SCC*mec*IV and SCC*mec*V), some specific SCC*mec* types have been described in *S. pseudintermedius*: SCC*mec*II-III is a composite element originating from a recombination between SCC*mec*II of *S. epidermidis* and SCC*mec*III of *S. aureus* (58); ΨSCC*mec*$_{57395}$ is a pseudo SCC*mec* cassette without *ccr* genes (63). These different SCC*mec* cassettes may have been acquired independently on multiple occasions by methicillin-susceptible *S. pseudintermedius* isolates (27, 38).

Other resistance genes in *S. pseudintermedius* were demonstrated mainly on transposons which contrasts with other staphylococci where plasmids play an important role in the transfer of antimicrobial resistance genes. In staphylococci different small plasmids carrying resistance genes like *cat*$_{(pC221)}$ and *tet(*K) have been detected, as well as plasmids able to transfer transposons (57). It has also been reported that smaller plasmids can recombine and get integrated within bigger plasmids, forming regions with multiple resistance genes (57). These plasmids can

be transferred across different staphylococcal species (57). In *S. pseudintermedius* genes like *cat(pC221)* and *tet*(K*)* are still commonly found to be located on small plasmids (43). Other resistance genes are found on a Tn*5405*-like element that can carry up to five resistance genes (*aphIII-3'*, *sat4*, *ant6-la*, *erm(*B*)* and *dfr*G). And recently the PRE25-like element carrying *cat*(pC221), *erm(*B*), aphIII-3', ant6-la* and *sat4* was detected in *S. pseudintermedius* (64). Both MGEs with multiple resistance genes may allow rapid evolution of multidrug resistances (65).

Population genomic analysis and screening for MGEs carrying resistance genes using WGS of a large set of MRSP and MSSP isolates originating from different hosts, combined with results of phenotypic susceptibility testing will reveal the diversity, epidemiological characteristics, host specificity and gene content of *S. pseudintermedius*.

Scope of this thesis

The aim of this thesis is to describe the population structure, mobile genetic elements, antimicrobial resistance genes presence, location, and phenotypic expression, in *Staphylococcus pseudintermedius* in canine and human infections.

Chapter 2: Evaluates the accuracy of online tools for resistance gene detection compared to MIC determination of antimicrobial resistance and studies the prevalence of antimicrobial resistance in different clonal complexes in MRSP.

Chapter 3: Analyses differences in β-lactam resistance levels, and their link with clonal complexes of MRSP.

Chapter 4: Compares the genomes of human and canine MSSP isolates to evaluate host specificity and analyses the emergence of CC241.

Chapter 5: Describes the similarities and differences of *S. pseudintermedius* isolates from two human cases and their household dogs.

Chapter 6: Analyses the role of mobile genetic elements in the evolution of *S. pseudintermedius* antimicrobial resistance.

Chapter 7: Discusses the findings of all studies from the thesis in a broader scope.

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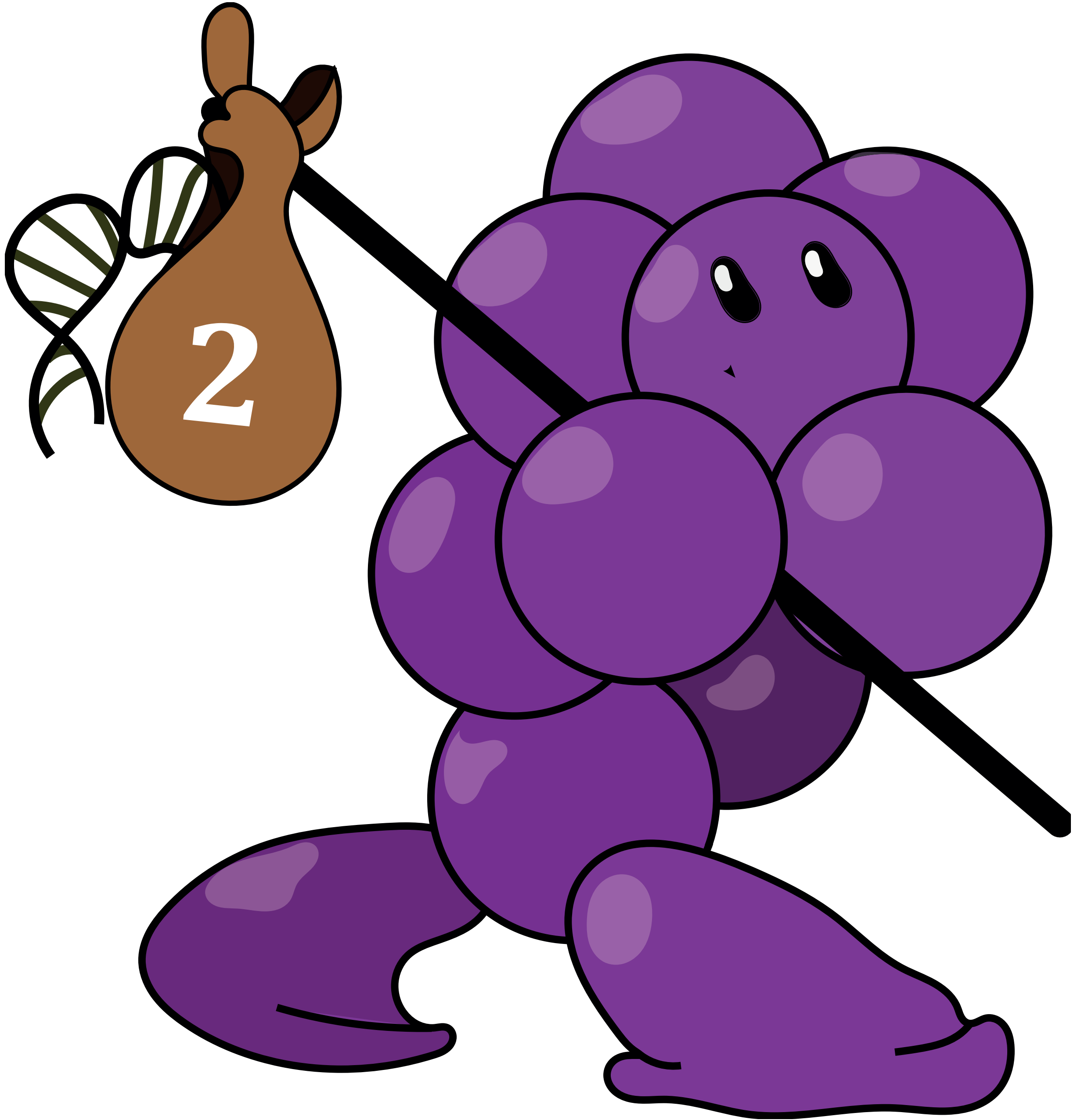
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CHAPTER 2

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 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.

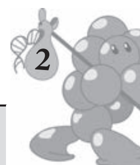


Table 1. Antimicrobial ranges and breakpoints

Antimicrobial class	Antimicrobial	Range tested	Breakpoint*	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*
streptogramins	quinupristin/ dalfopristin	0.5-4	>=2	CLSI M100S27(human)
lipopeptides	daptomycin	0.5-4	>1	EUCAST**
fosfomycins	fosfomycin	8-64	> 32	EUCAST**
steroials (fusidanes)	fusidic acid	1-2	>1	EUCAST**
oxazolidinones	linezolid	1-8	>=8	CLSI M100 S27 (human)
pseudomonic acids	mupirocin	1; 256	>256	EUCAST, 2018***
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)

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

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 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

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.

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.

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.

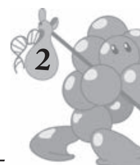
3.1.3 Lincosamides/macrolides

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

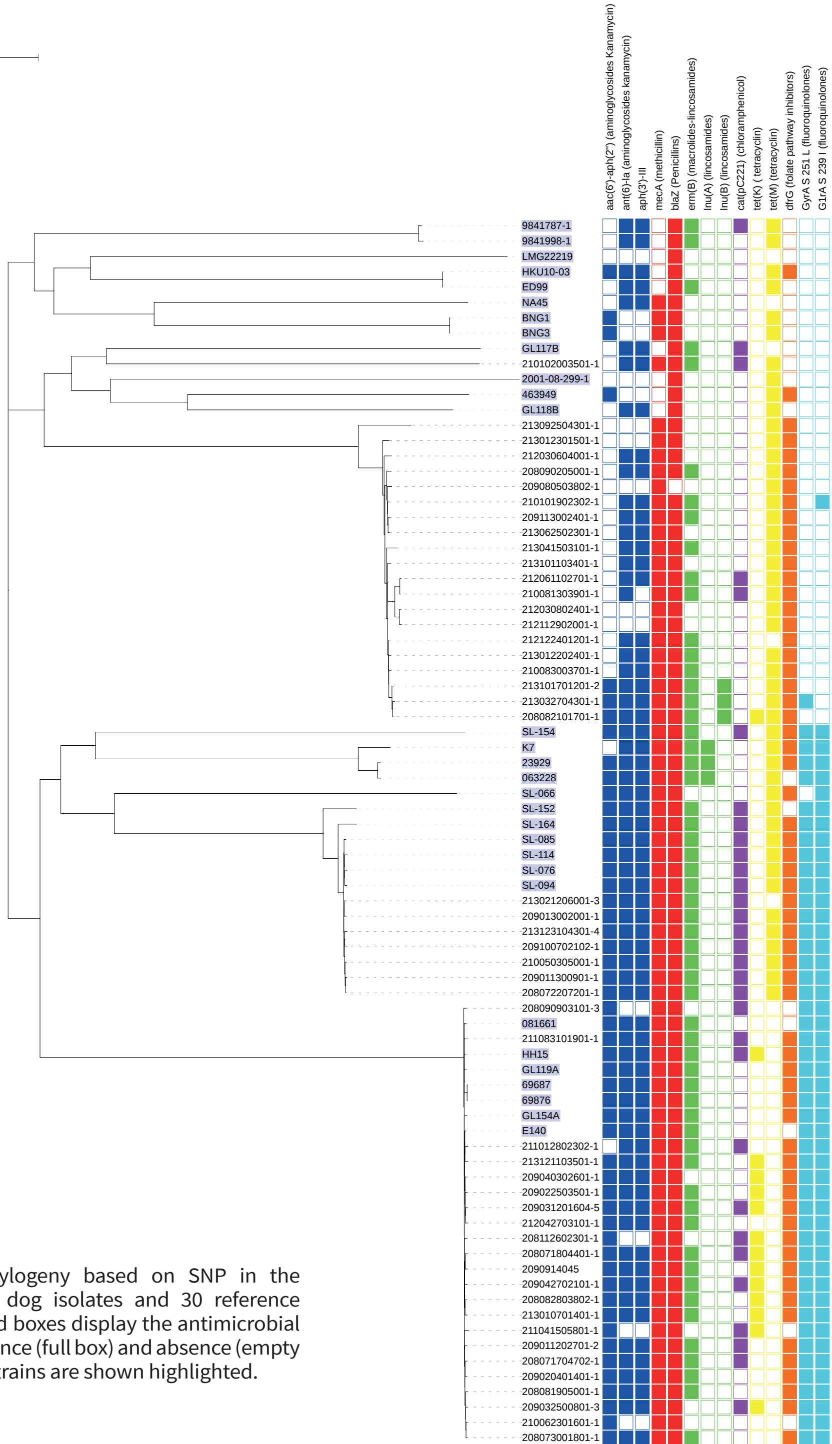


Fig. 1. Genome phylogeny based on SNP in the core genome of 50 dog isolates and 30 reference isolates. The coloured boxes display the antimicrobial resistance gene presence (full box) and absence (empty box). The reference strains are shown highlighted.

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 anti-microbials 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), *ant9-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).

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.

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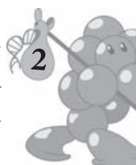

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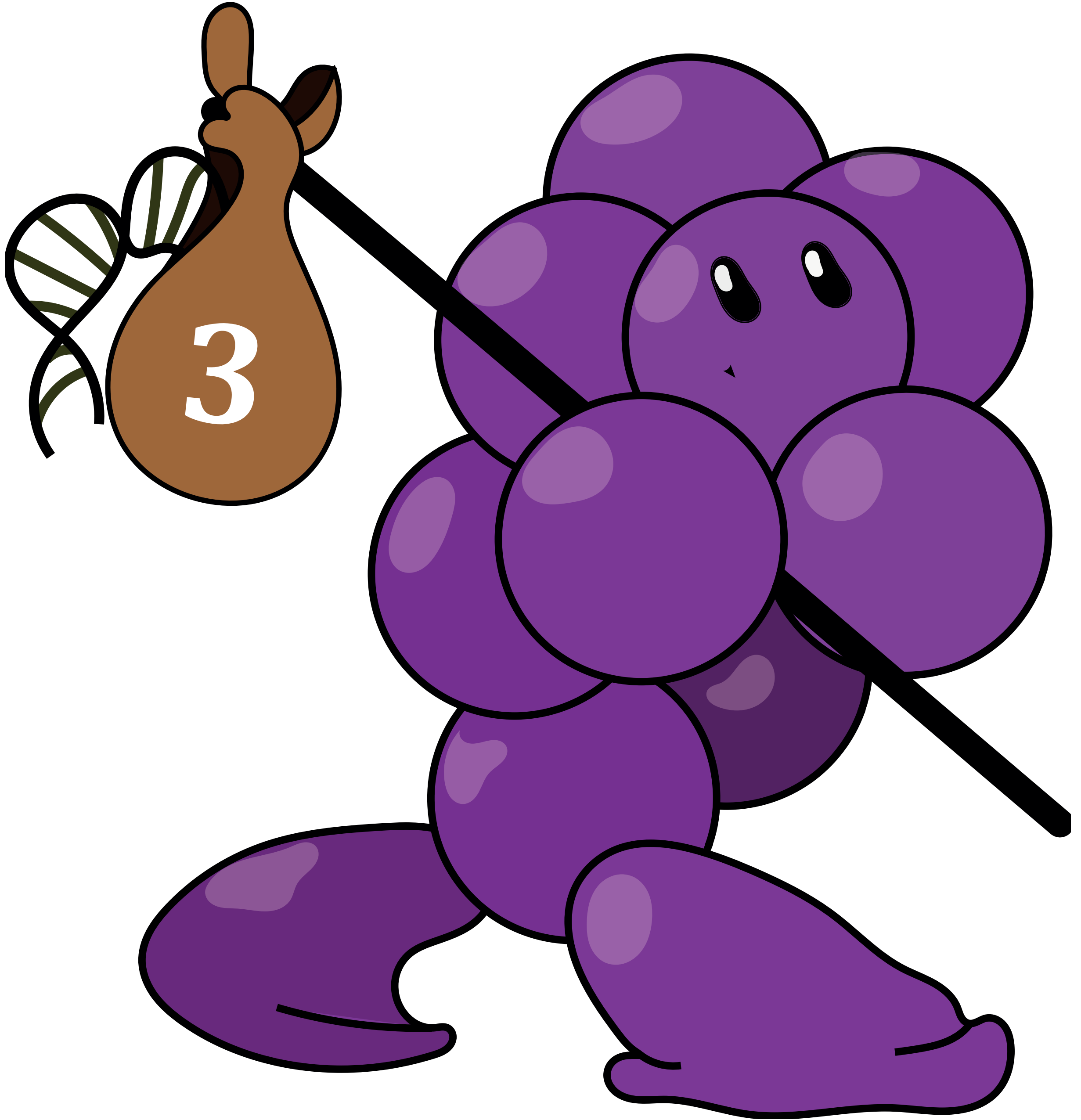
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CHAPTER 3

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 b-lactams.

Objectives: To identify possible associations between b-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 b-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 b-lactams for treatment of MRSP infections.

1. Introduction

Staphylococcus pseudintermedius is an opportunistic pathogen in dogs. Methicillin-resistant *S. pseudintermedius* (MRSP) has emerged worldwide over the past decade.[1,2] Methicillin resistance is associated with *mecA* located on staphylococcal cassette chromosome *mec* (SCC*mec*).[3] Various SCC*mec* types in *Staphylococcus aureus* are linked to different lineages, exhibiting different levels of oxacillin resistance.[4–6] In *S. pseudintermedius*, similar associations have been suggested.[7,8]

Screening for methicillin resistance in *S. pseudintermedius* is performed by oxacillin susceptibility testing using a considerably lower resistance breakpoint (R≥0.5 mg/L) than for *S. aureus* (R≥4 mg/L).[9] Oxacillin-resistant isolates should be reported as resistant to all b-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.[10]

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

2. Materials and methods

This study included 86 canine clinical MRSP (2008–14) isolates previously described in the Netherlands (*n* = 50) and Denmark (*n* = 36).[2,11,12] For Danish isolates, MLST was performed by PCR and Sanger sequencing and SCC*mec* 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 SCC*mec*Finder for SCC*mec* typing.[13] When no SCC*mec* type was detected, a local BLASTn search was performed against SCC*mec* elements previously identified in *S. pseudintermedius* using Geneious version 2019.0.3 (Biomatters, Auckland, New Zealand).

MICs of five b-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.[9]

Resistance was classified as high- or low-level to facilitate statistical analysis. Isolates displaying MICs of oxacillin ≥2 mg/L, of penicillin or ampicillin ≥8 mg/L and of amoxicillin/clavulanic acid ≥4 mg/L were defined as high-level resistant. Pearson's χ^2 test and Fisher's exact test (when sample size <5) were used to identify associations between CCs, SCC*mec* types and resistance levels. A *P* value for significance was determined by a 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.

3. 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 SCC*mec*II-III, which was significantly associated with this CC (*P* < 0.001) and not found in other STs. Thirty-one (94%) CC258 isolates harboured SCC*mec*IV, which was significantly associated with this CC (*P* < 0.001). SCC*mec*IV was also found in three other STs (268, 269 and 286). SCC*mec*V was found in four different STs and not significantly associated with any CC. SCC*mec* 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 SCC*mec* (*P* < 0.001). No significant difference was found in the distribution of CCs and SCC*mec* types between Denmark and the Netherlands, although SCC*mec*V 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 ≥0.5 mg/L). In all SCC*mec*II-III isolates, oxacillin

MICs were high (≥2 mg/L), whereas 77% of other isolates had low MICs (<2 mg/L) (Figure 1). SCC*mec*IV 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 SCC*mec* 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 ≤ 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 SCC*mec*II-III ($P < 0.001$) and low-level resistance with SCC*mec*IV ($P = 0.007$). 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 ≥0.5 mg/L). High-level resistance to amoxicillin/clavulanic acid (MIC ≥4 mg/L) was significantly associated ($P < 0.001$) with SCC*mec*II-III of which 68% had high-level resistance. Susceptibility (MIC ≤0.25 mg/L) and low-level resistance to amoxicillin/clavulanic acid (MIC <4 mg/L) were seen in 24% and 68% of SCC*mec*IV isolates, in 25% and 50% of SCC*mec*V isolates and in 21% and 57% of non-typeable SCC*mec* isolates, respectively. SCC*mec*IV 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 ≥4 mg/L). Resistance was primarily seen in SCC*mec*II-III isolates (65%). One isolate with SCC*mec*IV (3%), three isolates with non-typeable SCC*mec* types (21%) and none of the SCC*mec*V isolates displayed resistance. The associations of SCC*mec*II-III with resistance and SCC*mec*IV with susceptibility were significant ($P < 0.001$).

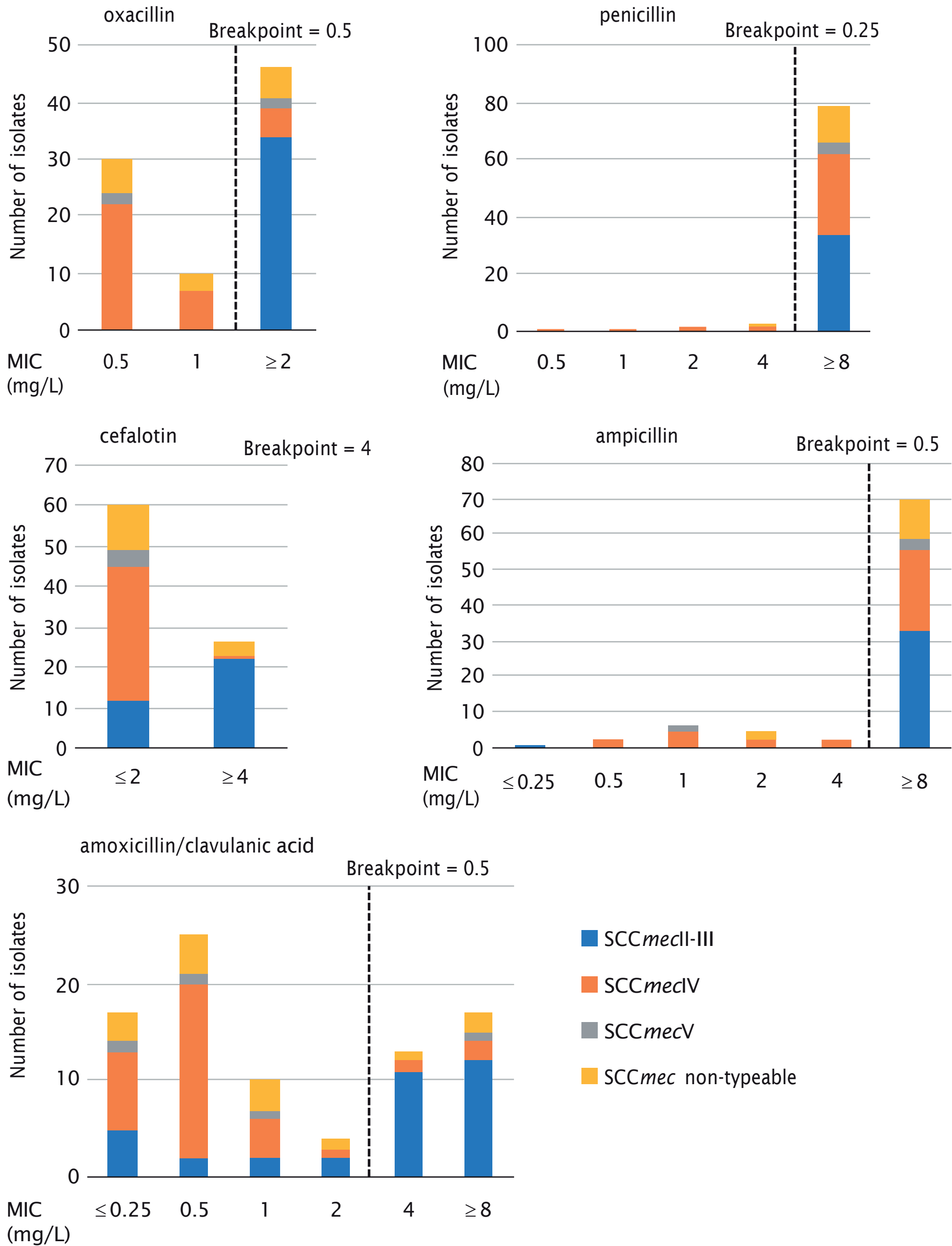

Figure 1. MIC distributions of b-lactam antibiotics for different SCC*mec* types. MICs of oxacillin, penicillin, cefalotin, ampicillin and amoxicillin/ clavulanic acid for SCC*mec*II-III, SCC*mec*IV, SCC*mec*V and non-typeable SCC*mec* types. The broken lines indicate the separation between high- and 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*.

4. Discussion

SCC*mec*II-III was only present in CC71, whereas SCC*mec*IV was associated with CC258 but also present in different STs. SCC*mec*V was shared by different STs. The SCC*mec* of the majority of CC45 isolates was non-typeable, which correlates with the previously described dissemination of novel SCC*mec* variants in CC45.[8,14] These results confirm a link between the SCC*mec* and CC in MRSP, as previously described.[8]

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

The association of SCC*mec*II-III and high MICs of b-lactams might have contributed to the success of CC71 globally. MIC differences of b-lactams between different SCC*mec* types have been previously reported for *S. pseudintermedius*, with SCC*mec*II-III having higher MICs of oxacillin and other b-lactams than SCC*mec*V.[7,8] This study confirms those findings while expanding the range of antimicrobials tested and the diversity of CCs and SCC*mec*.

It is unknown how SCC*mec* differences influence levels of b-lactam resistance in MRSP and why there are differences between b-lactams. In *S. aureus*, a role of the *mecA* promoter on the level of PBP2a production and oxacillin resistance level has been shown,[15] as well as overexpression of *blaZ* resulting in low-level methicillin resistance independently of *mec*A expression in borderline oxacillin-resistant isolates.[16] In *S. pseudintermedius*, *mecA* (*mecI* and *mecRI*) and *blaZ* (*blaRI* and *blaI*) regulators are known to modulate expression of *mecA*.[17,18] Altogether these studies suggest that the levels of b-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 b-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 b-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.[19] For urinary tract infections, anti-MRSA efficacy of high doses of amoxicillin/clavulanic acid has been shown.[20] For canine urinary tract infections, a higher breakpoint for *Staphylococcus* spp. exists (R >8 mg/L).[9] 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 b-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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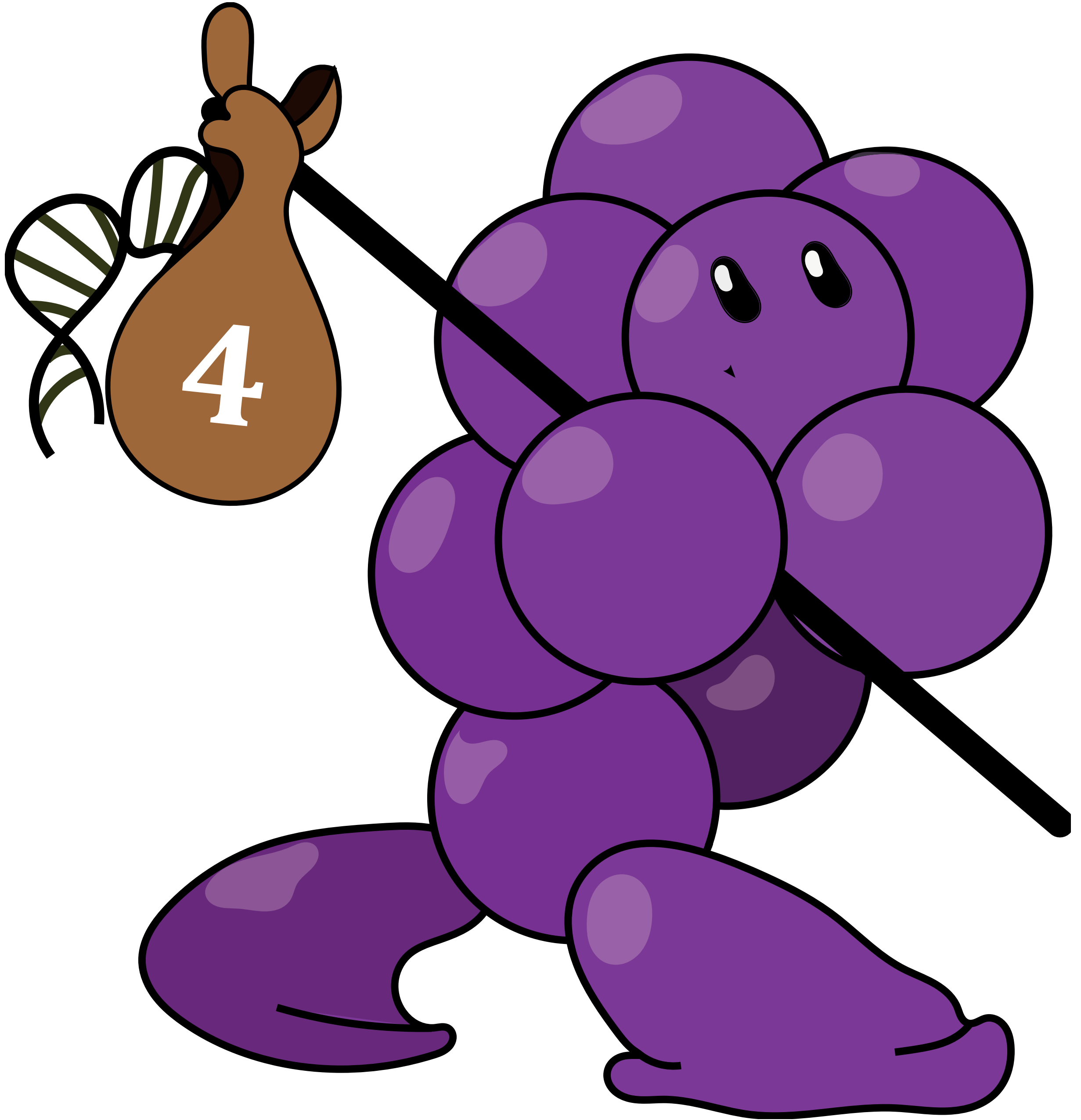
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CHAPTER 4

Absence of Host-Specific Genes in Canine and Human *Staphylococcus pseudintermedius* as Inferred from Comparative Genomics

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Abstract

Staphylococcus pseudintermedius is an important pathogen in dogs that occasionally causes infections in humans as an opportunistic pathogen of elderly and immunocompromised people. This study compared the genomic relatedness and antimicrobial resistance genes using genome-wide association study (GWAS) to examine host association of canine and human *S. pseudintermedius* isolates. Canine (n = 25) and human (n = 32) methicillin-susceptible *S. pseudintermedius* (MSSP) isolates showed a high level of genetic diversity with an overrepresentation of clonal complex CC241 in human isolates. This clonal complex was associated with carriage of a plasmid containing a bacteriocin with cytotoxic properties, a CRISPR-cas domain and a pRE25-like mobile element containing five antimicrobial resistance genes. Multi-drug resistance (MDR) was predicted in 13 (41%) of human isolates and 14 (56%) of canine isolates. CC241 represented 54% of predicted MDR isolates from humans and 21% of predicted MDR canine isolates. While it had previously been suggested that certain host-specific genes were present the current GWAS analysis did not identify any genes that were significantly associated with human or canine isolates. In conclusion, this is the first genomic study showing that MSSP is genetically diverse in both hosts and that multidrug resistance is important in dog and human-associated *S. pseudintermedius* isolates.

Keywords: *S. pseudintermedius*; comparative genomics; host association; antimicrobial resistance

1. Introduction

Staphylococcus pseudintermedius is found both as a commensal bacterium as well as an opportunistic pathogen in dogs. *S. pseudintermedius* in dogs is associated with skin, soft tissue and systemic infections similar to *S. aureus* infections in humans. Over the last decades, *S. pseudintermedius* is increasingly recognized as a potential zoonotic pathogen of canine origin in elderly and immunocompromised humans [1]. There is an increase in reports of *S. pseudintermedius* infections in humans, which might be at least partially explained by the implementation of MALDI-TOF MS in routine diagnostics facilitating proper identification of coagulase-positive staphylococci [2–5].

The epidemiology of *S. pseudintermedius* in human infections is poorly studied. Human infections have been reported to be mainly caused by methicillin-susceptible *S. pseudintermedius* (MSSP). This is often thought to result from transmission of MSSP between dogs and humans within the same household [6,7]. Transmission of methicillin-resistant *S. pseudintermedius* (MRSP) between dogs has been frequently observed, leading to long time carriage with possible re-infections. Dog-to-human transmission was infrequent and no long-term carriage in humans was observed [8]. A single case of human-to-human MRSP-transmission has been described [9].

In contrast with *S. aureus* which has been isolated from multiple host species and shows frequent acquisition, or loss of host-associated genes [10], *S. pseudintermedius* seems to be more host-restricted. However, information on the genetic variation and the mechanisms that allow adaption of MSSP to humans is scarce. A study on *S. pseudintermedius* adherence properties to corneocytes revealed a general preference for canine corneocytes compared to human corneocytes [11] and another study identified a cell-wall-associated protein with high binding strength to canine fibrinogen compared to fibrinogen of other host species [12]. In order to identify potential host-specific genes and clones we performed comparative genomics of methicillin-susceptible *S. pseudintermedius* from canine and human origin.

2. Results

2.1 MSSP Infections Determinants

Patient information was incomplete for 7/32 (22%) of the obtained human isolates. Most human MSSP isolates were from wound infections (*n* = 18). Other infections were ear (*n* = 2), joint (*n* = 2), skin (*n* = 2), systemic (*n* = 1), urinary tract (*n* = 1) and rectum infections (*n* = 1). The age of patients from 24 cases varied between 48 and 86 years; 1 patient was 6 years old (Supplementary Table S1). Patient isolates were obtained from hospitals located in six different provinces. The canine MSSP isolates were isolated from different body sites. The majority of canine isolates were from ear infections (*n* = 9), followed by skin infections (*n* = 8), wound infections (*n* = 5), urinary tract infections (*n* = 2) and a joint infection (*n* = 1) (Supplementary Table S1). All isolates were obtained between 2014 and January 2019. Isolates were isolated from different patients at different time points and are to the best of our knowledge unrelated epidemiologically.

2.2 *S. pseudintermedius* Phylogeny

The genetic relatedness of canine and human MSSP isolates is visualized in a phylogenetic SNP tree of the core genome (size of 2,170,170 bp) in comparison with included publicly available genomes [12–14] (Figure 1). This placed our results in a wide epidemiological context and shows that the isolates of this study are dispersed among the high genomic diversity of MSSP isolates.

To zoom in on the genome comparison of the dog and human isolates from this study the genotype details are shown in a phylogenetic tree in Figure 2. The extracted MLST types were superimposed, and the studied isolates belonged to 50 different sequence types (ST). The observed phylogenetic diversity between MSSP genomes was high overall and showed 1 cluster containing 10 genomes. This cluster, corresponding to clonal complex CC241 (comprising of ST241, ST941, ST1379, ST1350, ST1360), dominated, and consisted of seven human isolates (comprising of ST241, ST941, ST1379) and three canine isolates (comprising of ST241, ST1350, ST1360). While CC241 was overrepresented in humans, the difference in proportion of isolates from both hosts present in the CC241 cluster was not statistically significant ($p = 0.487$). Besides the genome cluster belonging to CC241, only two other genetically related pairs were identified, with 1 pair consisting of a canine and

a human isolate of ST989 with 55 SNP differences and 1 pair with 2 human isolates of ST985 from the same hospital with 8 SNP differences in their core genome. All other STs were represented by a single isolate (Figure 2).

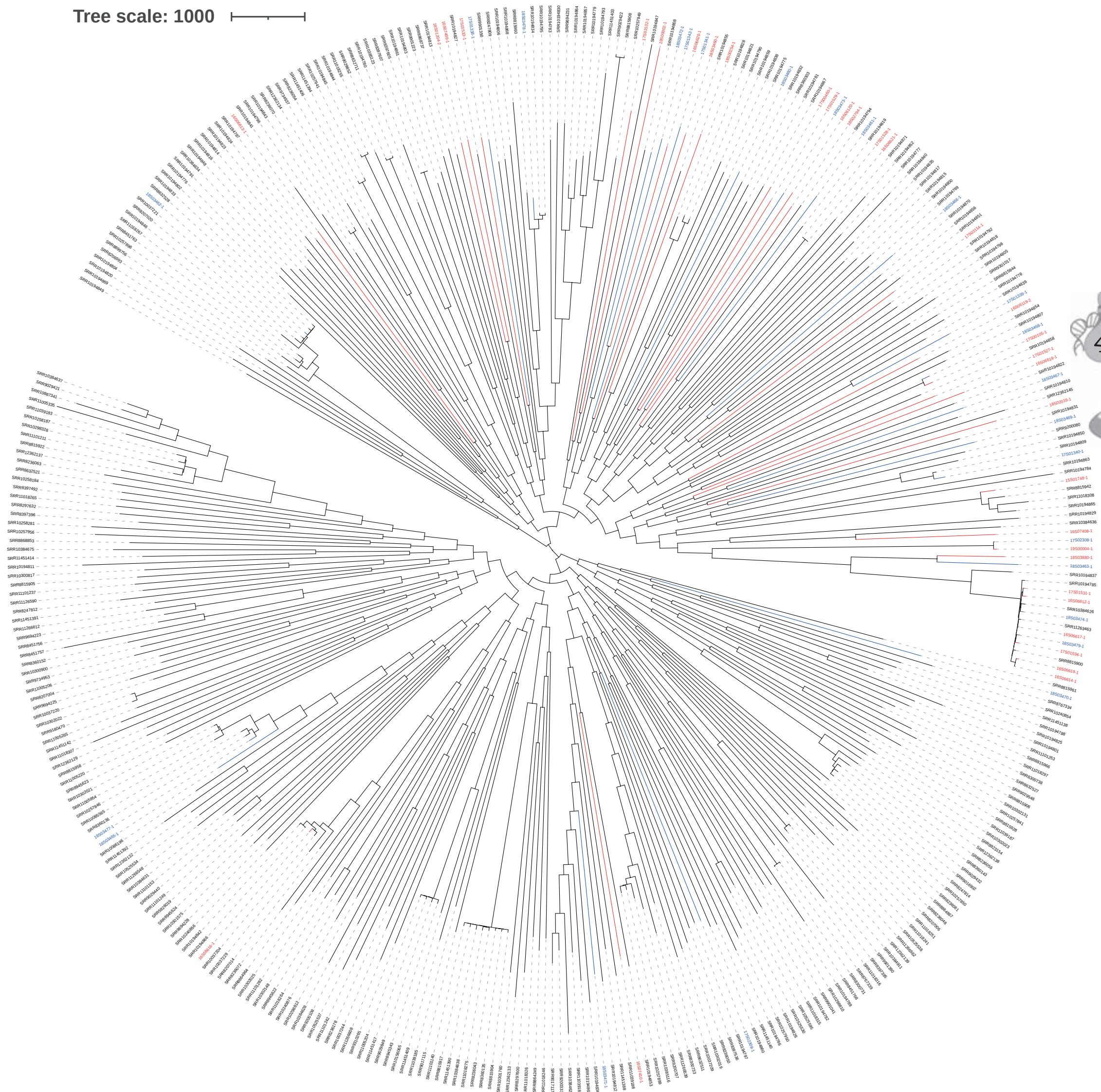

Figure 1. Phylogenetic tree based on the core genome SNPs of MSSP isolates. Publicly available MSSP genomes were compared with the MSSP isolates from this study, that have been marked red for human isolates and blue for dog isolates [12–14].

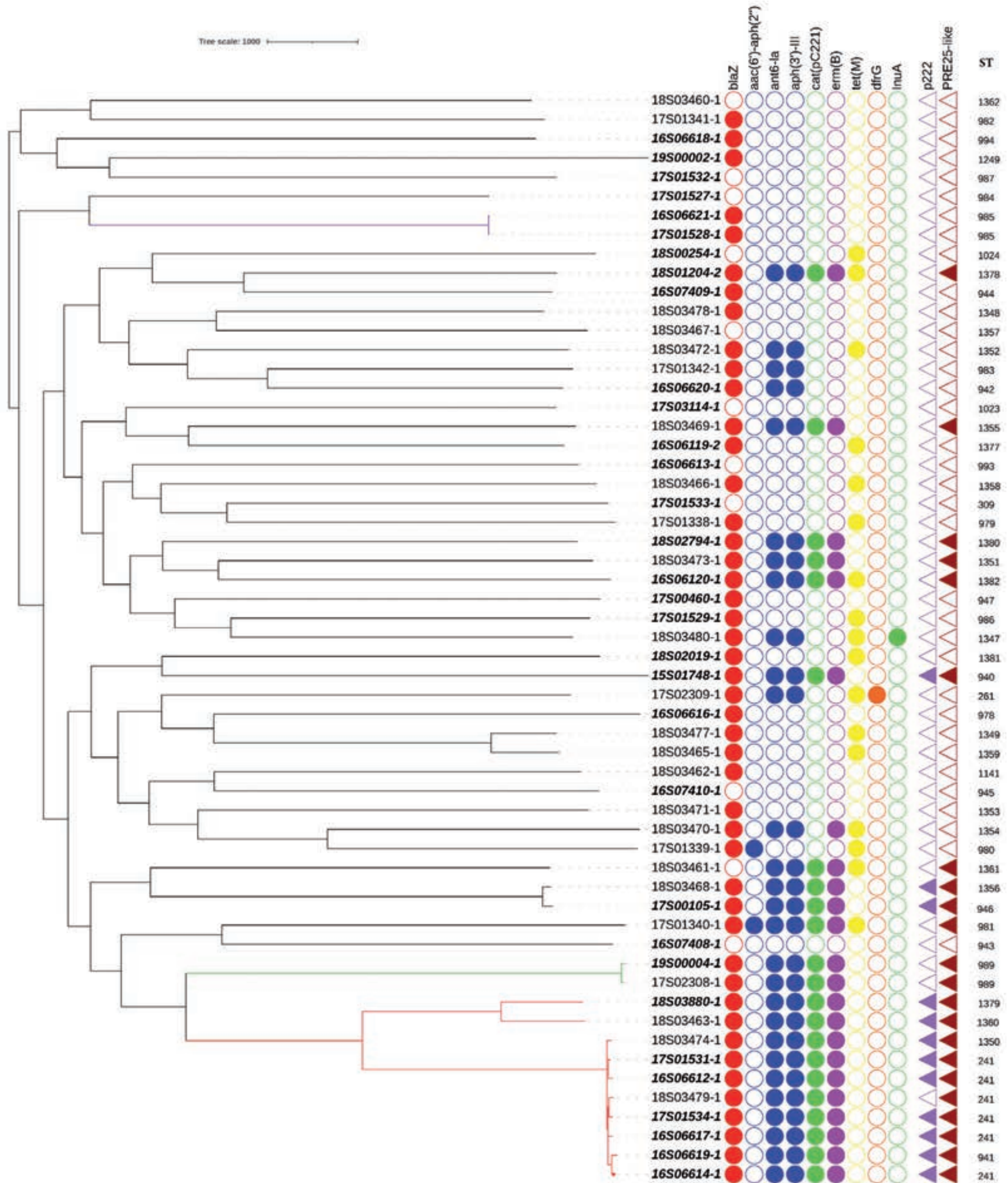

Figure 2. Isolate phylogeny and antimicrobial resistance genes.

Figure 2 shows a core-genome SNP tree of canine and humans (bold italic) isolates, presence (filled circles) or absence (empty circles) of antimicrobial resistance genes, presence of mobile genetic element PRE25-like [15] and plasmid P222 [16] (filled triangles), and sequence type (ST). Clones are shown by coloured branches (CC241 in red, ST989 in green, ST985 in purple). The length of the branch represents the number of SNPs.

2.3 Antimicrobial Resistance Genes

In *S. pseudintermedius* it has been shown that antimicrobial resistance genotypes can accurately predict phenotypical antimicrobial resistances [14,17,18]. Multidrug resistance (MDR) (i.e., carriage of resistance genes to 3 or more classes of antimicrobials) was identified in 13/32 (41%) human isolates and 14/25 (56%) canine isolates (Supplementary Table S1, Figure 1 and Table 1). No resistance genes were identified using Resfinder in 7/32 (22%) human isolates and in 2/25 (8%) canine isolates. Following the statistical analysis for host association, no statistically significant difference was found neither for the prevalence of predicted MDR in human versus canine isolates (p = 0.249), nor for isolates without resistance genes (p = 0.273).

Table 1. Antimicrobial resistance genes in human and canine *S. pseudintermedius* isolates.

Resistance	Gene	Human Isolates (n = 32)	Canine Isolates (n = 25)
β-lactam	*blaZ*	25 (78%)	23 (92%)
aminoglycoside	*ant6-Ia, aph(3′)-III*	14 (44%)	14 (56%)
	aac(6′)-Ie-aph(2″)-Ia	0	2 (8%)
chloramphenicol	$cat_{(pC221)}$	13 (41%)	9 (36%)
macrolide	*erm(B)*	13 (41%)	10 (40%)
tetracycline	*tet(M)*	6 (19%)	11 (44%)
lincosamide	*Inu(A)*	0	1 (4%)
folate inhibitor	*dfrG*	0	1 (4%)

The distribution of the identified antimicrobial resistance genes in MSSP from both hosts is presented in Table 1. The penicillin resistance gene *blaZ* was found in the majority of isolates from both hosts (48/57) (84%). Aminoglycoside resistance genes *ant6-Ia* and *aph(3')-III* were found in 14/25 (56%) of the canine isolates compared to 14/32 (44%) in human isolates. The gene *aac(6')-Ie-aph(2")-Ia* was detected in two canine isolates. The chloramphenicol resistance gene $cat_{(pC221)}$ was present in 13/32 (41%) of human isolates and 9/25 (36%) of canine isolates. The macrolide resistance gene *erm(B)* was present in 13/32 (41%) of human isolates and 10/25 (40%) of canine isolates. The tetracycline resistance gene *tet(M)* was present in 11/25 (44%) of the canine isolates and 6/32 (19%) of the human isolates. The folate pathway inhibitor resistance gene *dfrG* and lincosamide resistance gene *Inu(A)* were not present in human isolates and only in 1/25 (4%) of the canine

isolates. No statistically significant host association was found for the differences in resistance gene presence in canine and human isolates.

2.4 Host-Associated Genes

We did not identify gene presence or absence significantly associated with either canine or human *S. pseudintermedius* isolates using Roary (Table S2).

The genes *spsL* and *spsD* were studied specifically using alignments, as they were previously linked with host specificity [11,19]. The complete gene encoding the fibrinogen binding protein *spsD* was detected in 25 human isolates and 21 canine isolates. The fibronectin and fibrinogen binding protein *spsL* was present in 30 human isolates and 24 canine isolates. Alignment to reference genes *spsD* and *spsL* from ED99 showed a high level of sequence diversity across the gene with no clear clustering of variants of either dogs or human isolates (Figures S1 and S2).

As CC241 was overrepresented in human isolates, a GWAS study on orthologs associated with this clonal complex was performed that identified several genes shown in Table S3. In summary, 9 out of 10 CC241 isolates carried the plasmid p222 (99% identity and 97% coverage with the reference plasmid) encoding a bacteriocin with cytotoxic effect that was previously identified in a canine *S. pseudintermedius* isolate [16]. This p222 plasmid was also present in three (6%) non-CC241 isolates (two human (ST940 and ST946) and one canine (ST1356) isolate). The pRE25-like element that has been described as a chromosomal element carrying five resistance genes; *erm(B)*, *cat(pC221)*, *aph(3')-III*, *ant6-Ia*, *sat4* and a toxin antitoxin system [15] was also associated with CC241 isolates. Sequence homology (>90%) with this element was detected in all CC241 isolates (n = 10) as well as in seven non-CC241 human isolates belonging to other sequence types belonging to ST940, ST946, ST989, ST1378, ST1380, ST1382, and in six non-CC241 canine isolates belonging to ST981, ST989, ST1351, ST1355, ST1356, and ST1361. Despite the sequence homology, all isolates missed one or more transposase genes (Table S3). Manual investigation of the sequence assembly graphs using Bandage [20] revealed that collapsed repeats were the reason for these missing genes, a common issue with assembly from short-read sequences.

Next to the p222 plasmid and the pRE25-like element, a CRISPR-cas-type-III region was associated with CC241 and present in nine out of ten CC241 isolates. This CRISPR type was present as well in three other human isolates belonging to

ST309, ST943 and ST946. All genes associated with either presence or absence in CC241 in the GWAS analysis are presented in Table S3.

3. Discussion

Until a decade ago, human infections with *S. pseudintermedius* were only seldom reported, but the number of reports has markedly increased over the last decade, which could partially be linked to the use of MALDI-TOF [2–5]. Although zoonotic transmission from dogs to humans is generally suspected very few studies have compared the genetic characteristics of *S. pseudintermedius* isolates originating from human infections versus canine sources.

This study revealed that CC241 was overrepresented in the studied MSSP isolates and was composed mainly of human isolates. It is of interest to monitor the MDR CC241 clone as it carries genetic elements which can confer a number of selective advantages. First, the identification of a CRISPR-cas system associated with CC241 might increase genetic stability of CC241 which was shown for MRSP earlier [18], but not previously observed in MSSP. A second potential selective advantage could be the association of CC241 with plasmid p222, which contains BacSp222, a multifunctional peptide that functions as a bacteriocin against Gram-positive bacteria and is a virulence factor affecting eukaryotic cells [16]. A third potential selective advantage could be the multidrug resistance provided by the pRE25-like mobile element carrying five resistance genes coding for resistance to four antimicrobial classes [15]. This element with the IS1216 transposase on both ends is highly related to the pRE25 plasmid of *Enterococcus faecalis* and has recently been detected in canine isolates of *S. pseudintermedius* in Korea [15]. In the present study this pRE25-like element was present in all CC241 isolates, but was not limited to this clone, as it was present in 12 non-CC241 isolates as well. In the Korean study the STs of pRE25-like element containing isolates were diverse, but a selective advantage leading to dominance of clones containing this element was suggested [15]. To study in more detail the dissemination of MDR and the pRE25-like element we compared our isolate with two large studies with published canine MSSP genomes. The first one was in Europe where 8% of MSSP isolates were MDR and none had genes associated with pRE25-like (*erm(B)*, $cat_{(pC221)}$, *aph(3')-III*, *ant6-Ia*, *sat4*) [13]. The second in the USA showed 22.1% MDR among isolates containing *erm(B)*, $cat_{(pC221)}$, *aph(3')-III*, *ant6-Ia*, *sat4*, and 11

isolates carried the pRE25-like element [14]. Alignment with this element showed high homology with the pRE25-like elements identified in CC241 isolates in this study, and 4/11 isolates belonged to CC241 and the others belonged to various sequence types. The multiple findings of the pRE25-like element indicates that it is present in distinct MSSP lineages and most likely contributes to dissemination of resistance genes in MSSP, in the same way as has been observed for the resistance element carrying Tn5405 in MRSP [21]. The carriage of each of these elements will result in multidrug resistance and could limit treatment options for MSSP infections. The spread of this element among other human isolates could not be studied as none of the four publicly available MSSP genomes were multidrug resistant [12]. A similar finding of low phenotypic multidrug resistance (2 of the 24 tested isolates) has been reported for other human MSSP isolates [6].

Furthermore, we observed a high diversity in MSSP amongst both canine and human isolates, which confirms the diversity reported for canine MSSP isolates in a core genome MLST analysis [13]. To exclude that geographical differences are responsible for the observed diversity, a SNP phylogeny with publicly available MSSP genomes and our isolates was constructed. This confirmed the high diversity with small lineages, indicating that there is no geographical cluster of Dutch isolates, as they were dispersed over the tree. It also indicated that the lineage associated with CC241 was still overrepresented in human isolates [13,14] (Figure S3). This high diversity in MSSP is in contrast with the observed clonal dissemination of MRSP [13]. The phylogeny based on SNPs in the MSSP core genomes identified a deep-branched structure corresponding with a distinct MLST type for almost all isolates, whereas MRSP isolates mainly belong to a limited number of clonal complexes [13]. The genetic stability within certain MRSP clones might be explained by the carriage of lineage-specific prophages, restriction–modification or CRISPR/Cas systems hindering DNA uptake [18]. Furthermore, MRSP isolates often harbour resistance genes to multiple antimicrobial classes and use of any antimicrobial might co-select for the spread of these MDR isolates [21,22]. These elements might also explain the dominance of CC241 in our selection of MSSP isolates as all CC241 isolates were predicted MDR and contained lineage-specific elements, but were remarkably mainly found in human isolates (7/10). CC241 has also been described in a human infection in Spain [7], indicating a wider presence which is noticeable given the genetic diversity of MSSP.

Currently the number of *S. pseudintermedius* genomes from human infections is very low. This study provides the first genome comparison of human MSSP

isolates, and analysis of more human isolates will be needed to unravel the importance of CC241 or other lineages in human MSSP infections.

GWAS analysis based on gene presence/absence showed no host association of specific genes which could be explained by the limited sample size but could also be due to the absence of host adaptation. The high sequence diversity of MSSP found in both sources might also play a role in the difficulty of finding host association. As an example, the genes encoding fibrinogen binding proteins (spsD and spsL) that were previously shown to be host associated [11,19] showed a high level of diversity, in both canine and human isolates (Figures S1 and S2). While these genes have been reported to contain repeat regions which might account for some diversity [23], in our isolates genetic variation was not limited to those regions. This variability in fibronectin binding protein encoding genes has not been previously reported. Studying allele variants rather than analysing gene presence/absence could be necessary to identify host-associated genes using, e.g., k-mer approaches. Several hundred isolates would probably be required to detect statistically significant associations because of the more severe multiple testing correction, as was shown when evaluating a k-mer-based approach in *Streptococcus pneumoniae* [24]. Analysis of protein variants or differences in protein domains, such as has been observed for leucocidins in *S. aureus* and *S. pseudintermedius* [25,26], was beyond the scope of this study.

No statistically significant difference was found between dog and human isolates in the carriage of resistance genes. Multidrug resistance was present in 41% of human isolates and 56% of canine isolates. The *tet(M)* gene was found slightly more often in dogs than in humans even though this was not statistically significant. Resistance genes, not located on pRE25-like elements, encoding for resistance against aminoglycosides (*aac(6')-Ie-aph(2")-la*), lincosamides (*Inu(A)*) and folate pathway inhibitors (*dfrG*), were only found in non-CC241 canine isolates. However, the sample size of human isolates was rather small. The prevalence of these genes was low in canine isolates which might explain why these genes were not identified in human isolates in this study.

4. Materials and Methods

4.1 Bacterial Isolates

In total, 57 MSSP isolates were included, 32 *S. pseudintermedius* isolates from human infections obtained from 6 Dutch hospitals between 2014 and January 2019, and 25 *S. pseudintermedius* isolates from canine infections isolated at the Veterinary Microbiological Diagnostic Centre were selected to match the years of isolation of human isolates. All isolates were selected based on convenience sampling and epidemiologically unrelated. Species identification was confirmed by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF MS) (Bruker MALDI Biotyper, Bruker Daltonics, Billerica, MA, USA).

4.2 Genome Analysis

DNA was isolated using the Qiagen UltraClean Microbial DNA isolation kit (Qiagen, venlo, the Netherlands. DNA libraries were prepared with the Illumina Nextera kit according to manufacturer's instructions and sequenced using NextSeq sequencing with 150 base pairs reads (Illumina, San Diego, CA, USA). Reads-quality-check and adapter trimming was performed with Trim Galore v0.4.4 (https://www.bioinformatics.babraham. ac.uk/projects/trim_galore/) Last update: 24 March 2017. The genomes were assembled with SPAdes v3.10.1 [27], and contigs smaller than 200 base pairs and with a coverage lower than 10 were removed. Genome quality was assessed with CheckM v1.1.2 [28] for completeness (>95%) and contamination (<5%). The genomes were annotated using Prokka v1.13 [29]. The batch upload function, including Resfinder and MLSTFinder, from the Center for Genomic Epidemiology (CGE) (Copenhagen, Denmark) [30] was used to analyse the resistance gene content and sequence types of all isolates (last accessed on 12 March 2019). In case of new sequence types, the alleles for Multi Locus Sequence Type (MLST) were assigned an ST number by the curator of the PubMLST database (https://pubmlst.org/spseudintermedius/) (accessed on 5 March 2019).

The whole genome sequences were aligned, and the core genome size determined, using Parsnp v1.2 [31] for phylogenetic single-nucleotide polymorphism (SNP) analysis of the core genome that was visualised using ITOL v4 [32] and a minimum spanning tree was made with Phyloviz 2.0 [33] using the goeBURST algorithm to

assess the number of single-nucleotide polymorphisms between isolates. Orthology predictions of the annotated genomes were made using Roary 3.12.0 [34] and host and clonal complex associated genes were determined using Scoary 1.6.16 [35] applying a threshold of $p < 0.05$ for statistical significance using Bonferroni correction. Identification of regions with significant genes was performed with a BLAST search against the NCBI database, resulting in the identification of two mobile elements (identity > 90% coverage > 80%). Mobile elements with Genbank accessions CP011490 and MK775653 were used as reference sequences for alignment to the MSSP sequence contigs using Geneious version 2020.1.1 (Biomatters, Auckland, New Zealand). Annotation of the reference element was used to predict the annotation of orthologous genes; if annotation in the reference was absent the annotation of Prokka was used. Statistically significant genes annotated as coding for hypothetical proteins by Prokka were further analysed using NCBI BLASTn 2.10.1. Geneious was also used for the alignment of *spsL* and *spsD* genes to their references in ED99 strain CP002478 and for construction of the *spsL* and *spsD* trees.

Pearson's chi-square or Fisher's exact test (when sample size <5) were used for statistical analysis for host association of resistance genes. A *p*-value of <0.05 was considered statistically significant with Bonferroni correction.

4.3 Data Availability

Whole genome sequence reads and assembled contigs have been deposited in the NCBI Sequence Read Archive under project number PRJEB39511, accession numbers are available in Supplementary Table S1.

5. Conclusions

In conclusion, canine and human *S. pseudintermedius* from the Netherlands were genetically highly diverse, and no host-specific genes could be detected. CC241 was overrepresented in human isolates. The CC241 isolates carried BacSp222, a bacteriocin with cytotoxic activity and the mobile genetic pRE25-like element carrying five resistance genes, which was more widely present in the MSSP population. The dissemination of the pRE25-like element could pose a threat for treatment of human and canine *S. pseudintermedius* infections.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/antibiotics10070854/s1, Table S1: Isolate information, resistances and mobile elements in human and canine MSSP isolates, Table S2: Roary-Scoary analysis of dog and human isolates. Table S3: Genome wide association study of CC241 defined with Roary-Scoary analysis. Figure S1: Tree of *spsD* genes of dog and humans. Figure S2: Tree of *spsL* genes of dog and humans. Figure S3: Phylogenetic tree of MSSP isolates.

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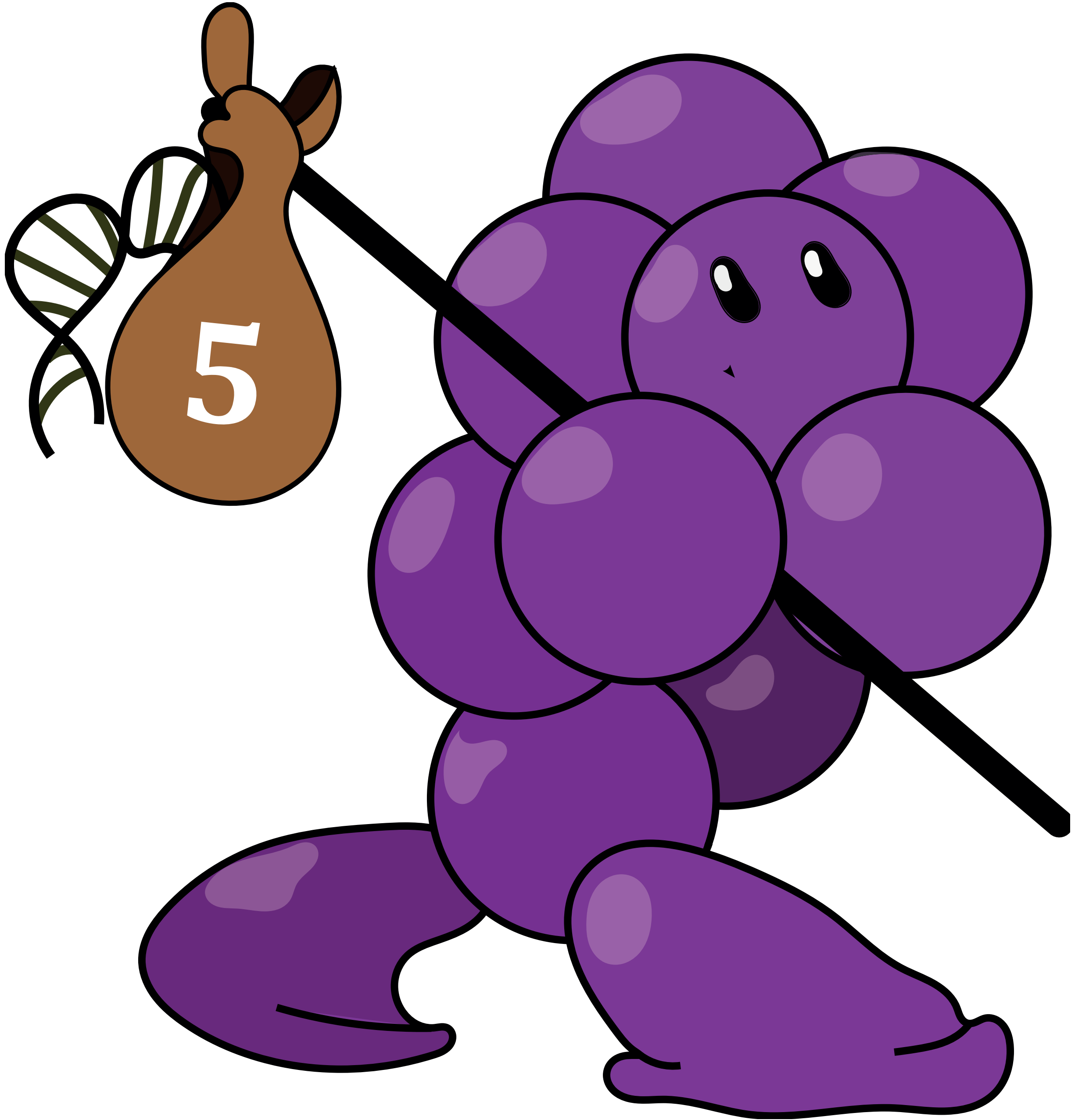
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CHAPTER 5

Within-household transmission and bacterial diversity of *Staphylococcus pseudintermedius*

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Abstract

Staphylococcus pseudintermedius can be transmitted between dogs and their owners and can cause opportunistic infections in humans. Whole genome sequencing was applied to identify the relatedness between isolates from human infections and isolates from dogs in the same households. Genome SNP diversity and distribution of plasmids and antimicrobial resistance genes identified related and unrelated isolates in both households. Our study shows that within-host bacterial diversity is present in *S. pseudintermedius*, demonstrating that multiple isolates from each host should preferably be sequenced to study transmission dynamics.

Keywords: *S. pseudintermedius*; transmission; One health; whole genome sequencing; zoonotic; bacterial diversity

1. Introduction

Staphylococcus pseudintermedius is both a commensal and opportunistic pathogen in dogs. Infections in humans are occasionally found; however, in humans, *S. pseudintermedius* might be underdiagnosed as it can be misidentified as *Staphylococcus aureus* or *Staphylococcus intermedius* [1,2]. Human infections with *S. pseudintermedius* are generally considered to be of zoonotic origin [3], although in exceptional cases no dog contact is reported [4]. Dog-to-human transmission of *S. pseudintermedius* has been reported, in which isolates from dogs and their owners were indistinguishable based on multi-locus sequence typing and pulsed field gel electrophoresis [4,5]. Nevertheless, carriage rates of *S. pseudintermedius* in humans remain very low compared to the carriage rates of dogs, even in dog owning households [6]. Longitudinal studies on methicillin-resistant *S. pseudintermedius* (MRSP) showed that dogs carried MRSP for prolonged periods of time (several months), whereas carriage in humans was rare and short-term. Human carriage is therefore considered to be contamination instead of colonization, though opportunistic infections in humans can occur [5,7]. In longitudinal studies, MRSP was found in the environment and in other dogs in the household [7,8]. Generally, isolates within one household belong to the same sequence type (ST), although occasionally different STs can be found in the same household [5]. Most studies on dog-to-human transmission of *S. pseudintermedius* include only a single isolate from each host. This approach might lead to misinterpretations when within-host bacterial diversity exists. We used whole genome sequencing of multiple isolates from dogs to investigate within-household transmission and bacterial diversity of *S. pseudintermedius* in two unrelated human infections caused by *S. pseudintermedius* and the dogs in these households.

2. Results

2.1 Household 1

Patient 1 was a 64-year-old woman with a wound infection on her foot in June 2016. One dog, suffering from a chronic skin condition, was present in the household. *S. pseudintermedius* was isolated from three sampling sites and multiple isolates were selected for genome analysis (n = 5 from each site) based on

morphological colony differences. This provided insight into the number of single nucleotide polymorphisms (SNP) in isolates from this dog. Dog isolates belonged to two clades that differentiated by 6913 core-genome SNPs. One clade consisted of six dog isolates (obtained from perineum and axillary) that displayed a very low level of diversity (differing by up to 5 SNPs) and belonged to ST387 (Figure 1). All six dog isolates carried the *blaZ* resistance gene and no plasmid was detected.

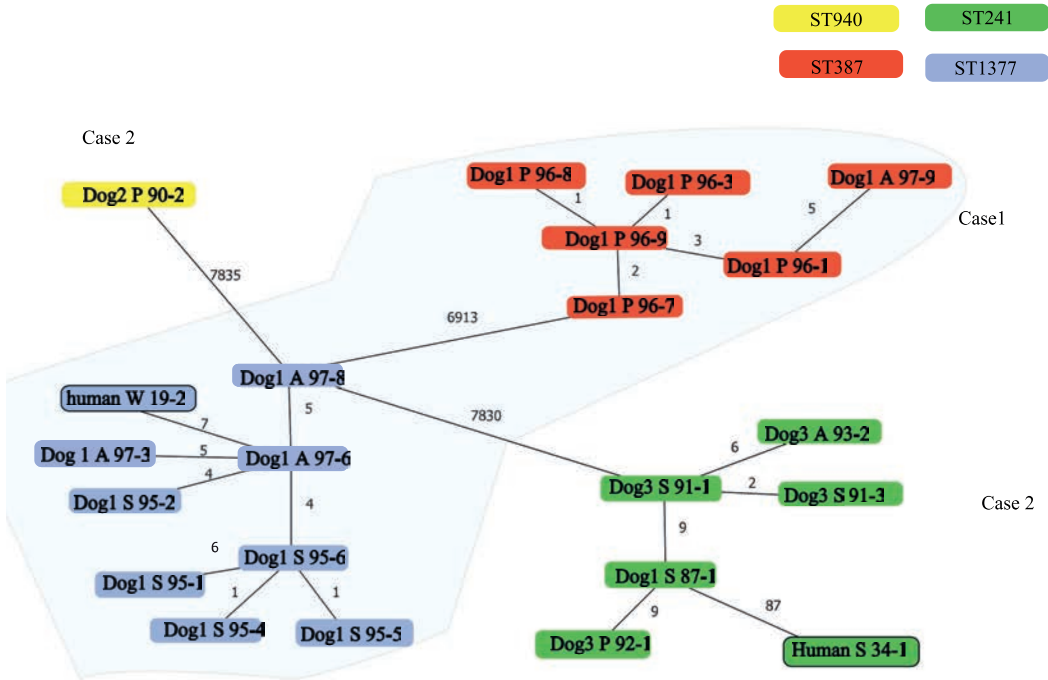

Figure 1. Minimum spanning tree of core-genomes showing the phylogenetic relationship between isolates from the two households, with the number of SNPs indicated on the branches. Isolates are identified by host species, followed by isolation site A = axillary, P = perineum, S = skin, W = wound, and lastly followed by the last three digits of their isolate number. Isolates from household 1 are shown against a blue background. Isolates from household 2 are shown against a white background. Isolates with no SNP differences are not shown.

In the other clade, the human isolate and nine of the dog isolates, obtained from the skin and axillary, differentiated between 0 and 7 core-genome SNPs and belonged to ST1337 (Figure 1). All isolates carried the resistance gene *tet(M)*, and all but one (16S06095-5) isolate carried the *blaZ* gene. The human isolate carried the *blaZ* and *tet(M)* resistance genes, no plasmid sequences, and differed by 7 SNPs from a dog isolate from the same household that also carried these genes and no plasmid sequences (Table 1).

2.2 Household 2

Patient 2 was a 63-year-old woman with an infected skin ulcer in July 2017. Three dogs were present in the household. No clinical conditions were reported for the dogs. All dogs were found to be positive for *S. pseudintermedius*, but not for all sites. Selection of morphologically different colonies resulted in one isolate from the skin of dog 1, one isolate from the perineum of dog 2, and 5 isolates from the skin (n = 3), the perineum (n = 1), and axillary (n = 1) of dog 3.

The human isolate, and all isolates from dog 1 and dog 3, belonged to ST241. The ST241 isolates from dog 1 and dog 3 differed by between 0 and 9 SNPs, whereas the human isolate showed 87 SNPs differed from its closest related canine isolate (dog 1). In the MS-tree, the SNPs were filtered for recombination and the 87 SNPs between dog and human isolates were not clustered in one location on the genome, indicating that these SNPs were not the result of a single recombination event. Isolate 17S01590-2 of dog 2 displayed 7835 SNPs compared to its closest relative, belonged to ST940, and was considered genetically unrelated to other isolates (Figure 1). All isolates of household 2 carried the p222 plasmid (coverage 97%, identity 99%) [9] and other predicted plasmid sequences. The BLASTn analysis of these contigs identified sequence homology with the PRE-25-like element [10], carrying *sat4*; *ant(6)-Ia*; *aph(3')-III*; *cat(pC221)*; and *erm(B)* resistance genes (coverage 67.9%, identity 99.9%) in all these isolates. A 2.7 kb plasmid sequence in all ST241 dog isolates belonged to the rep21 gene plasmid family (Table 1).

3. Discussion

Whole genome sequencing of *S. pseudintermedius* isolates from two unrelated human infections showed very low SNP diversity with canine isolates of colonized dogs in both households. The isolates retrieved from the human infections were considered genetically related to the isolates of the dogs. This is in accordance with longitudinal studies on MRSP showing that generally similar or indistinguishable *S. pseudintermedius* isolates can be present in humans, dogs, and environmental samples within the same household [5,7].

This study analyzed multiple dog isolates in one household, as it is known that inferring transmission by sequencing single colonies can be hindered by within-

Table 1. Isolate characteristics

Isolate	Origin	Isolation Date	Specimen	MLST	Resistance Genes					Mobile Elements
Household 1										
16S06119-2	human	June 2016	wound	1377	*blaZ*		*tet*(M)			
16S06095-1	dog 1	June 2016	skin	1377	*blaZ*		*tet*(M)			
16S06095-2	dog 1	June 2016	skin	1377	*blaZ*		*tet*(M)			
16S06095-4	dog 1	June 2016	skin	1377	*blaZ*		*tet*(M)			
16S06095-5	dog 1	June 2016	skin	1377			*tet*(M)			
16S06095-6	dog 1	June 2016	skin	1377	*blaZ*		*tet*(M)			
16S06097-3	dog 1	June 2016	axillary	1377	*blaZ*		*tet*(M)			
16S06097-6	dog 1	June 2016	axillary	1377	*blaZ*		*tet*(M)			
16S06097-7	dog 1	June 2016	axillary	1377	*blaZ*		*tet*(M)			
16S06097-8	dog 1	June 2016	axillary	1377	*blaZ*		*tet*(M)			
16S06096-1	dog 1	June 2016	perineum	387	*blaZ*					
16S06096-3	dog 1	June 2016	perineum	387	*blaZ*					
16S06096-7	dog 1	June 2016	perineum	387	*blaZ*					
16S06096-8	dog 1	June 2016	perineum	387	*blaZ*					
16S06096-9	dog 1	June 2016	perineum	387	*blaZ*					
16S06097-9	dog 1	June 2016	axillary	387	*blaZ*					
Household 2										
17S01534-1	human	July 2017	skin	241	*blaZ*	*sat4*	*cat*$_{(pC221)}$	*erm*(B)	*ant(6)-Ia,aph(3′)-III*	PRE25-like; p222
17S01587-1	dog 1	July 2017	skin	241	*blaZ*	*sat4*	*cat*$_{(pC221)}$	*erm*(B)	*ant(6)-Ia,aph(3′)-III*	PRE25-like; p222; 2,7 kb plasmid
17S01591-1	dog 3	July 2017	skin	241	*blaZ*	*sat4*	*cat*$_{(pC221)}$	*erm*(B)	*ant(6)-Ia,aph(3′)-III*	PRE25-like; p222; 2,7 kb plasmid
17S01591-2	dog 3	July 2017	skin	241	*blaZ*	*sat4*	*cat*$_{(pC221)}$	*erm*(B)	*ant(6)-Ia,aph(3′)-III*	PRE25-like; p222; 2,7 kb plasmid
17S01591-3	dog 3	July 2017	skin	241	*blaZ*	*sat4*	*cat*$_{(pC221)}$	*erm*(B)	*ant(6)-Ia,aph(3′)-III*	PRE25-like; p222; 2,7 kb plasmid
17S01592-1	dog 3	July 2017	perineum	241	*blaZ*	*sat4*	*cat*$_{(pC221)}$	*erm*(B)	*ant(6)-Ia,aph(3′)-III*	PRE25-like; p222; 2,7 kb plasmid
17S01593-2	dog3	July 2017	axillary	241	*blaZ*	*sat4*	*cat*$_{(pC221)}$	*erm*(B)	*ant(6)-Ia,aph(3′)-III*	PRE25-like; p222; 2,7 kb plasmid
17S01590-2	dog 2	July 2017	perineum	940	*blaZ*	*sat4*	*cat*$_{(pC221)}$	*erm*(B)	*ant(6)-Ia,aph(3′)-III*	PRE25-like; p222

host bacterial diversity [11,12]. The SNP diversity in the genomes between several of the studied dog isolates in household 1 was very low, most likely reflecting the diversity that occurs during colonization. However, the genomes with higher SNP diversity (6913 and 7835) indicated that dogs were colonized with genetically unrelated isolates. This highlights the need for sequencing multiple isolates from dogs to investigate household transmission. SNP diversity correlated with assigned MLST sequence types as isolates from the same ST generally carried less than 10 SNP differences, whereas isolates with different STs differentiated by either 6913 or 7835 SNPs. Sequence type and SNP differences between MSSP isolates of different body sites were also observed, with dogs being positive for either one or multiple body sites, with different frequencies for each site [13]. This study also showed that isolates presenting morphological differences can be very closely related.

Mobile genetic elements were identified in all isolates from household 2: the p222 [9] and the PRE25-like elements. The presence of these elements in CC241 isolates, and the presence of this clonal complex in human isolates, has been previously reported [14]. The plasmid present in dog isolates in household 2 was absent in the ST241 human isolate and shows that gain or loss of a plasmid occurred among highly genetically related isolates. This is in line with observed gene loss or acquisition events in *S. aureus,* which is involved in the host jump of CC398 from livestock to human, and there are other examples of gene acquisitions in *S. aureus* that have facilitated adaptations to other animal species [15,16]. The mobile elements in *S. pseudintermedius* carrying multiple resistance genes and potential virulence genes are important epidemiological markers to monitor, as they can act as a reservoir for transmission to humans [14]. Nevertheless, the genome comparison showed that ST241 isolates from dogs in household 2 were more closely related to each other (<10 SNPs) than to the human isolate (87 SNPs). The higher SNP diversity might suggest that evolution occurred over the course of infection, but as the patient was only sampled once this could not be confirmed. Furthermore, as the dogs in this study were sampled within the same month the patients were hospitalized, it is impossible to infer the direction and timing of transmission. It would be interesting to have multiple samples from the human to see if the diversity observed in the dog is also present in human hosts. Larger studies using whole genome sequencing combined with epidemiological data would be of interest to determine if SNP differences between related isolates are common and can indicate the direction of transmission.

4. Materials and Methods

4.1 Bacterial Isolates

S. pseudintermedius isolates from two human patients were obtained from the Amsterdam UMC location AMC in the Netherlands. Both patients were dog owners and gave their consent for samples from their dog(s) to be taken. Dogs (one in household 1 and three in household 2) were sampled by the owner at three body sites (skin, perineum, and axillary) within a month of confirmed infection of the owner. Samples were inoculated on sheep blood agar (bioTRADING, Mijdrecht, The Netherlands) and, after overnight incubation at 37 °C, presumptive colonies were identified as *S. pseudintermedius* by Maldi-TOF (Bruker MALDI Biotyper, Bruker Daltonics, Bremen, Germany). In each sample, all morphologically distinct colonies were selected for identification, resulting in multiple isolates per sample and per dog. The characteristics of the isolates are shown in Table 1.

4.2 Molecular Analysis

For whole genome sequencing, DNA was isolated using the Qiagen DNA isolation kit (Qiagen, Venlo, The Netherlands). DNA libraries were prepared with the Illumina Nextera kit according to manufacturer's instructions and sequenced with NextGen paired-end sequencing with 150 bp reads (Illumina, San Diego, CA, USA). Genomes were assembled with SPAdes v3.10.1 [17] and annotated using Prokka v1.13 [18]. Resistance genes were determined using Resfinder [19] and Multi Locus Sequence Type (MLST) was determined with MLSTFinder [20]. Core–gene alignment was performed using Parsnp v1.2 [21]. Gubbins was used to filter recombination regions [22]. SNPs were extracted from the core–gene alignment using SNP-sites v2.4.0 [23] and a minimum-spanning tree (MST) was constructed using the goeBURST algorithm and visualized using Phyloviz v2.0 [24]. Plasmid content was determined using RFPlasmid with a minimum plasmid prediction cut-off of 0.6 and a minimum length of 1 kb [25]. The plasmid contigs were characterized using BLASTn.

Isolates containing the combination of resistance genes *ant(6)-Ia*; *aph(3')-III*; *cat(pC221);* and *erm(B)* were analyzed for the presence of the pRE25-like element. This element has been previously described in *S. pseudintermedius* and was identified using Geneious version 2020.1.1 (Biomatters, Auckland, New Zealand).

4.2 Data Availability

Whole genome sequence reads of the canine isolates have been uploaded in ENA under bio project PRJEB53745 and the human isolates were previously uploaded under the following accession numbers: 17S01534-1 (GCA_903992455.1), 16S06119-2 (GCA_903991985.1).

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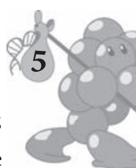

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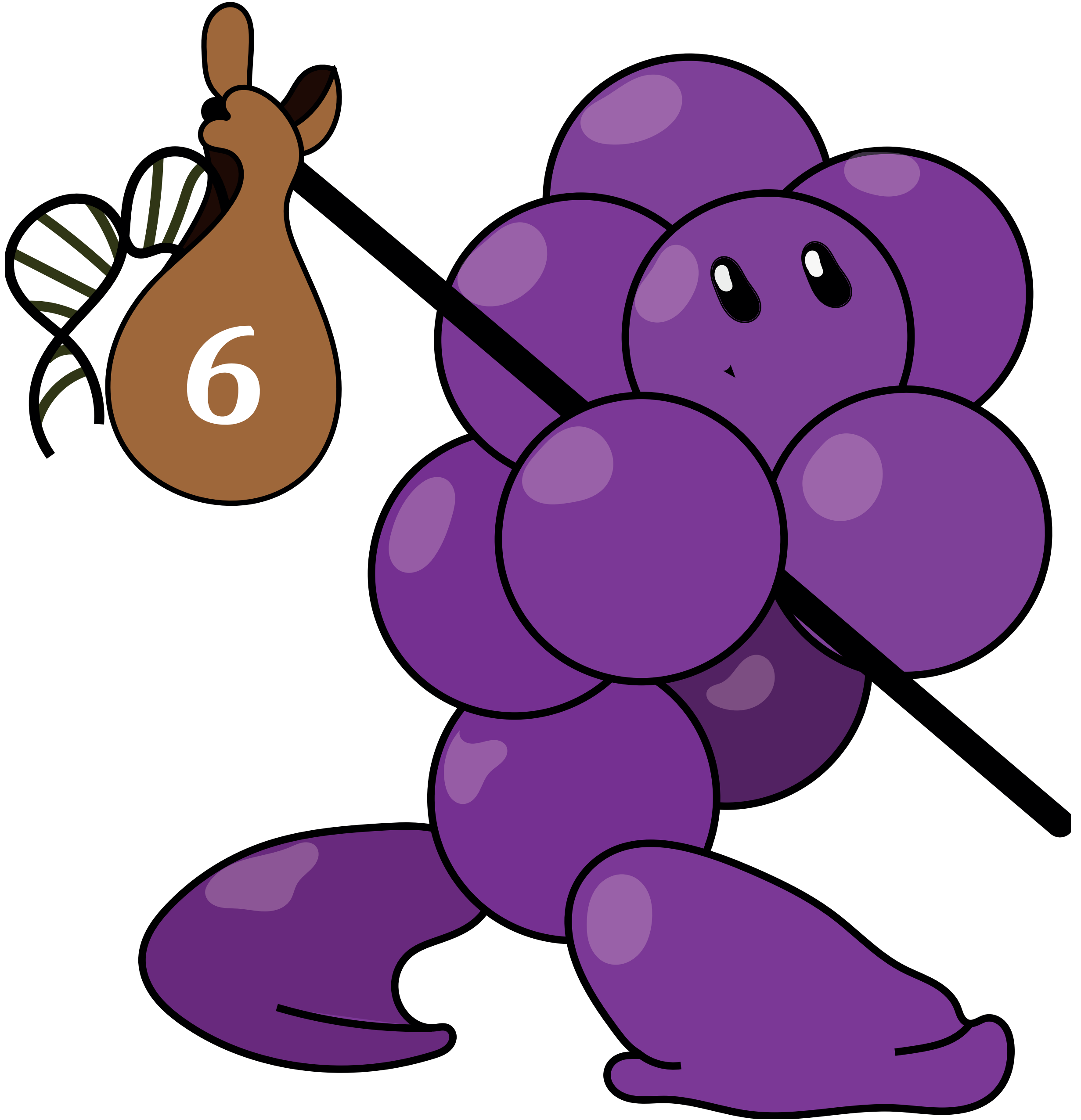
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CHAPTER 6

Microevolution of mobile elements carrying antimicrobial resistances in *Staphylococcus pseudintermedius*

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Abstract

Mobile genetic elements are important vehicles for dissemination of antimicrobial resistance (AMR) in *Staphylococcus pseudintermedius,* an opportunistic pathogen for dogs and occasionally humans and a reservoir of AMR genes for other pathogens. We performed a comprehensive genomic analysis on 223 clinical isolates from dogs and humans and resolved complete mobile genetic elements in 25 representative isolates using Nanopore long-reads. Phylogenetic reconstruction based on single nucleotide polymorphisms (SNP) showed that methicillin-resistant (MRSP) isolates diversified multiple times from methicillin-susceptible (MSSP) isolates. Multidrug-resistance (MDR) was associated with the integration or introduction of composite transposable elements carrying various resistance genes with Tn*554*, PRE25-like, Tn*4001*, Tn*5405*-like backbones, and small Staphylococcal plasmids. This underscores the importance of horizontal acquisition and subsequent microevolution of mobile resistance elements that may have shaped the local adaptation of highly resistant *S. pseudintermedius* isolates.

Keywords

Staphylococcus pseudintermedius, plasmids, mobile genomic elements, horizontal transfer, antimicrobial resistance, population genomics

1. Introduction

Staphylococcus pseudintermedius is a commensal and an important opportunistic pathogen in dogs and cats and can occasionally establish infections in humans[1,2]. The population consists of clonal methicillin-resistant isolates (MSRP) and genetically diverse methicillin-susceptible isolates (MSSP). Both are often multidrug resistant (MDR), which is limiting treatment options and provides a reservoir of antimicrobial resistance genes for other bacterial pathogens.

Like other staphylococci, *S. pseudintermedius* isolates harbour multiple antimicrobial resistance (AMR) determinants on mobile genetic elements (MGEs)[3]. The most significant MGE is the Staphylococcal Cassette Chromosome *mec* (SCC*mec*) element, which carries the *mecA* gene encoding methicillin resistance[4]. Multi-locus sequence typing (MLST) has demonstrated that MRSP isolates belong to a limited number of clonal complexes (CC)[5]. Worldwide several major MRSP clones have emerged, e.g. ST68 in North America, ST496 in Australia, and CC71 in Europe which declined over the years and was replaced by CC258[6–9]. More recently, whole genome phylogeny reconstruction demonstrated clonal expansion of multiple MRSP clonal lineages in a background of genetically highly diverse methicillin susceptible (MSSP) isolates[9–11]. Emergence of MRSP clonal lineages following independent SCC*mec* acquisitions by distinct MSSP isolates resulted in the association of specific SCC*mec* types with the major MRSP clonal lineages[6]. It has been proposed that acquisition of SCC*mec* combined with multiple antimicrobial resistance genes in chromosomal integrated transposons or IS elements may have accelerated the adaptation to antimicrobials or other environmental pressures and might have contributed to the success of CC71 in recent years[3]. Multiple genomic elements carrying transposases and often multiple antimicrobial resistance genes have been detected in MRSP isolates. The Tn*5405*-like element generally carries the resistance genes *aphA3-sat-aadE-dfrG-erm*(B) that confers resistance to four classes of antimicrobials[12] and the pRE25-like chromosomal element carrying five resistance genes, *erm*(B), *cat*$_{(pC221)}$, *aph*(3')-III, *ant*6-Ia, *sat*4 and a toxin antitoxin system[13].

The contribution of plasmids in the success of MRSP clones is thought to be limited, although some plasmid-associated antimicrobial resistance genes have been identified in both MRSP and MSSP isolates[3,14]. The *tet*(K) gene, which is commonly carried on small plasmids in other staphylococci, has been identified on a 4.5 kb plasmid, and the *erm*(C) gene has been detected on a 2.5 kb plasmid in *S. pseudintermedius* isolates[15]. Furthermore, the chloramphenicol resistance gene *cat*$_{pC221}$ is commonly

present in MRSP isolates and was demonstrated to be located on small plasmids that vary in size and composition and have been observed in combination with the *str* gene that confers streptomycin resistance[14,15].

These plasmids, transposons and IS-elements are easily exchanged by horizontal gene transfer (HGT) between staphylococcal species which may provide rapid adaptation of clonal lineages of *S. pseudintermedius*. Also, in *S. aureus* it has been demonstrated that frequent acquisition and loss of mobile genetic elements occur in successful methicillin-resistant *S. aureus* clones[16]. To study MGE elements involved in acquisition of antimicrobial resistance genes in *S. pseudintermedius* and clonal expansion of MRSP, we used a multilevel phylogenetic analysis to discern the importance of these elements in shaping the population structure and dissemination of antimicrobial resistances in *S. pseudintermedius.*

2. Methods

2.1 Whole genome sequencing

In total 223 *S. pseudintermedius* isolates were included in this study that were isolated from clinical samples from dogs between 1993 to 2018 at the Veterinary Diagnostics laboratory of Utrecht University, and from patients in three hospitals in the Netherlands **(Supplementary Table 1)**. Carriage of methicillin-resistance determinants was performed by qPCR as previously described, resulting in a set of 98 MRSP and 92 MSSP canine isolates and 33 MSSP human isolates.

Whole genome sequencing was performed in a previous study for 103 isolates[11] and 120 isolates were sequenced in this study as follows; DNA was isolated using the Qiagen UltraClean Microbial DNA isolation kit (Qiagen, Venlo, the Netherlands). DNA libraries were prepared using NexteraXT (Illumina, San Diego, CA, USA) according to manufacturer's instructions and pooled libraries were sequenced on the Illumina NextSeq platform with 150 base pairs paired-end reads. Illumina reads were trimmed using Trim Galore v0.4.4 (https://github.com/FelixKrueger/TrimGalore). The genomes were assembled using SPAdes v3.10.1[17] and contigs smaller than 200 base pairs and with a coverage lower than 10 were removed. Genome quality was assessed with CheckM v1.1.2[18] for completeness (>95 %) and contamination (<5 %) and the genomes were annotated using Prokka (version 1.13)[19].

Based on the short-read data 25 isolates were selected that carried multiple AMR

genes on the predicted plasmids with different *rep* types (**Table 1 and Supplementary Table 1**). The same DNA was sequenced using Oxford Nanopore technology to fully resolve the plasmids and integrated chromosomal elements. Twenty-five isolates carrying multiple resistance genes, or different replication genes were selected for Nanopore sequencing. This was performed as outlined in the genomic DNA by ligation protocol (SQK-LSK109), with sequencing on a MinION device using flow cell type R9.4.1 (FLO-MIN106D) (Oxford Nanopore, Oxford, United Kingdom). Reads were filtered with options minimal length of 5000 and keep percentage of 90 % using filtlong (https://github.com/rrwick/Filtlong). Reads were assembled using Flye v2.7[20] using options -nano hq -min overlap 1000 -meta, into a single scaffold and used as existing long read assembly in Unicycler v. 0.4.7[21] (with the Illumina and Nanopore reads). Additionally, for two isolates (18S02770 and 18S03480) the genomes were closed by filtering the long-reads with filtlong (https://github.com/rrwick/Filtlong) by keeping the best reads up to 300Mbp (option -t 300000000) and using Trycycler v0.5.1[22] using assemblers Flye v2.7[20], Raven v1.6.1[23], Canu v2.2[24] and minimap2 v2.22-r1101/miniasm v0.3-r179[25], followed by 5 rounds of Pilon (version 1.22) (https://github.com/broadinstitute/pilon) for polishing the assemblies with the Illumina short-reads. Genomes were aligned using Parsnp v1.2[26] for phylogenetic single-nucleotide polymorphism (SNP) analysis of the core genome. SNPs from recombination events were removed from the core alignment using Gubbins v2.3.4[27]. Multilocus sequence typing was detected using MLSTFinder[28], and the alleles of a novel Sequence Type (ST) were assigned by the curator of the PubMLST database (https://pubmlst.org/organisms/staphylococcus-pseudintermedius). The core genome tree was visualised using the Interactive Tree of Life[29].

2.2 Antimicrobial resistance gene detection

Antimicrobial resistance genes were detected with ABRicate (https://github.com/tseemann/abricate) using the CARD and AMRFinder databases using 60% coverage, 90 % identity. Isolates were considered multidrug resistant (MDR) if they contained resistance genes to three or more antimicrobial classes[30]. The names of resistance genes were annotated according to CARD. SCCmecFinder v1.2 was used to type the SCC*mec* element[31]. These elements were classified to types previously described and acknowledged by the International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements (www.sccmec.org), and to the SCC*mec* composites that have been described in *S. pseudintermedius*[4,32,33]. When an

SCC*mec* type could not be assigned, the *mec* and *ccr* gene complexes were searched using BLASTN with identity and coverage threshold of 90 and 60 %, respectively.

2.3 Mobile elements

Plasmid-associated contigs were predicted in the short read assemblies using the tool RFPlasmid[34] with a cut-off for plasmid vote ≥ 0.6 and a minimum contig length of 1,000 bp. Plasmid sequences were characterized using the types of replication genes and the relaxase genes[35,36]. With PlasmidFinder v2.1, replication (*rep)* genes were identified using identity threshold of 60 % and coverage of 90 %[37]. The relaxase genes were detected with the MOBsuite database (https://github.com/phac-nml/mob-suite) using default thresholds. As all relaxase genes were identified as MOBV, the accession was assessed to characterize the gene match. Insertion elements (IS element) were identified using ISFinder (https://isfinder.biotoul.fr/) with an E-value cut-off of ≤ 1-E05 and BLASTn NCBI searches. The identified mobile elements and plasmids were manually characterized and annotated using NCBI GenBank BLASTn and BLASTp and was visualized using clinker[38]. A plasmid was predicted as multi-copy when the plasmid contig was detected when the sequencing depth of reads was 10 times higher than the average coverage.

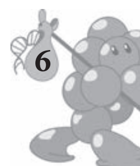
3. Results

3.1 Phylogenetic reconstruction of diverse *S. pseudintermedius* clades

The reconstructed phylogenetic tree of the core genomes, with an average core genome size of 2.039,995. bp, showed deep rooted branches and revealed three distinct *S. pseudintermedius* clades. The clades contained three major MRSP clonal lineages and two small MSSP clonal lineages that correlated with previously assigned clonal complexes with multi-locus sequence typing, CC71, CC45 and CC258 and for MSSP CC241[6]. A novel MSSP clonal lineage, assigned CC442, and consisted of 11 isolates of which one was a multidrug-resistant isolate from 1999, that did not share a specific resistance gene set with the other isolates (**Figure 1**). The isolates were assigned to 131 different MLST STs types, of which 123 isolates belonged to a Clonal Complex (CC); CC45 (n = 8), CC71 (n = 43), CC241 (n = 11), CC258 (n = 35), CC442 (n = 11), and 115 isolates with unique ST types.

MRSP diversified from MSSP isolates in two clades **(Figure 1)**. In clade 1 this is shown for the MRSP CC258 clonal lineage, and in clade 3 for MRSP isolates of CC71 and CC45 and smaller clusters of MRSP isolates that each diversified from MSSP isolates. Additionally, there are two MSSP clonal lineages, represented by MSSP CC241 in clade 3 and MSSP CC442 in clade 2, that both diversified from distantly related MSSP isolates. The isolates in this study were recovered over a period of 15 years, including the first MRSP CC71 methicillin resistant clone in clade 3. This showed an ongoing diversification of MSSP into MRSP that might select for potentially novel emerging clonal lineages.

Antimicrobial resistance genes were detected in silico searching for horizontally acquired AMR determinants[39,40]. Remarkably, were there only 11 isolates, divided over each clade that circulated in 2008 and 2016 without any horizontally acquired AMR gene. Ninety-eight % of the MRSP isolates (n = 96/98 isolates) and 40 % of the MSSP isolates (n = 50/ 125 isolates) were classified as MDR[30]. In many draft genomes genes encoding resistance against aminoglycosides were present (**Supplementary Table S1**).



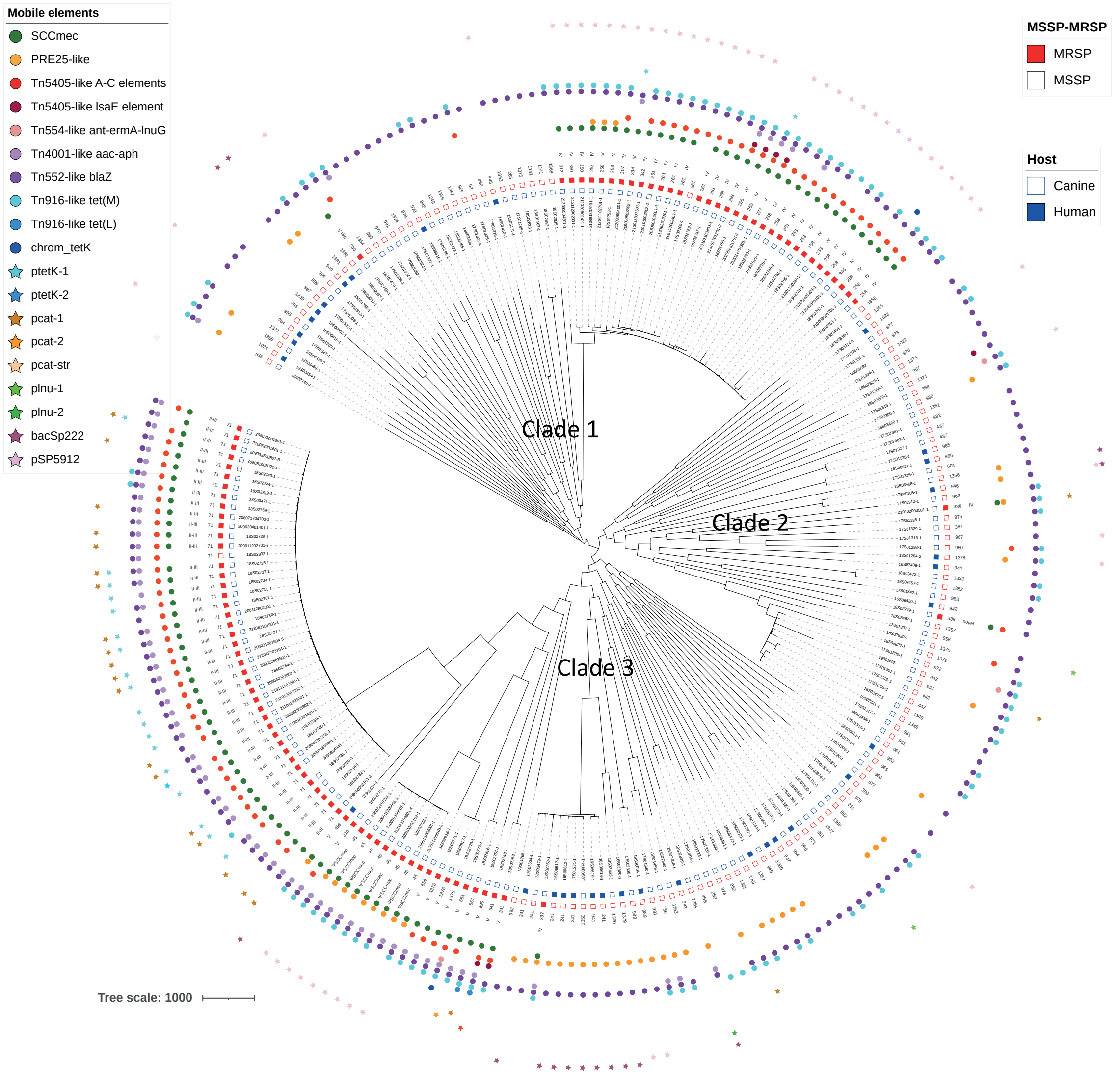


Figure 1. Phylogenetic clades of *S. pseudintermedius* based on core genome single nucleotide polymorphisms. MRSP genomes are marked with red-coloured boxes and MSSP with empty, red-coloured boxes. Depicted is the isolation year, multi-locus sequence type, SCC*mec* type, resistance genes and mobile genetic elements. Branch lengths are proportional to the number of SNPs.

3.2 Predicted mobile genetic elements in the *S. pseudintermedius* draft genomes

To elucidate mobile elements that are important for the dissemination of AMR genes in the population we predicted the plasmid-associated genetic content in the *S. pseudintermedius* genomes, using RFPlasmid. Plasmid-associated sequences (further described as plasmid content) were identified in all but two MRSP genomes (n=96); all CC258, CC45, C241 isolates and 98 % of CC71 isolates contained plasmid content. Of the 125 MSSP isolates 60 % (n=74) contained plasmid-content (Supplemental Table 1). To classify the plasmid content in *S. pseudintermedius*, a plasmid type was assigned based on the plasmid backbone, consisting of the replication (*rep*) gene with a relaxase (*mob*) gene (**Supplementary Table S2**). Among the 223 *S. pseudintermedius* isolates, four *rep* gene families were detected that matched previously described rep_7, rep_{13}, rep_{21}, and rep_{US12} genes[35,36,41]. The *rep* gene type was combined with a relaxase type and when they were located on the same contig we could distinguish 18 different plasmid-backbone types. A total of 117 isolates contained one or multiple plasmid-backbone types, of which 28 isolates (20 MRSP and 8 MSSP) contained two plasmid-backbone types (**Supplementary Table 1).** Of the plasmids that were predicted in 171 isolates, a contig carrying a plasmid replicon, similar to replicon types mainly detected in *S. aureus*[36,41], was detected in 120 isolates (20,21). The plasmid content of 51 isolates (42.5 % of the studied genomes) could not be characterized. A prophage detection analysis of the contigs using Phaster also did not resolve the origin of these replicon types (data not shown), and it is possible that the replicon types of the non-typable plasmid-predicted contigs are novel.

3.3 Complete plasmid sequences show extensive plasmid plasticity

In the complete genomes, small plasmids were reconstructed that carried tetracycline, lincosamide or chloramphenicol resistance genes. These were often related to plasmids that have been described before[14,42].

3.3.1 Tetracycline resistance gene carrying plasmids

The *tet*(K) gene, encoding resistance to tetracycline, was situated on plasmid sequences and on a chromosomally integrated plasmid which all carried a rep_{7_1} gene (*repC* (Cassette)) replication genes in the same plasmid backbone, but there was

Table 1. Long-read assembled mobile genetic elements and plasmids in *S. pseudintermedius.*

						AMR chromosomal elements						AMR plasmids			
Isolates	**contig**	**contig size (bp)[1]**	**coverage depth**	**Clade**	**SCCmec**	**pRE25 -like**	**Tn*4505* -like[2]**	**Tn*4001* - like[3]**	**Tn*554* - like**	**blaZ**	***tet* (M)**	***tet* (K)**	***lnu* A**	***cat***	**non-AMR plasmid**
16S06614-1	chr	2571427	1	CC241	-	+				+					
	p1	15203	7.56												BacSp222
	p2	2453	15.35												pSP_11304-3A_2
16S06620-1	chr	2507103	1	clade 2	-		B			+					
17S01301-1	chr	2545238	1	442	-	+					B				
17S01307-1	chr	2536299	1		-					+					
	p1	2359	11.84										plnu-2		
17S01333-1	chr	2600049	1	clade3	-	+				+	E				
18S02729-1	chr	2750832	1	CC71	II-III			A		+					
	p1	3785	9.71											pcat-1	
18S02731-1	chr	2747891	1	II-III	II-III		C	A		+					
	p1	17354	3.4												UN[4]
	p2	4439	96.99									ptetK-1			
18S02741-1	chr	2640298	1	CC258	IV		C			+	A				
	p1	3043	13.09												pSP5912
18S02748-1	chr	2680876	1	clade3	V		C**IsaE	D		+	C				
	p1	5137	19.54											pcat-str	
18S02749-1	chr	2669663	1	clade2	novel		C			+					
	p1	11965	1.7												BacSp222
18S02750-1	chr	2754967	1	CC258	IV		C**IsaE	B		+	A				
	p1	2453	7.45												pSP_11304-3A_2
18S02761-1	chr	2831518	1	CC71	II-III		A	A		+					
18S02769-1	chr	2700218	1	clade1	V -like			C		+	B				
	p1	2743	5.76												pSP5912
18S02770-1	chr	2796642	1	clade3	V		C	A	+	+	C	c_tetK			
18S02816-1	chr	2734896	1	clade3	V		C	A		+	C				
	p1	4615	13.91											pcat-2	
	p2	1487	13.69												UN
18S02820-1	chr	2680530	1	CC258	-		C			+					
	p1	3043	11.8												pSP5912
18S02828-1	chr	2562314	1	clade2	-		B**IsaE			+					
	p1	17353	3												UN[4]
18S02839-1	chr	2727658	1	clade3	-	+		B		+					
	p1	15.203	2.13												BacSp222
	p2	2.482	5.04										plnu-3		
18S03459-1	chr	2723024	1	clade3	-	+		A		+	A and B				
18S03480-1	chr	2722684	1	clade3	-		B			+	A				
	p1	3.101	7.38												UN
	p2	2.361	5.44										plnu-1		
208082803802-1	chr	2751067	1	CC71	II-III		C	A		+					
	p1	4455	55.94									ptetK-2			
208090903101-3	chr	2693529	1	CC71	II-III			A		+					
	p1	3785	87.79											pcat-1	
209011300901-1	chr	2599442	1	CC45	ΨSCCmec$_{57395}$	+		A		+	D				
	p1	3.043	205.65												pSP5912
211083101901-1	chr	2834878	1	CC71	II-III		A	A		+					
	p1	3785	41.4											pcat-1	
213101701201-2	chr	2726214	1	CC258	IV		B**IsaE	B		+	A				
	p1	2453	21.07												pSP_11304-3A_2

[1] all were circular. ** elements carrying UN unidentified plasmids, 4 carry the same unidentified plasmids.

sequence diversity in their plasmid recombination genes and hypothetical genes, as they differed slightly in their gene organization and plasmid sizes (**Table 1, Figure 2**). The *tet*(K) resistance plasmid of 4,439 bp of genome 18S02731 (ptetK-1 with *rep*$_{7\text{-}1\text{-}e}$) was with 100 % coverage and >99 % identity similar to the *S. aureus tet*(K) resistance plasmid pKH6 or other unnamed staphylococcal plasmids in the NCBI GeneBank. Based on the sequence depth is ptetK-1 most likely a high copy plasmid. PtetK-1 was also identical to the *tet*(K) encoding region of a composite SCC*mec* III element

Tetracycline resistance plasmids and chromosomal insertion

208082803802_p1_tetk: rep, tetk, pre

18S02731_p2_tetk: rep, tetk, pre, hypothetical protein

18S02770_chrom_tetk: hypothetical protein, hypothetical protein, hypothetical protein, group II intron reverse transcriptase/maturase, rep, tetk, pre, hypothetical protein, group II intron reverse transcriptase/maturase, sttH

2.5kb

Chloramphenicol resistance plasmids

18S02816_p1_cat: rep, cat, hypothetical protein, hypothetical protein, putative 27.7 kDa protein

208090903101_p1_cat: rep, cat, pre

18S02729_p1_cat: rep, cat, pre

211083101901_p1_cat: rep, cat, pre

18S02748_p1_catstr: rep, cat, str, pre, copG

2.5kb

Lincosamide resistance plasmids

18S02839_p2_lnu: rep, lnuA

17S01307_p1_lnu: rep, lnuA

18S03480_p2_lnu: rep, lnuA

2.5kb

Figure 2. Structural organization of *tet*(K) gene in plasmids and chromosomal location, and the *cat* and *lnu*A genes in plasmids from *S. pseudintermedius*. The arrows indicate the positions of the genes and their direction of transcription.

described in *S. aureus*, where the complete plasmid sequence is inserted between IS-341 repeats (accession AB037671)[43]. When using the complete plasmid sequence as reference for mapping the whole set of draft genomes, the plasmid was detected in 18 CC71 isolates from 2004 onwards, and in one CC258 isolate (Figure 1). The second complete *tet*(K) resistance plasmid, with a plasmid length of 4,455 bp of genome 208082803802-1 (ptetK-2 with rep7-1-b) was detected to have 84 % sequence identity to an unnamed *tet*(K) carrying plasmid of *S. pseudintermedius* (CP051150) over 97 % of its length. The rest of the sequence of this plasmid was unique. This plasmid was detected in only one CC71 isolate in the total set of draft genomes. The integrated ptetK with $rep_{7\text{-}1\text{-}a}$ plasmid in isolate 18S027701 was 100% identical to the integrated plasmid in the genome of *S. pseudintermedius* HSP240 (CP083203.1) and showed 98 % coverage with 99 % sequence identity and 98 % coverage with unnamed plasmids identified in *S. condimentie* (CP068076.1) and *S. equorum* (CP068072.1). Of 7 *tet*(K) genes the plasmid or chromosomal location could not be confirmed due to contig breaks in the draft genomes.

3.3.2 Lincosamide resistance gene carrying plasmids

Lincosamide resistance can be conferred by several genes, and in the analyzed genomes the resistance gene *lnu*(A) was present, that generally confers low-level lincosamide resistance. It has been detected on small plasmids in several Staphylococcal species[44]. Three *lnu*(A) resistance plasmids were detected with identical or reshuffled sequences that were similar to previously described rolling-circle replication plasmids in coagulase negative staphylococci[45]. Isolate 18S03480-1 carried a *lnu*(A) resistance plasmid of 2,631 bp with a $rep_{13.3}$ gene that was 100 % identical to the *S. chromogenes* pLNU1 plasmid (plnu-1). Another *lnu*(A) resistance plasmid of 2,359 bp (plnu-2) of isolate 17S01307-1 carried a $rep_{13.4}$ gene and showed 98 % sequence identity to the *rep* gene of plasmid pLNU9, and the segment encoding *Inu*(A) was identical to plasmid pLNU1. A larger plasmid of 2,484 bp in genome 18S02839-1, caried *Inu*(A) and $rep_{13\text{-}1}$ genes (plnu-3) and showed of approximately 1,800 bp with >97 % sequence identity with plasmid pl1 of *S. pseudintermedius* 063228[46] and low sequence identity with several pLNU plasmids[45] (**Figure 2, Table 1**). No other isolates in the analyzed set of shortread assemblies carried *lnu*(A) resistance genes.

3.3.3 Chloramphenicol resistance gene carrying plasmids

The gene coding for chloramphenicol acetyltransferase (*cat*) was detected on three fully sequenced plasmids. In *S. pseudintermedius* the *cat* gene is commonly associated with the *S. aureus* plasmid pC221 and sequence variants of this plasmid **(Figure 2, Table 1)**. However, in the three resolved plasmids only the *cat* gene was identical to plasmid pC221 and there was a higher sequence identity to the *rep* and *cat* region of the *S. aureus* chloramphenicol resistance plasmid pKH7. The three pcat-1 plasmids of 3,785 bp detected in 18S02729-1, 208090903101-, 211083101901-1 were similar, but two carried a *rep*$_{7\text{-}7}$ replication gene and the plasmid of 211083101901-1 carried a rep$_{7_12}$ showing some SNP differences that may have purged over time. The pcat-1 plasmids were identical to a chloramphenicol resistance plasmid of an unpublished *S. aureus* plasmid UP_1500 in GenBank (CP047814.1). Based on the sequencing depth it is most likely a high copy plasmid. Mapping of this plasmid as reference to the entire set of draft genomes showed the presence in 19 of 43 CC71 isolates **(Figure 1)**. A larger plasmid pcat-2 of 4,615 bp in 18S02816-1 (pcat-2) also carried a *rep* $_{7\text{-}7}$ gene and was highly similar to an unpublished *S. epidermidis* plasmid with the same size (LR735423). Mapping of this plasmid to the draft genomes detected this plasmid in no other isolate. The third *cat* carrying plasmid pcat-str with 5,137 bp in 18S02748-1 was larger and additionally carried the streptomycin resistance encoding *str* gene. This resistance combination has been previously reported in *S. pseudintermedius*, but this is the first time a complete plasmid analysis identified a mosaic organized plasmid. The *rep* and *cat* genes had a high sequence identity of 99 % to the *S. aureus* chloramphenicol resistance plasmid pKH7, but the remaining sequences showed no significant identity to plasmid sequences present in GenBank. The plasmid pcat-str was present in a single ST341 isolate in clade 3. Overall, the *cat* gene was detected in the short-read sequences of 74 isolates, that could be linked to a plasmid in 21 isolates. In 46 isolates the *cat* gene was present on a chromosomal contig, as will be described in the following section. Of seven isolates the *cat* gene location could not be resolved using short-read contigs.

3.4 Multiple antimicrobial resistance genes are associated with genomic mobile elements

Mobile elements are considered most important for transfer of antimicrobial resistance genes in *S. pseudintermedius*. However, as these elements are difficult

to assemble from short-read sequences, due to transposon induced repeat sequences, information on the heterogeneity of elements and their distribution in the *S. pseudintermedius* population is limited. With long-read sequencing, multiple genomic elements known to be involved in the acquisition of AMR: the SCC*mec* elements, several transposon-mediated elements, and the widely integrated *bla*Z genes, were completed with high resolution in this study.

3.5 SCC*mec* elements are linked to clonal MRSP lineages and acquisition of novel elements

The SCC*mec* elements of the 98 sequenced MRSP isolates were almost all classified according previously reported SCC*mec* elements in *S. pseudintermedius*, SCC*mec*II-III, IV, V and ΨSCC*mec*$_{57395}$ (**Supplemental Table S1**). Two novel SCC*mec* elements were identified using long-read sequencing. In isolate 18S02769-1 a mosaic SCC*mec* element similar to a type V (5C2) element was detected, with a *mec* gene complex C and the recombinase gene *ccrC*6, however here the orientation of *mecA* was inverted and the element contained integrated arsenic resistance genes downstream of *orfX* and copper resistance genes downstream of *mecA*. The sequence of this element was identical to the published element of isolate NA45 with ST84[46]. In isolate 18S02749-1 the element consisted of a *mec* gene complex A, with the *mecA*, *mecR*1 and *mecI* genes, and contained a recombinase gene *ccrC*8, a combination that has not been described before (**Supplemental Table S1**).

Two SCC*mec* elements appeared to be unique for a clonal lineage. One is the composite SCC*mec*II-III described in 2009 that originated from a recombination between SCC*mec*III from *S. aureus* and SCC*mec*II from *S. epidermidis* and was unique for clonal lineage CC71 **(Figure 1)**. In one CC71 isolate (18S02833) no *ccr* or *mecA* genes or remnants were detected and it is possible that this isolate or its ancestor never acquired an SCC*mec* element. The second clonal lineage-associated element is the pseudo-SCC*mec* element ΨSCC*mec*$_{57395}$ that was first described in CC45 isolates in Thailand in 2013 (31). In our isolates the SCC*mec*$_{57395}$ pseudo element was unique for CC45. In clonal lineage CC258 two SCC*mec* elements were acquired, although only the SCC*mec*IV element expanded since 2008 as it was observed in 32 isolates while the SCC*mec*V element was detected in a single isolate (**Figure 1**). In one CC258 isolate (17S02309-1) a remnant of an SCC*mec*IV element was present and was limited to a *ccrA2* gene adjacent to *orfX*, but no IS*341*, *mecA* and *mecRI* genes

were present. The SCC*mec*IV element is the most common element in community-associated MRSA and is considered the most widely distributed SCC*mec* element[47]. It has been suggested that size reduction or altered gene expression could contribute to the fitness of isolates carrying the SCC*mec* element[48]. We observed expansion of SCC*mec*IV in CC258 and single introduction of SCC*mec*IV in clade 2 and in CC241 in clade 3. Isolates with SCC*mec*V expanded since 2013, as it was detected in clade 3 in small groups of clustered MSRP isolates that shared a common ancestor with CC45 and CC71 isolates, as well as in a single CC258 isolate (**Figure 1**).

3.6 Acquisition of multiple antimicrobial resistances by transposon mediated elements

3.6.1 PRE25-like element

The conjugative pRE25-like element carrying *cat*, *erm*(B), *aph*A-3, *aad*E, *sat* resistance genes, toxin–anti-toxin genes, IS*1216* and two rep_{7} and rep_{US12} replication genes was observed in the closed genomes of six isolates. The element was highly conserved and highly homologous to the described pRE25-like element (GenBank MK775653)[13]. The element was inserted into the same chromosomal integration site, at the left flanked by replication genes and on the right flanked by a gene encoding a peptidoglycan-binding domain protein (position 2,575.180 bp to 2,597.552 bp in isolate 18S02839-1) (**Figure 3, Table 2**). This conserved sequence was used to map to the total genome set and identified the PRE25-like element in 35 (28 %) of the MSSP isolates (10 CC241 in clade 3, 15 other isolates in clade 3, four clade 2, five clade 1 and one CC442 isolate in clade 2), and to a lesser extend in 14 (14 %) of the MRSP isolates (all CC45 isolates in clade 1, four CC258 isolates in clade 1 and two clade 3 isolates). A considerable proportion of chromosomes carried two *rep* genes, indicating that recombined plasmids in the PRE25-like element are stably maintained in the *S. pseudintermedius* population.

Table 2. The distribution of mobile genetic elements in 223 *S. pseudintermedius* genomes.

	MRSP	MSSP	CC45	CC71	CC241	CC258	CC442	Clade 1	Clade 2	Clade 3
number of isolates	98	125	8	43	11	35	11	37	36	42
chromosomal mobile elements										
SCC*mec*	98	0								
PRE25-like	15	35	8		11	4	1	5	4	17
Tn*5405* -like[2]	73	6		39		27		2	3	8
Tn*554* -like_ant(9)-erm(A)-lnu(G)	1	2					1		1	1
Tn*916*-*tet* (M)[1]	54	47	7	3	1	32	9	12	12	25
Tn*4001* _like_aac(6')-aph(2")	63	6	8	39	1	6		2		12
Tn*552* _blaZ	95	112	8	41	11	34	11	33	33	36
Tn*554* _blaZ	7	6				8		1	4	
c-tetK	2					1				1
Plasmids										
ptetK-1	20			18		2				
ptetK-2	1			1						
plnu-1		2							1	1
plnu-2		1							1	
plnu-3										
pcat-1	19			19						
pcat-2	1									1
pcat-str	1									1
bacp222	1	14			9			2	2	2
pSP5912	41	13	8			32		4	6	4
pSP_11304-3A_2										

[1] one isolate carried two tet(M) chromosomal insertions

[2] types A, B, C

Figure 3. The genetic variants of PRE25-like elements carrying antimicrobial resistance genes in *S. pseudintermedius*. Antimicrobial resistance genes are indicated in red, IS elements in blue, helix-turn-helix modifying genes in purple, plasmid-associated genes in yellow and hypothetical genes in grey.

3.6.2 Novel composite Tn4505-like multi-resistance elements

Highly diverse chromosomal elements with a Tn*5405* backbone and IS*1182*[3] were identified that carried genes encoding resistance to aminoglycosides (*aphA3*, *sat4*, *aadE, spw*), resistance to macrolides and/or lincosamides (*erm*(B), *lnu*(A), *lnu*(B), *lsa*(E)), and resistance to trimethoprim-sulfamethoxazole (*dfrG*). The complete genomes revealed variants of this element carrying up to 11 antimicrobial resistance genes, that were inserted in three different insertion sites on the chromosome and were named element types A, B, and C (**Figure 4).** Element type A was flanked by *gltP*, a glycerol-3-phosphate transporter and *sdrG*, a fibrinogen-binding adhesin gene. The sequences of these elements were identical in two isolates and carried five resistance genes *aphA3-sat4-aadE-erm(*B*)-dfrG, a* Tn*504,* two copies of IS*1182*, a truncated Tn*552* and *repR* replication genes. This element type is present in *S. pseudintermedius* 69687[3] and was similar to a Tn*5405* composite element with integrated plasmid described in *S. aureus*[49]. Element B was flanked by the gene encoding the transcriptional repressor LexA, carried aminoglycoside-streptothricin encoding resistance genes (*aphA3-sat4-aadE*), flanked on the right by GMP reductase encoding *guaC*. In element B of isolate 18S03828, the downstream region with IS*1182* and the resistance genes *erm(B)* and *dfrG* was absent, instead two segments with resistance genes were observed. The segment with the pleuromutilins-lincosamides-streptogramin A resistance genes *lnu*(B)-*lsa*(E) was linked to a segment with aminoglycoside resistance encoding genes *spw-apt*-ant(6*)-la*. A similar element with *lnu*(B)-*lsa*(E) and *spw-apt-ant*(6)-la was present on the *lsa*(E)-carrying resistance island in *Enterococcus faecalis* (MG765453.1). This sequence was also present in the *lnu*(B)-*lsa*(E) segment of element C. Of this element C, 10 sequence variants were detected, that were all flanked by *comK* encoding a competence protein (WP_014614331.1) gene and a fibrinogen-binding protein encoding gene *sdrG* (WP_199274233.1). The main composition of element C is related to element A, with the resistance genes *aphA3-sat4-aadE-erm(B)-dfrG,* transposon Tn*5405* with two IS*1182* and a truncated Tn*552*. Of the 10 genomes carrying element C, three were lacking the trimethoprim resistance gene *drfG* and in three genomes shared a larger element linked to the *E. faecalis* resistance island carrying *lnu*(B)-*lsa*(E) and *spw-apt-ant*(6)-Ia, as mentioned above. Element C in chromosome 18S02748 is the most extended one, as it additionally carried bifunctional 6'-aminoglycoside acetyltransferase 2"-aminoglycoside phosphotransferase *aac(6')-aph(2"*) genes (described below) and GCN5-related *N*-acetyltransferases family (GNAT) genes.

To elucidate the presence of type A and C elements in the draft genomes, we mapped these elements to the draft genomes, but with the high number of contig breaks due to repeat-sequences in Tn*5405*-like elements, it was not possible to pinpoint the different element types. However, in general a Tn*5405*-like element was predominantly present in CC71 and CC258 MRSP isolates, and more often in MRSP (73 of 98 genomes) and only in 6 of 92 MSSP genomes (**Figure 1**, **Table 2**). No Tn*5405* transposon was present but the *drfG* gene was inserted with a putative IS1 family element, in seven CC258 draft genomes, in all eight CC45 and in two clade 3 genomes. In CC45 *dfrG* was flanked by a single-stranded DNA binding protein, and *fabZ* encoding a 3-hydroxyacyl dehydratase on the other side. In the CC258 and Clade 3 genomes *dfrG* was flanked by a putative IS element and was present in three chromosomal insertion sites: between *spEX* encoding an exotoxin and *panDCB* encoding a pantothenate synthetase gene or flanked by *yit*T encoding a family protein

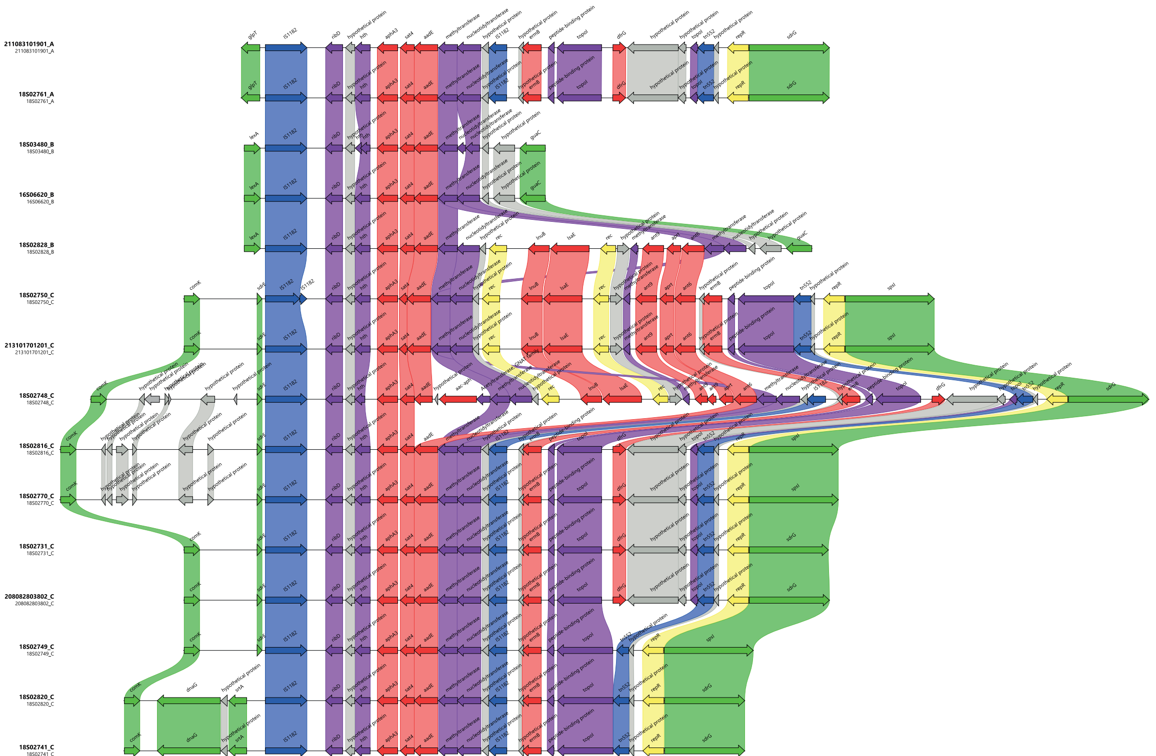


Figure 4. Genetic variants of composite Tn*4505-like* multi-resistance elements in *S. pseudintermedius*. Antimicrobial resistance genes are indicated in red, IS elements in blue, helix-turn-helix modifying genes in purple, plasmid-associated genes in yellow and hypothetical genes in grey.

and a tRNA gene (isolate 18S02757), or between a putative lipoprotein encoding gene and *lctP* encoding L-lactate permease (isolate 17S01530).

3.6.3 Tn554-like aminoglycoside-macrolide-lincosamide resistance element

In one complete genome 18S02770 a chromosomal mobile element carrying the AMR genes *ant(9)-erm*(A)*-lnu*(G) was identified. The *erm*(A) gene was unique for this isolate and was situated with *ant(9)* in a Tn*554* backbone with downstream *Inu*(G)[50,51] (**Figure 5**). The AMR gene *lnu*(G) was present in two draft genomes, which carried most likely a remnant of this element as Tn*554* and the *ant(9)-erm*(A) resistance genes were absent (**Supplementary Table 1**).

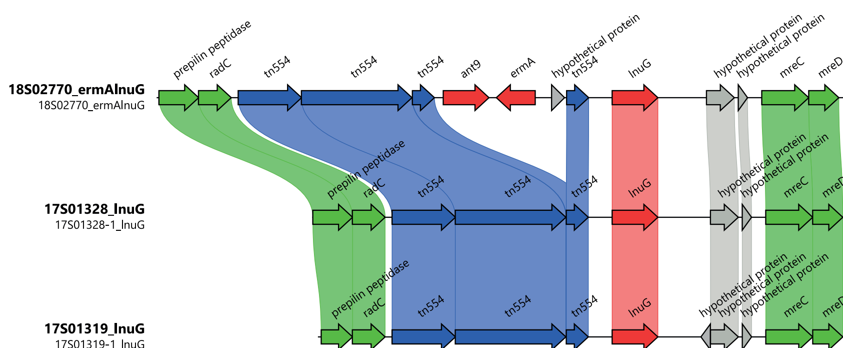
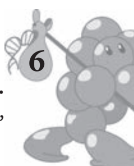


Figure 5. The genetic variants of Tn554 carrying macrolide and aminoglycoside resistance in *S. pseudintermedius*. Antimicrobial resistance genes are indicated in red, IS elements in blue, helix-turn-helix modifying genes in purple, plasmid-associated genes in yellow and hypothetical genes in grey.



3.6.4 Tn4001-like bifunctional aac(6')-aph(2") element

The *aac(6')-aph(2")* genes encode bifunctional aminoglycoside modifying enzymes, a 6'-aminoglycoside acetyltransferase and 2"-aminoglycoside phosphotransferase often in a Tn*4001* backbone with IS*256*[52]. Sequence variants of this element were present in 15/25 complete genomes (**Figure 6, Table 1**). The *aac(6')-aph(2")* genes were inserted in different chromosomal positions. In 10/15 genomes they were flanked with genes encoding phage proteins (integration site A) and contained in seven genomes, IS*256* with *aac(6')-aph(2")* genes of Tn*4001*. However, IS*256* was absent in integration site B, where the genes were flanked by *dtd3* encoding D-aminoacyl-tRNA deacyclase and *metG* encoding a methionine-tRNA ligase. In integration site C were the genes flanked by IS*257*, a glycerophosphoryl diester phosphodiesterase

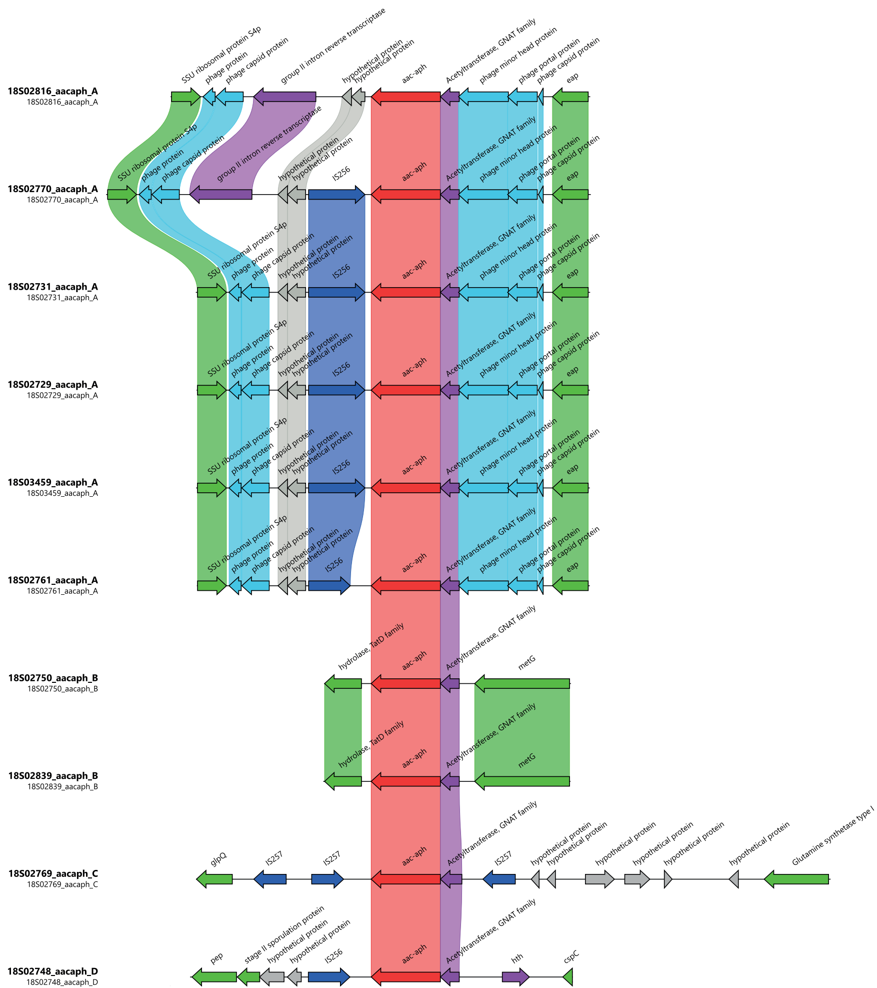


Figure 6. Genetic variants of Tn*4001-like* bifunctional *aac(6')-aph(2")* elements in *S. pseudintermedius*. Antimicrobial resistance genes are indicated in red, IS elements in blue, helix-turn-helix modifying genes in purple, plasmid-associated genes in yellow and hypothetical genes in grey.

and a glutamine synthetase *femC* gene. In integration site D were the genes bounded by IS*256* and a gene encoding a helix-turn-helix domain protein, and a cold shock protein encoding gene *cspC* (**Figure 6**). By mapping the short-reads to the *aac(6')-aph(2")* element, the genes were detected in 69 genomes. Additional *aac(6')-aph(2")* genes were detected in the Tn*4505*-like element of isolate 18S02748. However, due to contig breaks, the contigs were too small and the exact chromosomal location in the draft genomes could not be defined.

3.6.5 Tn916 tetracycline resistance element

In the closed genomes, the tetracycline resistance gene *tet*(M) gene was integrated in five different chromosomal sites A-E and was flanked by genes of the conjugative transposon Tn*916*. (**Figure 7**) Transposon Tn*916* was identified with a NCBI BLASTn search and this element was highly conserved in various chromosomal insertion sites. The Tn*916* element carrying *tet*(M) is widespread, and segments of 18kb ranging from the *fstK* gene to final downstream Tn*916* gene, are 100 % identical to *tet*(M) elements in multiple *Streptococcus* spp., e.g. *S. canis* (strain HL_77_2, CP053790.1) and *S. agalactiae* (CP051004.1).

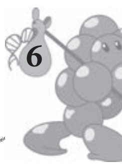


Figure 7. Genetic variants of Tn*916* tetracycline resistance elements in *S. pseudintermedius*. Antimicrobial resistance genes are indicated in red, IS elements in blue, helix-turn-helix modifying genes in purple, plasmid-associated genes in yellow and hypothetical genes in grey.

The genome of isolate (18S02748) carried *tet*(M), and *tet*(L) genes and a plasmid recombination gene. This sequence had high sequence identity to an unpublished *E. faecium* plasmid carrying Tn*6248* flanked *tet*(M)-*tet*(L)-*cat* sequences (GenBank KP834592). In the draft genome assemblies, *tet*(L) was found once more in genome 18S02757-1 on a short contig with the same plasmid recombination gene but with a different plasmid *rep* gene flanked by IS*1216* which is a member of the IS*6*-family insertion sequences with enterococcal origin, similar as has been proposed for the PRE25-like element[3]. The *tet*(M) gene was detected in the draft assemblies of 101 isolates. The gene was present in 54 MRSP and 47 MSSP but we could not resolve the chromosomal location in the draft genomes.

3.6.6 The Tn552 beta-lactamase resistance element

The ß-lactamase gene *blaZ* was present in a locus with the regulator gene *blaR1* and repressor *blaI* in all complete genomes. In 24 genomes, the *blaZ-blaR1-blaI* locus was flanked by Tn*552* and a putative ATP-binding protein and inserted in four different chromosomal sites (**Figure 8**). In 18S03459 the *blaR1* open reading frame was split by insertion of IS*256*, and there was copy of the locus present in another chromosomal location flanked by hypothetical genes. Mapping of this locus to the draft genomes showed *blaZ* gene in 207 genomes of which 13 genomes contained two *blaZ* genes (**Figure 1**).

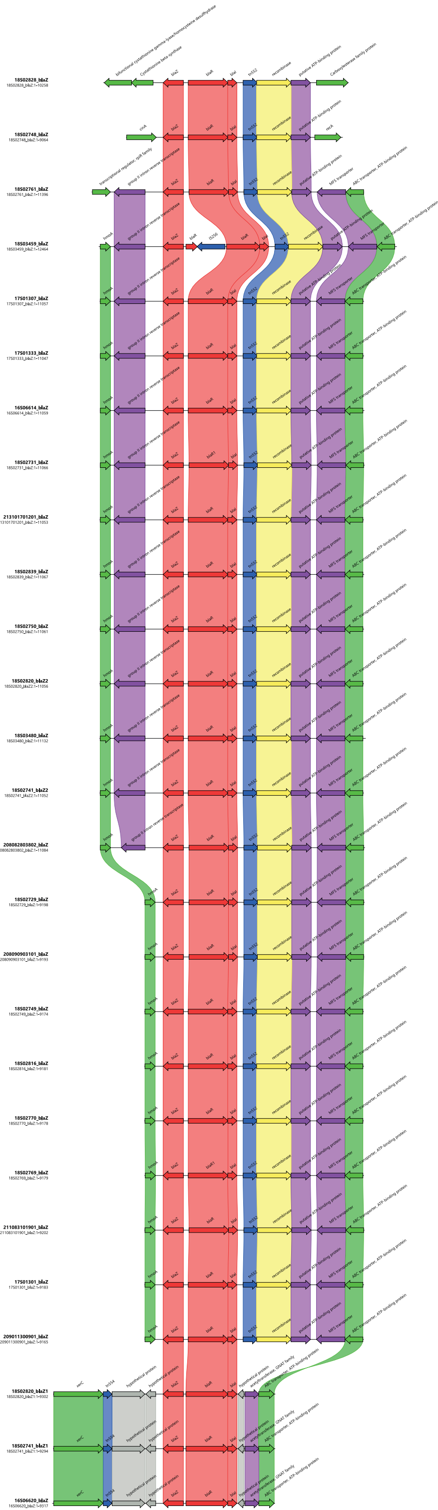


Figure 8. Genetic variants of *Tn552* ß-lactamase resistance elements in *S. pseudintermedius*. Antimicrobial resistance genes are indicated in red, IS elements in blue, helix-turn-helix modifying genes in purple, plasmid-associated genes in yellow and hypothetical genes in grey.

3.6.7 Other mobile antimicrobial resistance gene

AMR detection using CARD identified the macrolide efflux pump associated gene *mef(E)* with 79 % DNA sequence identity in 38 of the draft genomes. This gene has been associated with macrolide resistance in *Streptococcus pneumoniae*[53], and has been described on a plasmid in *Micrococcus* species[54]. In *S. pseudintermedius* the gene was flanked in all genomes with a adenylate cyclase gene and a mutR transcriptional regulator in the core genome. This is the first study describing *mef*(E) in *S. pseudintermedius*, and whether this gene is associated with macrolide resistance must be established.

3.7 Plasmids not carrying antimicrobial resistance genes

Multiple plasmids that did not carry antimicrobial resistance genes were detected in the closed genomes (**Table 1**). The Sp222 plasmid[55] of 12-15 kb was identified in two closed genomes (16S06614-1 and 18S02839-1) and carried a replication gene (EGQ1590623.1) that could be not be defined with PlasmidFinder, as 57 % identity and 80 % coverage with rep_{21_14}_rep(pKH21) was below the applied threshold. Plasmid Sp222 is known for encoding bacteriocin activity and has been associated with MSSP isolates and was detected in the draft genomes in 14 MSSP isolates of CC241 (9/14) and 1 MRSP isolate. A small plasmid of 2.7-3 kb was detected in four closed genomes. This plasmid carried a rep_{13_3} replication gene and shared high identity (99 %) with plasmid pSP5912 of *S. pseudintermedius* (CP009121), carrying a putative multi-drug transporter gene and a hypothetical protein with PFAM domain of a transcriptional regulator. In the draft genomes, this plasmid was present in 53 genomes (41 MRSP and 13 MSSP) and was predominant in CC258 (n = 32 of 35) and CC45 isolates (n = 8 of 8). Another small plasmid of 2.45 kb was present in 3 complete genomes that showed high identity (96 % coverage, 84 % identity) with *S. pseudintermedius* plasmid pSP_11304-3A_2 (CP065923.1) This plasmid contained a rep_{7_3} replication gene and mainly carried genes encoding hypothetical proteins. Two other small plasmids were detected, one of 1.5 kb that carried a replication gene of unknown type and a ribbon-helix-helix domain-containing protein, and a 3.1 kb plasmid also with an unknown replication gene and three genes encoding hypothetical proteins. In two closed genomes a larger plasmid of 17 kb contained multiple conjugal transfer genes (*tra*), and multiple hypothetical genes.

4. Discussion

Complete genome assemblies revealed multiple chromosomally integrated AMR elements in *S. pseudintermedius*. This provided insight in the complex organization of these elements and addressed again the importance of these elements in the dissemination of AMR genes. This is consistent with previous studies that antimicrobial resistances in *S. pseudintermedius* is mainly a result from integrated transposons, phages and conjugative elements[12,56]. Larger conjugative plasmid might also play a role in the transfer of AMR genes as they can carry transposons with resistance genes which can integrate in the chromosome[51,57]. These plasmids can also mobilize and transfer elements carrying AMR genes already inserted in the chromosome, even when they are not themselves conjugative[57]. The aminoglycoside resistance in some isolates was attributable to co-integrated distinct conjugative transposons (Tn*5405*-like, Tn*554*, PRE25-like, Tn*4001*) (**Figure 1**).

The role of small plasmids in AMR genes transmission has been considered limited in *S. pseudintermedius*[14], but we identified multiple small plasmids carrying, *tet(K), cat*, or *lnuA* genes. The fact that most of the isolates carried plasmid content suggests that these might be important vehicles for AMR acquisition in *S. pseudintermedius*. In other staphylococci larger conjugative plasmids carrying resistance genes have been characterized that encode their own conjugative transfer. An example is the pSK41 plasmid that contains IS*257* flanked segments. Variants of this plasmid carry IS*257* in multiple copies and cointegrated copies of smaller plasmids[58]. In the *S. pseudintermedius* genomes the IS*257* flanked segments were present on the Tn5404-like element inserted in different genome locations and were not observed in plasmids in this study. The Tn*5405*-like elements varied in their gene compositions with up to 11 resistance genes in a common Tn*5405* and IS*1182* backbone and plasmid replication genes. This suggest that *S. pseudintermedius* is capable of accumulating AMR genes from smaller plasmids into a composite transposon-like element acting as a hotspot for insertion, by homologous recombination between IS copies[48]. Another integrated MGE is the PRE25-like element that was remarkably conserved in gene composition and present in the same chromosomal insertion site. The *cat* gene in this element may be derived from a recombined plasmid, and it may have resulted from recombinants of elements present in other species, but it is still highly homologous to the PRE25 plasmid in enterococci[13]. Remarkable, is the observation that its presence is dispersed over MRSP and MSSP isolates of all clades but is not present in CC71 isolates and genomes carrying this element do not carry Tn*5405*-like

elements. Currently is it unclear whether these elements are mutually exclusive and if so, by which mechanism, however brooks et al.[59] have described lineage associated RM systems that may form a barrier to HGT, similar to what has been observed in *Escherichia coli* [60].

Phylogenetic reconstruction of MRSP and MSSP revealed three clades consisting of isolates that all carried mobile elements with AMR genes. The general concept is that MRSP clonal lineages emerged after acquisition of an SCC*mec* element conferring methicillin resistance. In clade 3 several MRSP clones have emerged, and for the successful European CC71 SCC*mec* II/II clone it has been proposed that the Tn*5405*-like mobile element carrying multiple resistance genes enhanced the success of this clone, but other factors that might have contributed are the high copy ptetK-1 and pcat-1 plasmids associated with this clonal complex, identified in this study. Expansion of CC45 from a clade 3 ancestor could be explained by acquisition of a SCC*mec*$_{57395}$ pseudo element, the PRE25-like element [13] and transposable elements Tn*916* and Tn*4001*, adding tetracycline and additional aminoglycoside resistance (Figure 1). Another acquisition of the PRE25-like element combined with the bacSp222 encoding plasmid is thought to be associated with the clonal expansion of CC241 in clade 3[11]. This clade harbored two MRSP isolates (18S027481; 18S02770) which carried multiple resistance elements and plasmids, and with acquisition of an SCC*mec* element without fitness costs they may evolve as high-risk AMR clones.

For CC71 and to a lesser extent CC45 it has been described that a phage insertion in the *comGA* gene might hamper HGT[59]. This supports the concept that some *S. pseudintermedius* isolates are more likely to undergo recombination than others, and therefore are more likely to acquire AMR determinants. In other clades the clonal expansion seems less frequent, and clones are more genetically diverse like CC258 in Clade 1. Isolates of this clonal complex carried multiple MGEs (Tn*5405*-like, Tn*916*, pSP5912) or CC442 carrying Tn*916* in Clade 2. All these MGEs carried multiple AMR genes and may indicate that these genes have been acquired in response to selective pressure from used antimicrobials. This are adaptive mutations that might support clonal expansion. Clonal lineage CC71 was successful over years and was replaced by the recently emerging CC258 clone showing higher SNP and gene diversity, and carriage of complex MGE, that was also observed for individual MRSP isolates. This could mean that new isolates can become clonal or disappear. While this temporal variation in clones has been previously shown in different countries[5,7] its evolution is most likely dependent on the acquisition of AMR genes that arises in response to the use of antimicrobials. Nevertheless, MGEs can be adaptive or lost when they have

fitness costs. In *S. aureus* deletion of SCC*mec* compensates for fitness costs in high level vancomycin resistant isolates[61]. So far, the SCC*mec* loss in *S. pseudintermedius* seems rare[62]. In this study a possible SCC*mec* loss was detected, as one isolate carried a SCC*mec*IV remnant. SCC*mec*IV has been shown to have a lower fitness cost than larger SCC*mec* elements[57] providing easier transfer which may explain why it entered the population multiple times. The balance between fitness cost and evolutionary advantage might account for acquisition and loss of other MGEs carrying AMR genes.

In conclusion, the mobile genetic elements in *S. pseudintermedius* can be shared with other Staphylococcal, Enterococcal and Streptococcal species that can be present in the same niche. We highlight the AMR genes in *S. pseudintermedius* as a reservoir for interspecies HGT of resistance genes, and drivers for local adaptation of highly resistant *S. pseudintermedius* isolates.

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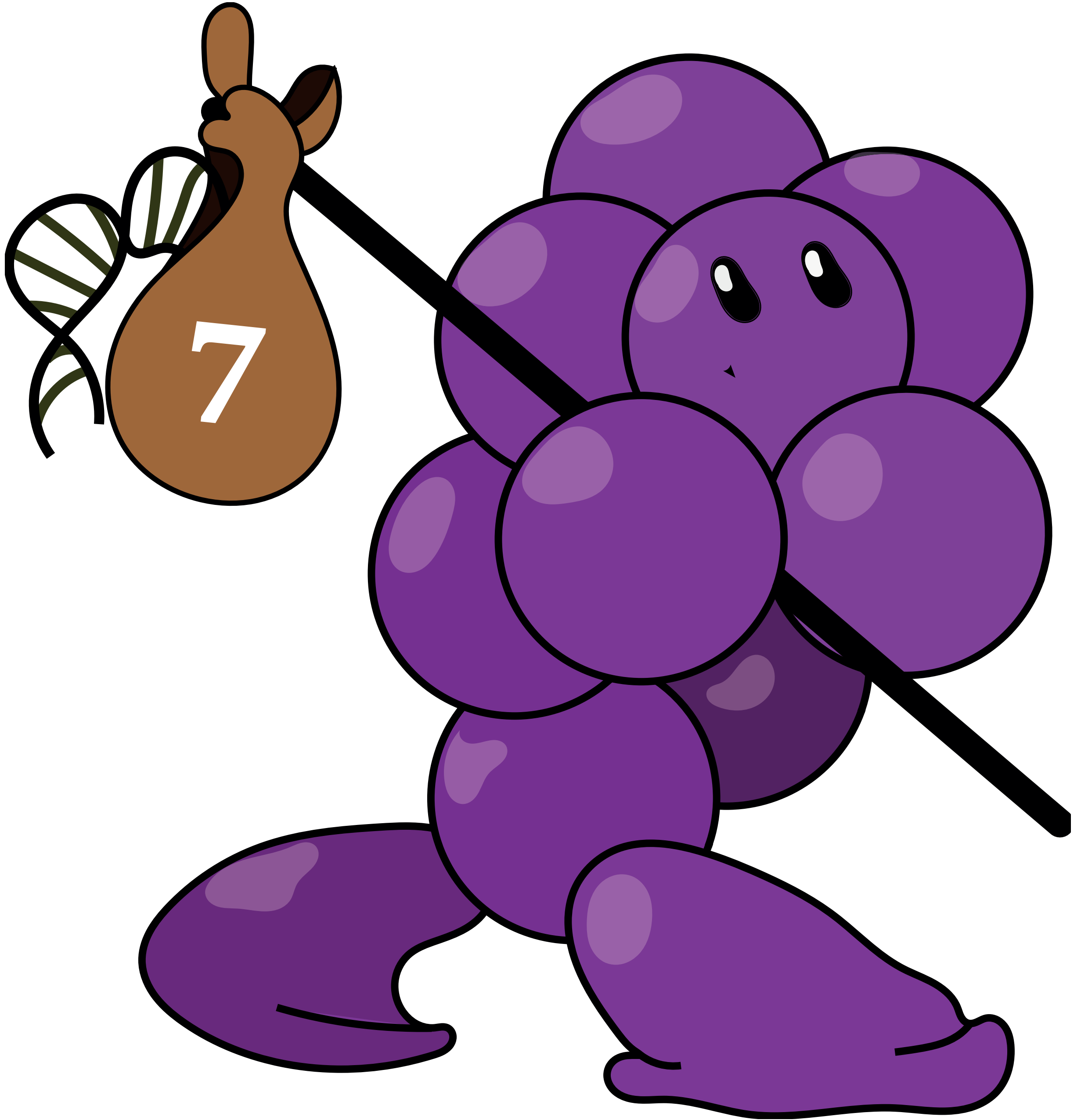
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CHAPTER 7

General Discussion

Research questions at the start of the PhD-study

Staphylococcus pseudintermedius is strongly associated with dogs as a commensal and opportunistic pathogen (1). Infections have occasionally been detected in humans (2), where *S. pseudintermedius* was regularly misidentified as *S. aureus,* prior to the introduction of MALDI-TOF in routine diagnostics (3). Methicillin-resistant *S. pseudintermedius* (MRSP) isolates have emerged over the past decades. For susceptibility testing, the expert rule that is used for methicillin-resistant *S. aureus* (MRSA) stating that methicillin-resistant isolates should be considered resistant to all β-lactam antibiotics, is also applied to MRSP. This consideration is nevertheless questionable as it has not been verified in *S. pseudintermedius* (4). Therefore, analysis of possible low-level resistance or even susceptibility to a selection of β-lactams in MRSP is needed. Furthermore, for some antimicrobial resistance genes their presence has been associated to phenotypic resistance in *S. pseudintermedius* (5), but a systematic analysis of genotype-phenotype association by using whole genome sequencing (WGS), and therefore the significance of resistance gene presence has not been evaluated.

Several studies have described carriage of mobile genetic elements (MGEs) in *S. pseudintermedius*, but the contribution of these elements to the dissemination of antimicrobial resistance genes in the population has not been studied. Neither has possible genetic host specificity of canine and human isolates of *S. pseudintermedius* extensively been studied.

To elucidate those aspects, in this thesis several aspects of *S. pseudintermedius* were studied. The described studies focussed on the molecular epidemiology of antimicrobial resistance in *S. pseudintermedius*, on host association, zoonotic risk and diversity in β-lactam susceptibility of isolates.

Main findings

Chapter 2 shows that phenotypical resistances can be reliably predicted by identifying resistance genes and that resistance gene patterns in MRSP are linked to clonal complexes.

Chapter 3 shows that different SCC*mec* elements are linked to specific clonal complexes that express different levels of phenotypic β-lactam resistances.

Chapter 4 shows the overrepresentation of multidrug resistant clonal complex CC241 in human isolates, but no specific host associated factors could be detected in either dog or human isolates.

Chapter 5 is a case study, which shows genetic relatedness between canine and human isolates within households, as well as high diversity in isolates colonising the same dog.

Chapter 6 identifies high plasmid diversity and novel genomic elements showing that antimicrobial resistance genes are often carried on MGEs carrying multiple resistance genes, that vary between isolates from different clonal complexes.

Zoonotic importance of *S. pseudintermedius*

The prevalence of *S. pseudintermedius* infections in humans, its evolution over time, and epidemiology are difficult to evaluate, because until recently *S. pseudintermedius* was still often misidentified as *S. aureus* (3). Since the implementation of MALDI-TOF in routine diagnostics, there has been an increase in reporting of *S. pseudintermedius* in humans (6, 7). Therefore, it is unknown whether human infections with *S. pseudintermedius* are actually emerging or rather better detected. Studies in humans are relatively limited and are often presented as case studies with only a limited number of isolates from dogs and humans (2, 8). However, two larger retrospective studies on *S. pseudintermedius* in human clinical cases in hospitals in North America showed that *S. pseudintermedius* infections in humans are mostly caused by MSSP (and not MRSP) and have a low prevalence (9, 10). Of the infections in human cases, it was estimated that *S. pseudintermedius* represented 0.05% of soft tissue and wound infections (10). MRSP has been previously detected in human cases only occasionally (2). In our collection only one human MRSP isolate was present. The relatively low number of human cases suggests that *S. pseudintermedius* does not represent a major challenge for public health.

In this thesis, we present one of the largest collections (n=33) of human *S. pseudintermedius* isolates, collected from various hospitals in different regions of the Netherlands over a period of four years. This study provided a wider view of *S. pseudintermedius* in humans and potential transmission between dogs and humans. Nevertheless, this study does not address the prevalence of *S. pseudintermedius* in humans, which is currently unknown and which impact could therefore not be

estimated. To get a better understanding of the prevalence and potential sources of *S. pseudintermedius* in human infections, it would be interesting to perform a questionnaire based study collecting data from human (hospital) microbiology laboratories on the number of *S. pseudintermedius* isolates, the type of infection and the risk factors for infections, compared to other staphylococci. Such a study could be done on a national or preferably European level.

In *S. aureus*, several host-specific mutations and acquisition or loss of mobile genetic elements have been detected, allowing adaptation to diverse (animal) host species (11, 12). Genomic studies on host-specificity of *S. pseudintermedius* had not been performed before the research presented in this thesis. In Chapter 4, we present the first large genomic study of human and canine isolates, analysing possible host specificity, using phylogenetic analysis, as well as a genome wide association analysis. A high genetic diversity was identified among human and canine MSSP isolates (Chapter 4 and 6) and no host-specific genes could be detected in either canine or human isolates. The relatively low prevalence of *S. pseudintermedius* in human infections and the lack of host-specific genes supports the hypothesis that human infections are most likely a spill over from the dog population in the same way as *S. aureus* in dogs is considered a spill over from human to dog in most cases (11, 13). While no lineage was statistically host associated, a single clonal lineage (CC241) was overrepresented in humans. This is particularly interesting as it emerged among a genetically diverse MSSP background (Chapter 6, dog and human combined).

CC241 was not present in our isolate collection before 2015 and it might have appeared recently as an emerging clone in the Netherlands. Host-associated genes were not detected (chapter 4) but there might be a selective advantage for CC241 through the carriage of two mobile elements. Plasmid p222 expresses a highly cationic bacteriocidal peptide BacSp222 that inhibited growth of Gram-positive bacteria including staphylococci and showed dose-dependent cytotoxicity for human keratinocytes (14). The peptide also demonstrated immunomodulation responses *in vitro*, and verification of cytotoxicity for dog skin or in an *in vivo* model is needed to unravel if the peptide is host specific. Overall, this may allow CC241 isolates to compete with other commensal staphylococci on the skin, in particular *S. aureus* or *S. epidermidis* in humans, and *S. schleiferi* or other *S. pseudintermedius* isolates in dogs. The second MGE present in this clone is a PRE25-like element responsible for multidrug resistance, with resistance genes encoding for aminoglycosides, chloramphenicol, macrolides, and streptothricin resistance (15). In Chapter 6, a stable chromosomal integration of this element was observed, which might suggest

that antimicrobial selection could favour the spread of CC241. Targeted surveillance of the CC241 prevalence over the coming years would be of interest to see whether it emerges further in the human and canine *S. pseudintermedius* population.

Acquisition or loss of host-specific genes or several mutations in *S. aureus* have been considered important drivers for host adaptation (11). In *S. pseudintermedius* no significant host-associated genes could be detected, but gene mutations have not been studied in *S. pseudintermedius*. Previous studies have linked the adherence proteins spsL and spsD of *S. pseudintermedius* to host specificity (16), although in our isolates these proteins showed a high level of diversity in both canine and human isolates. The number of isolates studied in Chapter 4 remains limited, which together with the high variability in human and canine MSSP isolates, causes lack of power in identifying host specific elements. Therefore, larger studies are needed to confirm the absence of host specificity. It is also important to keep monitoring *S. pseudintermedius* by ongoing collection and characterisation of canine and human isolates, as host specific clones might emerge over time.

It is unclear whether all isolates from dogs are equally transmissible, and if the transmission of a canine isolate can further lead to human-to-human transmission. Currently only one case of probable human-to-human transmission of MRSP (ST71) has been reported in a hospital setting (17). In studies on human clinical cases more than 90% of patients had contact with dogs (9, 10). Previous longitudinal studies on MRSP transmission showed that humans generally carry the same isolate as their dog, and that the environment was often found positive for MRSP. MRSP carriage in humans appeared to be rare and short lived (18, 19). Another study showed that veterinary personnel and clinics can be positive for MRSP, which suggest that transmission is also taking place in veterinary settings (20). The sharing of isolates between dogs and humans has equally been shown in MSSP (8), but studies on transmission are limited to MLST analysis and antimicrobial resistances, which has a very low resolution compared to WGS and SNP comparison, therefore differences between isolates could be missed. In Chapter 5, we present a case study with the genomic SNP comparison of canine and human isolates in households with human *S. pseudintermedius* infections. In both cases genetically related isolates were found in dogs and owners. In one household, the canine isolates of two different dogs were genetically more closely related to each other than to the human isolate, although all three isolates belonged to ST241. This could indicate that genomic evolution occurred during human infection. It is equally relevant to mention that genetically unrelated

canine isolates were present in both households, with one dog carrying multiple sequence types (ST). It is not clear whether isolates belonging to different STs co-colonized the owner, as these isolates could be missed because only one isolate was taken from the positive culture of the human sample. It is currently unknown whether humans carry genetically different *S. pseudintermedius* isolates simultaneously. A large longitudinal genomic comparison study of *S. pseudintermedius* in households, covering six months to a year, taking multiple samples of dogs as well as humans in each sampling, and picking more than one colony after culture would allow a better understanding of transmission dynamics of *S. pseudintermedius*. A microbiome study of *S. pseudintermedius*-positive dogs and owners would equally help to gain insight into the diversity of *S. pseudintermedius* genotypes, AMR and virulence genes, and the composition of the microbiome will show if there is stable or transient colonisation of *S. pseudintermedius*.

As a conclusion, *S. pseudintermedius* infections in humans are mainly due to a spill over from canine isolates. Nevertheless, *S. pseudintermedius* can cause human infections similar to those caused by *S. aureus* and isolates can be equally multidrug resistant. Therefore, it remains important to survey the epidemiology of this species in the future, to monitor the presence of *S. pseudintermedius* infections and the possible emergence of host-specific clones in human infections.

***S. pseudintermedius* in dogs**

Dogs are the major host of *S. pseudintermedius,* therefore the population dynamics are well studied in this animal species. It has been previously suggested that MSSP and MRSP represent two different populations (21). MSSP is genetically highly variable (22), whereas MRSP is mainly clonal (23). Our study on a large collection of canine isolates (MRSP and MSSP) confirms these observations (Chapter 6). MRSP is represented in the Netherlands by three main clonal complexes (CC45, CC71 and CC258), and in recent years occasionally isolates with other genotypes appeared. It is also important to note that when looking at clustering in the phylogenetic tree, three different clusters are visible of which two contain both MSSP and MRSP isolates where the MRSP clonal complexes emerge from independent MSSP ancestors. For one MRSP clonal complex (CC71) it was possible to estimate that this clone was introduced into the population in 2001-2002 (unpublished results). This could be the first date of introduction of MRSP in the Netherlands, however, the first observation of CC71 MRSP in a clinical sample was in 2004 (24). No quantitative data could be

found on the use of β-lactams in these years, nevertheless it has been shown in later studies that β-lactams are the most used antibiotics in companion animals (25) and it is likely that this was already the case in 2001-2002 and might have selected for the emergence of MRSP. CC45 and CC258 emerged more recently and seem to partially replace CC71, however no date could be determined for the introduction of these clonal complexes due to high level of recombination and limited number of isolates. In MSSP a minority of the isolates are clustering in two clonal complexes (CC241 and CC442), whereas most isolates belonged to unique STs. It is plausible that clonal complex CC241will undergo further clonal expansion in the future.

Sampling strategy and biases

In this thesis only clinical isolates were analysed, which could have biased our analysis towards the more virulent and resistant part of the *S. pseudintermedius* population. Furthermore, the analysed dog isolates represented only a fraction of the total number of *S. pseudintermedius* isolated each year. The selection was done with the aim of obtaining a representative population by randomly selecting isolates, attention was also paid to including similar numbers of MRSP and MSSP isolates, as well as balancing the number of isolates over the selection period. However, it was not possible to obtain the exact same number of isolates per year, as in the first years the numbers were limited, in particular because isolates without resistance were often not stored. In humans the prevalence and diversity of *S. pseudintermedius* were probably highly underestimated since in earlier years routine diagnostics were inadequate to distinguish *S. pseudintermedius* from other coagulase-positive staphylococci, and *S. pseudintermedius* isolates were not always stored. All these issues may have biased the results obtained with the genomic studies in this thesis. Furthermore, the level of metadata (patient age, gender, infection site, animal contact) varies depending on hospitals and / or isolates. During the collection time, a sample information form was used to remedy to this problem, but it was only available for the more recent isolates. This did not allow to study the association of infections with different risk factors.

Drivers of multidrug resistance and clonal expansion

The emergence of MRSP clones is considered to have evolved by the acquisition of a MGE, the staphylococcal chromosomal cassette *mec* (SCC*mec*), carrying the *mecA* gene coding for an alternative penicillin binding protein. Multiple independent

SCC*mec* acquisitions occurred resulting in genetically distinct MRSP lineages with their own SCC*mec* type (7, 21). In our collection all CC71 isolates carried the SCC*mec*II-III, which suggests a single acquisition followed by clonal spread, which is also observed for CC45 carrying ΨSCC*mec*$_{57395}$. In contrast, CC258 has mainly acquired SCC*mec*IV, while SCC*mec*V was also detected. Both SCC*mec* types were present in different STs and were not linked to a clone. The fact that SCC*mec*IV and SCC*mec*V are found in multiple isolates suggest that they have entered the population multiple times. SCC*mec*IV and SCC*mec*V were also found in different clones in *S. aureus* (26, 27). For SCC*mec*IV it has been suggested to have a reduced fitness cost compared to other SCC*mec* types (26), which could be linked to the fact that this SCC*mec* type is shorter and therefore possibly more easily transmitted. In one case a probable loss of SCC*mec*IV was observed in CC258, which shows a dynamic process of gain and loss of SCC*mec* with gain being more frequent, as has been previously suggested (7). SCC*mec*II-III on the other hand is a recombination between SCC*mec*II of *S. epidermidis* and SCC*mec*III of *S. aureus* and is, until now restricted to *S. pseudintermedius* CC71.

Next to resistance to β-lactam antimicrobials encoded by the *mecA* gene, MRSP isolates are often multidrug resistant (MDR) limiting treatment options. In Chapter 2 we suggested that resistance gene patterns in MRSP were most likely associated with the CC. However, through full genome analysis and additional long-read sequencing of MRSP as well as MSSP isolates, we observed more variability in the carriage of AMR genes (Chapter 6). MDR was observed in MRSP as well as in MSSP and was linked to the spread of MGEs. MGEs are genetic elements which can be transmitted horizontally between bacteria within and across species, these include plasmids, transposons, genetic cassettes, pathogenicity and resistance islands. These elements often carry one or multiple resistance genes. In *S. pseudintermedius* MDR was linked to several large conjugative MGEs carrying multiple resistance genes, of which the PRE25-like element was remarkably stable in gene composition and insertion site, where others were highly diverse in particular the Tn5405-like element, which was shown to carry up to eleven resistance genes in 3 different insertion sites (Chapter 6). Recombination within MGEs is thought to occur frequently in Gram-positive bacteria. It is also common that different transposons insert within the same insertion site (26). This can lead to an accumulation of MGEs in the same location which can in turn form larger composite MGEs. The fact that most elements are inserted in the chromosome is important as this makes it more difficult for isolates to lose those elements and could facilitate the accumulation of diverse elements

over time. Resistance genes can be transferred on a large variety of elements (plasmids, transposons, phages, large conjugative elements), all are detected in *S. pseudintermedius*. Recombination and thereby diversification of MGEs seems to take place in MRSP as well as in MSSP but the mechanisms and extent of recombination remain understudied. The acquisition of multiple MGEs seems to be an important factor for the emergence of successful clones in *S. pseudintermedius*. Indeed, most successful MRSP clonal lineages (CC71, CC45, CC258) and MSSP (CC241) have emerged from a diverse MSSP background after acquisition of multiple MGEs carrying antimicrobial resistance genes. However, the proportion of clones seems to vary over time, with clones emerging or declining in prevalence, showing that the population of *S. pseudintermedius* is dynamic. Therefore, active surveillance could monitor the possible emergence of new MDR clones.

The fact that multiple resistance genes can be present in an isolate increases the risk of co-selection of present resistance genes when using antimicrobials. Thereby leading to the selection of MDR isolates as well as a possible increase of exchange of antimicrobial resistance genes within the *S. pseudintermedius* population or transfer to other species.

When using short-read sequencing it was not possible to elucidate the MGE composition or location due to frequent contig breaks of these elements. Therefore, long-read sequencing should be used routinely to gain insight in the dissemination of these elements. Furthermore, functional studies on MGE transfer, within and across species, to study transmissibility of MGEs between species, would be of great added value to understand the dynamics of AMR spread.

Different levels of β-lactam resistance

All MRSP isolates are considered resistant to all β-lactam antimicrobials. This is based on an expert rule which was set up for *S. aureus* to avoid major errors (i.e. resistant isolates reported as susceptible), and runs as follows: if resistant to isoxazolyl-penicillins (as determined with oxacillin, cefoxitin, or by detection of *mecA*-gene or of PBP2a), then isolates should be reported as resistant to all β-lactams, except those specifically licensed to treat infections caused by methicillin-resistant staphylococci owing to low affinity for PBP2a (28). When screening for methicillin resistance in *S. pseudintermedius* using disk diffusion or MIC testing, oxacillin should be used as cefoxitin does not reliably detect the presence of *mecA* in this staphylococcal species (29). In Chapter 3 we showed that the expert rule might not be valid for *S.*

pseudintermedius, as differences in β-lactam resistances were observed, with isolates displaying low level resistance or even susceptibility to amoxicillin/clavulanic acid and cefalotin. These results could be of clinical importance, as infections with low level β-lactam resistant isolates might be treated effectively using these antimicrobials. Similar results have already been shown in MRSA in humans, with good clinical cure rates being reported after cefalotin treatment for uncomplicated MRSA skin infections (30) and for high doses of amoxicillin/clavulanic acid for urinary tract infections (31). Clinical trials of β-lactams treatment of MRSP in dogs will be necessary to confirm whether these antimicrobials could result in effective treatment of infections caused by low level resistance isolates.

The mechanism causing differences in β-lactam susceptibility is unknown. Previous studies suggested that these MIC differences in MRSP were associated with different SCC*mec* types (32, 33), which was confirmed in this thesis (Chapter 3). SCC*mec*II-III was associated with overall higher MICs for β-lactams, and SCC*mec*IV, SCC*mec*V and ΨSCC*mec*$_{57395}$, with lower MICs and even susceptibility to cephalosporins (Chapter 3). In a large collection of MRSA isolates harbouring SCC*mec*IV, susceptibility to penicillin/clavulanic acid was observed (34). Rapid screening assays that can be implemented in daily routine that are able to differentiate between the different SCC*mec* types might be helpful in predicting susceptibility to β-lactams. However, SCC*mec* type cannot be followed blindly as a predictor of β-lactam resistance, as other regulatory genes or mutations might be involved in the phenotypic expression. Resistance to methicillin is associated with the expression of *mecA*, which is regulated by three regulatory genes in *S. aureus*: the repressor *mecI,* the sensor *mecR1,* and antirepressor *mecR2* (35). In *S. pseudintermedius mecI*, *mecR1* and a *mecR2*, are present in SCC*mec*II-III (36, 37), which is associated with higher β-lactam resistances, and are absent in other SCC*mec* types. In one isolate with a new SCC*mec* type the *mecR1* and *mecI* genes were present but the *mecR2* gene was absent, this isolate also showed a D7E substitution in the MecI protein and had a low MIC for all β-lactams (unpublished results). It would require more isolates with deletion of *mecR2* to unravel if *mecR2* absence and/or mecI substitutions lead to lower resistance. *MecA* expression is also regulated by *blaZ* and its regulating genes *blaR1* and *blaI* (38). The analysed isolates often carried multiple and diverse *blaZ* genes, making it difficult to draw conclusions on their contribution (unpublished results). It has been shown that the expression of *mecA* in *S. aureus* is also regulated by mutations in the promotor sequence, that were shown to be associated with lower β-lactam resistance in the presence of clavulanic acid (a β-lactamase inhibitor)

(34). These mutations were not present in our isolates. In addition, in *S. aureus* substitutions in the PBP2a protein were associated with an increase in susceptibility in the presence of clavulanic acid (34). In our isolates only one of these mutations E246G, was associated with high MICs. Other mutations in 27 identified genes and 3 intergenic regions have previously been shown to lead to an increase of the level of β-lactam resistance in the case of heterologous resistance in *S. aureus* (35).

None of the SCC*mec* elements or the genomic comparisons studied in this thesis identified a gene composition completely explaining the level of β-lactam resistance and it is highly plausible that the regulation of β-lactam resistance is a complex and multifactorial process. Further experimental studies are needed to elucidate these mechanisms. An attempt was made to study the effect of the function of *mecA* regulators, by using allelic exchange to add *mecI*, *mecR1*, *mecR2* genes in isolates where they were absent and to determine if this would increase resistance levels. This failed due to the unsuccessful transformation of *S. pseudintermedius*. It would be interesting to measure the transcription level of the *mecA* gene in isolates carrying different SCC*mec* types, although it has previously been shown that the level of *mecA* transcription could not explain the MICs in ST71 and ST68 isolates (38). Studying the PBP2a affinity of isolates with high or low MIC levels could also increase the understanding of β-lactam resistance. Additionally, a genomic wide association study would be of interest to identify potential genes involved in low-level resistance.

Global conclusion

S. pseudintermedius is a common dog pathogen with occasional spill over to humans. Infections in humans are caused by isolates closely related to those of their pets, even if the pets often carry multiple heterogenous isolates. MSSP isolates are genetically more diverse than MRSP isolates that show a more clonal population structure. The population of human isolates showed the same overall diversity as the canine population and no host related genes could be detected. Most successful clones are MRSP which have emerged after the acquisition of SCC*mec*, but the acquisition of multiple MGEs other than SCC*mec* seems another important driver of clonal success and can occasionally lead to the emergence of MDR MSSP clones such as CC241 that appeared to be overrepresented in human isolates. It remains important to monitor *S. pseudintermedius* infections in humans as MDR isolates could complicate treatment and new MDR clones may emerge.

The observed low level β-lactam resistance and even susceptibility for cefalotin

and amoxicillin-clavulanic acid in isolates carrying SCC*mec*IV, requires functional studies to elucidate the mechanism of low-level resistance, as well as clinical evaluation of using β-lactams antimicrobials for treatment of dogs.

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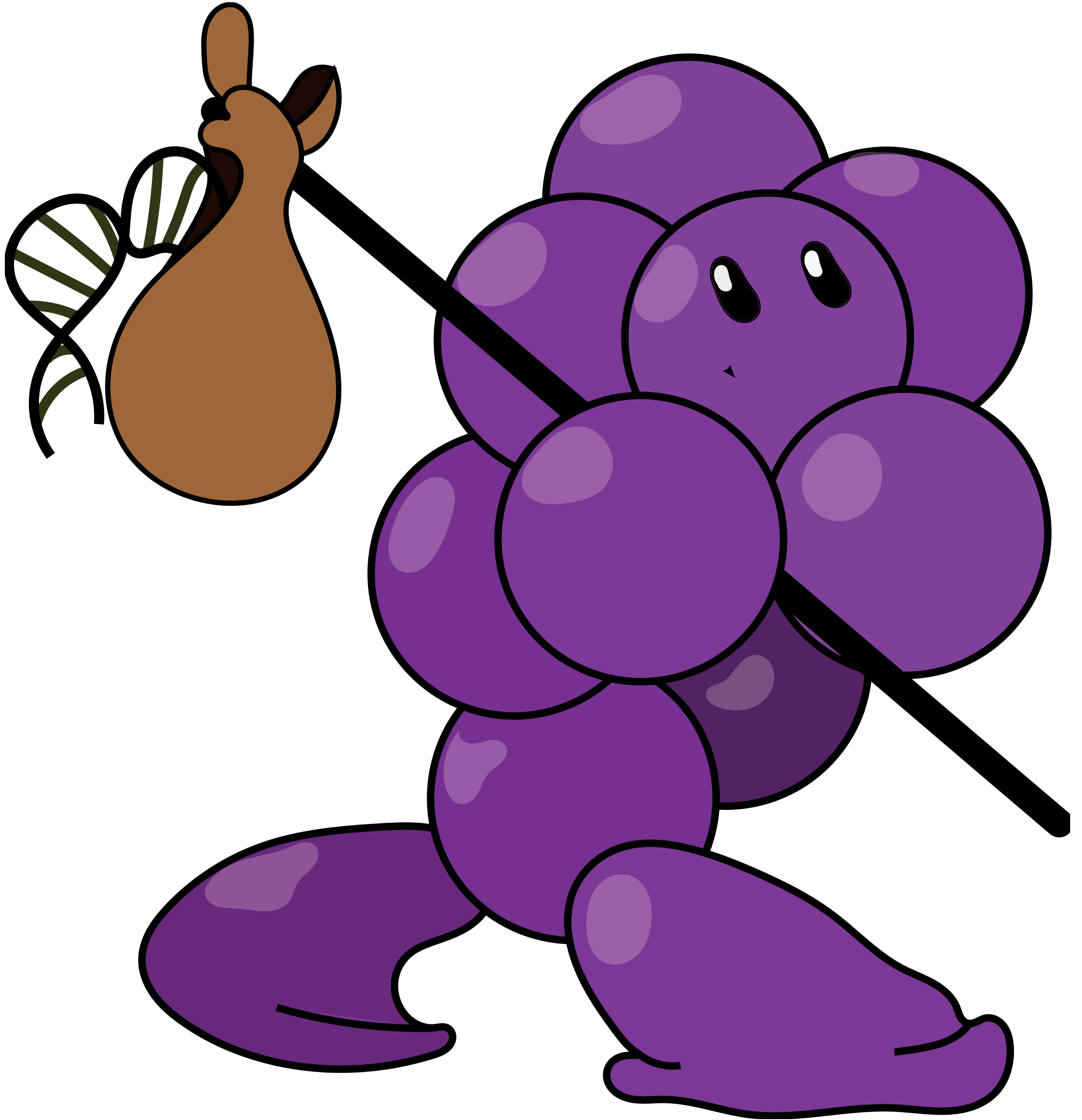

Appendix

Summary

Staphylococcus is a genus of Gram-positive bacteria of clinical importance for humans as well as animals. Staphylococci are traditionally separated in two groups based on the production of coagulase, an enzyme coagulating plasma. Coagulase-positive staphylococci are considered the major pathogens, whereas coagulase-negative staphylococci are considered relatively minor pathogens, although some coagulase-negative species are causing bloodstream infections in humans. The most clinically relevant staphylococci in animals are coagulase-positive staphylococci associated with specific infections in animal hosts, e.g. *Staphylococcus aureus* causing mastitis in ruminants, *S. hyicus* causing dermatitis in pigs and *S. pseudintermedius* causing pyoderma in dogs. *S. pseudintermedius* is a commensal in dogs and colonizes different body sites (e.g. skin, ear, nose, mouth, perineum). *S. pseudintermedius* can cause opportunistic infections that occur predominantly in the ear, skin, and surgical wounds.

An important and clinically relevant property of staphylococci, including *S. pseudintermedius*, is their ability to acquire antimicrobial resistance. These antimicrobial resistances are mostly associated with mobile genetic elements (MGEs), although point mutations, conferring resistance towards e.g. fluoroquinolones, fusidic acid and rifampicin may occur. The most significant acquired antimicrobial resistance from staphylococci is methicillin resistance encoded by the *mecA* gene, which is carried on a distinct mobile genetic element, the Staphylococcal Chromosomal Cassette *mec* (SCC*mec*). Isolates harboring this SCC*mec* are called methicillin-resistant *S. pseudintermedius* (MRSP).

Similarly, as in *S. aureus* methicillin resistance has emerged in *S. pseudintermedius* over the past decades. MRSP is often multidrug resistant (MDR) implying resistance to three or more antimicrobial classes. Antimicrobial resistance can be assessed phenotypically by determining the minimum inhibitory concentration (MIC), and its interpretation relies on clinical breakpoints specific to the antimicrobial and bacterial species. For *S. pseudintermedius*, clinical breakpoints are lacking for many antimicrobials, which complicates the interpretation.

Antimicrobial resistance can also be inferred from whole genome sequences by detecting resistance genes or chromosomal mutations leading to resistances.

However, the databases to detect resistance genes in sequenced genomes were developed for Gram-negative bacteria or for *S. aureus*, but not specifically for *S. pseudintermedius*. The reliability of using the online databases to infer resistance in *S. pseudintermedius* was assessed in **Chapter 2.** For this study 50 isolates were sequenced using whole genome sequencing (WGS). The majority (i.e. 98.3%) of the resistances detected phenotypically (using MICs and clinical breakpoints) could be explained by the presence of an associated resistance gene or point mutation. It appeared that the antimicrobial genotype from whole genome sequencing is highly predictive of the resistance phenotype in MRSP, and that errors result in considering susceptible isolates as resistant, mostly due to genetic variations inactivating the resistance genes.
To study the potential association of resistance gene patterns with genetic lineages of MRSP a phylogenetic analysis was performed, also in **Chapter 2**. This analysis showed a clonal distribution in MRSP and an association between resistance gene content and clonal complex (CC). In CC45 genomes a very conserved resistance gene profile was present, while it was only partly conserved in CC71, whereas in CC258 resistance gene patterns were highly diverse. This analysis, using more genome sequences, was further extended in **Chapter 6** where it was studied whether resistance genes could be associated to mobile genetic elements (MGEs) in methicillin-susceptible *S. pseudintermedius* (MSSP) as well as MRSP.

Chapter 3 delved deeper into potential associations between clonal complexes (CC) and the resistance levels against β-lactam antimicrobials. SCC*mec* types were associated with different CCs and with either high- or low-level resistance against oxacillin, penicillin, amoxicillin/clavulanic acid and cefalotin. CC71 isolates harbouring SCC*mec*II-III showed higher resistance levels to β-lactam antimicrobials. Isolates harbouring SCC*mec*V were showing variable resistance levels and CC258 isolates with SCC*mec*IV were associated with low-level resistance or even susceptibility to several β-lactams. Susceptibility was detected to amoxicillin/clavulanic acid and cefalotin. The finding of amoxicillin/clavulanic acid and cefalotin *in vitro* susceptibility across all CCs might have clinical implications and requires pharmacokinetic/pharmacodynamic studies on the clinical outcome to evaluate the *in vivo* efficacy of these β-lactams for treatment of MRSP infections.

While *S. pseudintermedius* seems to be rather host-restricted to dogs, incidental opportunistic infections in humans caused mostly by MSSP are increasingly

reported over the last decades, in particular in elderly and immunocompromised people. This might partially be explained by improved identification of bacterial species since the implementation of MALDI-TOF MS in microbiology laboratories, but host adaptation and genetic variation might play a role as well. In case of human infections with *S. pseudintermedius,* dog-to-human transmission is the suggested route, which is confirmed by indistinguishable isolates from dogs and their owners in several case reports.

To identify possible host adaptation of *S. pseudintermedius*, isolates from canine and human origin were compared in **Chapter 4**, while **Chapter 5** focused on the analysis of within household variability between canine and human isolates.

In **Chapter 4** a phylogenetic and genome wide association study was performed on the genomes from canine and human isolates. This showed high phylogenetic diversity among MSSP from both canine and human isolates, and no host associated genes could be detected. However, one clonal lineage, CC241 was overrepresented in human isolates and was associated with several MGEs carrying multiple resistance genes and virulence factors. Given its multi-resistance and potential virulence, it is important to monitor its epidemiological spread.

Within-household transmission was studied in **Chapter 5** using WGS of isolates from two unrelated human infections and their household dogs. In both cases the isolates from the human infections were considered genetically related to the isolates of their dogs confirming dog-to-human transmission. Several dogs carried multiple unrelated *S. pseudintermedius* isolates as well, demonstrating that within-host bacterial diversity exists, and shows the importance of analysing multiple colonies when transmission events are being studied.

In other staphylococci and in particular *S. aureus* multiple MGEs are known, including plasmids and transposable elements carrying antimicrobial resistance genes, but for *S. pseudintermedius* the knowledge of MGEs and their role in dissemination of antimicrobial resistance genes was limited.

In **Chapter 6** the MGEs in *S. pseudintermedius* were studied more in depth. The analyses identified high plasmid diversity and novel genomic elements. It provided insight in the complex organization of MGEs and addressed their importance in

the dissemination of antimicrobial resistance genes and clonal expansion of MRSP. MRSP was shown to have evolved multiple times from a highly diverse MSSP background and have undergone clonal expansion after acquisition of SCC*mec* and other MGEs facilitating clonal success. Composite MGEs carrying up to 11 different resistance genes were detected. Besides MGEs that were integrated in the genome, small plasmids carrying antimicrobial resistance genes were present, of which a tetracycline resistance encoding plasmid was associated with clonal expansion of CC71 MRSP. A dynamic process of acquisition as well as the microevolution of MGEs were shown. Several MGEs were identified that can be shared with other staphylococcal, enterococcal and streptococcal species highlighting the potential of dissemination of AMR genes beyond *S. pseudintermedius*.

In summary, *S. pseudintermedius* is a common dog pathogen with occasional spill over to humans. The population of *S. pseudintermedius* consists of a genetically diverse MSSP population and a limited number of clonal MRSP lineages. Human and canine isolates showed high phylogenetic diversity and no host associated genes were detected. Successful MRSP clonal lineages have emerged after the acquisition of SCC*mec* and multiple MGEs carrying resistance genes. MGEs that can integrate in the genome are important drivers of clonal expansion of MDR lineages, as was shown for CC241 which was overrepresented in human isolates. Adequate diagnostics of *S. pseudintermedius* infections in humans and animals is required as MDR isolates could complicate treatment. This study showed that the acquisition of mobile transposable elements and plasmids can drive the dissemination of novel MDR lineages. The observed low level β-lactam resistance and even susceptibility for cefalotin and amoxicillin-clavulanic acid in isolates carrying SCC*mec*IV indicates that these antimicrobials can be used for treatment of infection, but it requires functional studies to elucidate the mechanism of low-level resistance, as well as clinical evaluation of using β-lactams antimicrobials for treatment of dogs.

Samenvatting

Staphylococcus behoort tot de Gram-positieve bacteriën welke klinisch van belang zijn voor zowel mens als dier. Stafylokokken worden traditioneel ingedeeld in twee groepen op basis van de productie van coagulase, een enzym dat de omzetting van fibrinogeen naar fibrine katalyseert. Coagulase-positieve stafylokokken worden beschouwd als belangrijke pathogenen, terwijl coagulase-negatieve stafylokokken worden beschouwd als weinig pathogeen, hoewel sommige coagulase-negatieve soorten bloedbaaninfecties bij mensen kunnen veroorzaken. De meest klinisch relevante stafylokokken bij dieren zijn coagulase-positieve stafylokokken welke geassocieerd worden met specifieke infecties bij dierlijke gastheren, bijvoorbeeld *Staphylococcus aureus* de verzoorzaker van mastitis bij herkauwers, *S. hyicus* welke dermatitis veroorzaakt bij varkens en *S. pseudintermedius* de veroorzaker van pyodermie bij honden. *S. pseudintermedius* is een commensaal bij honden en koloniseert verschillende plaatsen op het lichaam (bijv. huid, oor, neus, mond en perineum). *S. pseudintermedius* kan opportunistische infecties veroorzaken die voornamelijk voorkomen in het oor, de huid en chirurgische wonden.

Een belangrijke en klinisch relevante eigenschap van stafylokokken, waaronder *S. pseudintermedius*, is hun vermogen om antimicrobiële resistentie te verwerven. Deze antimicrobiële resistenties worden meestal geassocieerd met mobiele genetische elementen (MGE's), hoewel ook puntmutaties die resistentie veroorzaken tegen bijvoorbeeld fluoroquinolonen, fusidinezuur en rifampicine kunnen optreden. De belangrijkste verworven antimicrobiële resistentie van stafylokokken is methicilline resistentie gecodeerd door het *mecA gen*, dat aanwezig is op een mobiel genetisch element, de Staphylococcal Chromosomal Cassette *mec* (SCC*mec*). Isolaten die een SCC*mec* bevatten worden methicilline-resistente *S. pseudintermedius* (MRSP) genoemd.

Net als bij *S. aureus*, is methicilline resistentie in de afgelopen decennia ontstaan in *S. pseudintermedius*. MRSP is vaak multiresistent (MDR) wat inhoudt dat er resistentie tegen drie of meer antimicrobiële klassen aanwezig is. Antimicrobiële resistentie kan fenotypisch worden vastgesteld door de minimaal inhiberende concentratie van een antibioticum (MIC) te bepalen, en de interpretatie ervan is gebaseerd op klinische breekpunten die specifiek zijn voor het antibioticum en de bacteriesoort. Voor *S. pseudintermedius* ontbreken van veel antibiotica de klinische breekpunten wat de interpretatie bemoeilijkt.

Antimicrobiële resistentie kan ook worden afgeleid uit genoom sequenties door resistentiegenen of chromosomale mutaties te detecteren. De databases om resistentiegenen in genoom sequenties te kunnen detecteren zijn echter ontwikkeld voor Gram-negatieve bacteriën of voor *S. aureus*, maar niet specifiek voor *S. pseudintermedius*. De betrouwbaarheid bij het gebruik van de online databases om resistentie te detecteren in *S. pseudintermedius* werd onderzocht in **hoofdstuk 2.** Voor deze studie werden 50 isolaten gesequenced met behulp van whole genome sequencing (WGS). De meerderheid (d.w.z. 98,3%) van de fenotypisch gedetecteerde resistenties (met behulp van MIC's en klinische breekpunten) kan worden verklaard door de aanwezigheid van een geassocieerd resistentiegen of puntmutatie. Het bleek dat het antimicrobiële genotype bepaald met whole genome sequencing zeer voorspellend is voor de fenotypische resistentie in MRSP, en dat fouten ertoe kunnen leiden dat gevoelige isolaten als resistent worden beschouwd, meestal als gevolg van genetische variaties die de resistentiegenen inactiveren.

Om te onderzoeken of het gevonden resistentie profiel geassoccieerd is met genomische lijnen van MRSP, werd in **hoofdstuk 2** ook een fylogenetische analyse uitgevoerd. Deze analyse toonde aan dat er een duidelijke verband bestond tussen een bepaald resistentie patroon en klonaal complex (CC) van MRSP. In CC45 genomen was een zeer geconserveerd resistentiegenprofiel aanwezig, terwijl het slechts gedeeltelijk geconserveerd was in CC71, en zeer divers was in CC258. De analyse werd uitgebreid met meer genoomsequenties in **hoofdstuk 6**, waarin tevens werd bestudeerd of resistentiegenen konden worden geassocieerd met mobiele genetische elementen (MGE's) in methicilline gevoelige *S. pseudintermedius* (MSSP) en MRSP.

Hoofdstuk 3 ging dieper in op de mogelijke associatie tussen klonale complexen (CC) en de mate van resistentie tegen β-lactam antibiotica. SCC*mec* typen werden geassocieerd met verschillende CC's en met een hoge of lage resistentie tegen oxacilline, penicilline, amoxicilline/clavulaanzuur en cefalotine. CC71 isolaten met SCC*mec*II-III vertoonden hoge resistentieniveaus tegen β-lactam antibiotica. Isolaten met SCC*mec*V daarentegen vertoonden variabele resistentie niveaus en CC258 isolaten met SCC*mec*IV waren geassocieerd met lage resistentie niveaus of zelfs gevoeligheid voor verschillende β-lactams, zo werd er gevoeligheid voor amoxicilline/clavulaanzuur en cefalotine vastgesteld. Deze *in vitro* gevoeligheid voor amoxicilline/clavulaanzuur en cefalotine binnen enkele CC's kan klinische implicaties hebben en vereist farmacokinetische/farmacodynamische en klinische

studies om de *in vivo* werkzaamheid van deze β-lactams voor de behandeling van MRSP infecties te evalueren.

Hoewel honden voor *S. pseudintermedius* de specifieke gastheer zijn, worden incidentele opportunistische infecties bij mensen veroorzaakt door met name methicilline gevoelige *S. pseudintermedius* (MSSP). De laatste decennia wordt dat steeds vaker gemeld, met name bij ouderen en immuungecompromitteerde mensen. Dit kan gedeeltelijk worden verklaard door een verbeterde identificatie van bacterie soorten door de implementatie van de MALDI-TOF MS technologie in microbiologische laboratoria, maar gastheeraanpassing en genetische variatie kunnen ook een rol spelen. In het geval van humane infecties met *S. pseudintermedius* is overdracht van hond op mens de meest waarschijnlijke route. Dat blijkt ook uit verschillende casuïstieken waarbij isolaten van honden en hun eigenaren identiek waren.

Om mogelijke gastheeraanpassing van *S. pseudintermedius* te onderzoeken werden isolaten van honden en humane oorsprong met WGS vergeleken in **hoofdstuk 4**, terwijl **hoofdstuk 5** zich richtte op de analyse van de diversiteit tussen honden en humane isolaten in hetzelfde huishouden.

In **hoofdstuk 4** werd onderzocht of er gastheer geassocieerde genen konden worden gevonden in genoom sequenties van MSSP isolaten van honden en mensen. Een fylogenetische genoom analyse toonde een grote diversiteit aan met meestal multiresitente isolaten van zowel honden als mensen, en konden er geen genen aangetoond worden die geassocieerd waren met de gastheer (mens cq. hond). Eén van de klonale complexen CC241, was echter ovarvertegenwoordigd in de humane isolaten en was geassocieerd met verschillende MGE's welke meerdere resistentiegenen en virulentiefactoren bevatten. Door de multiresistentie en potentiële virulentie is het belangrijk om de epidemiologische verspreiding van dit klonale complex te monitoren.

Overdracht binnen het huishouden werd bestudeerd in **hoofdstuk 5** met behulp van WGS van isolaten van twee niet-gerelateerde humane infecties en de honden in de huishoudens. In beide gevallen waren de isolaten van de humane infecties genetisch verwant aan de isolaten van hun honden, wat de overdracht van hond op mens bevestigt. Verschillende honden droegen ook meerdere niet-verwante *S.*

pseudintermedius isolaten bij zich, wat aantoont dat er binnen de gastheer bacteriële diversiteit bestaat. Dit toont het belang aan van het analyseren van meerdere kolonies wanneer transmissie routes worden bestudeerd.

In andere stafylokokken en in het bijzonder van *S. aureus* zijn meerdere MGE's bekend waarmee antimicrobiële resistentiegenen overdragen kunnen worden, zoals plasmiden en elementen met een transposon, maar voor *S. pseudintermedius* was de kennis van MGE's en de rol bij de verspreiding van antimicrobiële resistentiegenen beperkt.

In **hoofdstuk 6** werden de MGE's in *S. pseudintermedius* diepgaander bestudeerd. De analyses identificeerden een hoge diversiteit van plasmiden en nieuwe genomische elementen. Het gaf inzicht in de complexe organisatie van MGE's en ging in op hun belang bij de verspreiding van antimicrobiële resistentiegenen en klonale expansie van MRSP. MRSP bleek meerdere keren te zijn geëvolueerd uit een zeer diverse MSSP-achtergrond, waarna klonale expansie optrad na opname van een SCC*mec* element en andere MGE's. Er waren samengestelde MGE's die tot 11 verschillende resistentiegenen konden bevatten. Naast de MGE's die in het genoom waren geïntegreerd, waren er kleine plasmiden met antimicrobiële resistentiegenen aanwezig, waarvan een plasmid met tetracycline resistentie geassocieerd was met klonale expansie van CC71 MRSP. De analyse liet een dynamisch proces van acquisitie en de micro-evolutie van mobiele genetische elementen zien. De verschillende MGE's die werden gekarakteriseerd kunnen uitgewisseld worden met andere stafylokokken-, enterokokken- en streptokokkensoorten, wat het potentieel van verspreiding van AMR-genen buiten *S. pseudintermedius* benadrukt.

Kortom, *S. pseudintermedius* is een veel voorkomende hondenpathogeen met af en toe overloop naar mensen. De populatie van *S. pseudintermedius* bestaat uit een genetisch diverse populatie van MSSP isolaten en enkele klonale lijnen met MRSP. De isolaten van mensen en honden vertoonden een hoge fylogenetische diversiteit en er werden geen gastheergerelateerde genen gedetecteerd. Succesvolle MRSP kloons zijn ontstaan na de verwerving van SCC*mec* en meerdere MGE's met resistentiegenen. MGEs die in het genoom kunnen worden geïntegreerd, zijn belangrijke aanjagers van klonale expansie van MDR-kloons, zoals werd aangetoond voor CC241, welke oververtegenwoordigd was in humane isolaten. Adequate diagnostiek van infecties met *S. pseudintermedius* bij mens en dier is vereist omdat multiresistentie

de behandeling van infecties kan bemoeilijken. Deze studie toonde aan dat de verwerving van mobiele elementen en plasmiden de verspreiding van nieuwe MDR-klonen kan stimuleren. Het waargenomen lage niveau van β-lactamresistentie en zelfs gevoeligheid voor cefalotine en amoxicilline-clavulaanzuur in isolaten met SCC*mec*IV geeft aan dat deze antibiotica kunnen worden gebruikt voor de behandeling van infecties, maar het vereist functionele studies om het mechanisme van lage resistentie op te helderen, evenals klinische evaluatie van het gebruik van deze β-lactams voor de behandeling van infecties bij honden.

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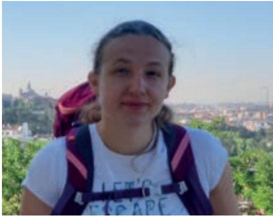
About the author



Alice Wegener was born on 26/07/1991 in Châtellerault (France). After graduating from the Abi-Bac program at the Lycée international Georges Duby (Luynes, France), she went to Lyon for a biology Bachelor. After the first lesson of microbiology, it became obvious to her that this would be her career path. This passion for bacteria was confirmed during an internship at Cardiff University in the laboratory of Dr. Berry on the project "Extraction and purification of the cry6 protein from *Bacillus thuringiensis* in order to work out its structure". In 2012, she graduated from the microbiology program of the Biology Bachelor at University Claude Bernard Lyon 1 (France). She carried on with a Master in Biology of Micro-organisms again, with a specialisation in microbiology. After an internship at the Mycobacterial Molecular Pathology team at IBPBS Toulouse (France), she graduated the Master with honours in 2014. In 2016, she started a PhD research project in England in Dr. Hwang's laboratory within the Imagine: Imaging Life project, at the University of Sheffield (UK), on the "study of the spatial organization of chromosomes in Vibrio cholerae". This project was ended after 10 months. After this Alice searched for a PhD project across Europe and found a perfect match with the project "Genomic dynamics of antimicrobial resistance in canine and human derived *Staphylococcus pseudintermedius*" under the supervision of Prof. Wagenaar, Dr. Duim and Dr. Broens, at the clinical infectiology department of the veterinary Faculty at Utrecht University. The product of this project is presented in this thesis.

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