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Clonal spread of methicillin-resistant *Staphylococcus pseudintermedius* in Europe and North America: an international multicentre study

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Objectives: The aim of this study was to determine the phenotypic and genotypic resistance profiles of methicillin-resistant *Staphylococcus pseudintermedius* (MRSP) and to examine the clonal distribution in Europe and North America.

Methods: A total of 103 MRSP isolates from dogs isolated from several countries in Europe, the USA and Canada were characterized. Isolates were identified by PCR–restriction fragment length polymorphism (RFLP), antimicrobial susceptibility was determined by broth dilution or gradient diffusion, and antimicrobial resistance genes were detected using a microarray. Genetic diversity was assessed by multilocus sequence typing (MLST), PFGE and *spa* typing. Staphylococcal cassette chromosome *mec* (SCC*mec*) elements were characterized by multiplex PCR.

Results: Thirteen different sequence types (STs), 18 PFGE types and 8 *spa* types were detected. The hybrid SCC*mec* element II–III described in a MRSP isolate was present in 75 (72.8%) isolates. The remaining isolates either had SCC*mec* type III ($n=2$), IV ($n=6$), V ($n=14$) or VII-241 ($n=4$) or were non-typeable ($n=2$). The most common genotypes were ST71(MLST)-J(PFGE)-t02(*spa*)-II–III(SCC*mec*) (56.3%) and ST68-C-t06-V (12.6%). In addition to *mecA*-mediated β-lactam resistance, isolates showed resistance to trimethoprim [*dfr*(G)] (90.3%), gentamicin/kanamycin [*aac(6′)-Ie–aph(2′)-Ia*] (88.3%), kanamycin [*aph(3′)-III*] (90.3%), streptomycin [*ant(6′)-Ia*] (90.3%), streptothricin (*sat4*) (90.3%), macrolides and/or lincosamides [*erm*(B), *lnu*(A)] (89.3%), fluoroquinolones (87.4%), tetracycline [*tet*(M) and/or *tet*(K)] (69.9%), chloramphenicol (cat_{pC221}) (57.3%) and rifampicin (1.9%).

Conclusions: Two major clonal MRSP lineages have disseminated in Europe (ST71-J-t02-II–III) and North America (ST68-C-t06-V). Regardless of their geographical or clonal origin, the isolates displayed resistance to the major classes of antibiotics used in veterinary medicine and thus infections caused by MRSP isolates represent a serious therapeutic challenge.

Keywords: antimicrobial resistance, genotyping, MLST, PFGE, *spa*, dog

Introduction

Since its first description in 2005,[1] *Staphylococcus pseudintermedius*, and not *Staphylococcus intermedius*, has been shown to be the species of the *Staphylococcus intermedius* group (SIG) that predominantly colonizes dogs and cats.[2–4] In addition, *S. pseudintermedius* is a leading cause of skin infections and post-operative infections in dogs and cats (reviewed by Weese and van Duijkeren[5]) and has zoonotic potential.[6] During the past 5 years, methicillin-resistant *S. pseudintermedius*

(MRSP) has posed a therapeutic challenge because of the limited treatment options.[7–11] MRSP isolates are characterized by the presence of the *mecA* gene, which is located on staphylococcal cassette chromosome *mec* (SCC*mec*) elements and confers resistance to β-lactam antibiotics. Three SCC*mec* types (SCC*mec* II–III, SCC*mec* V and SCC*mec* VII-241) of MRSP have been completely sequenced.[8,12] SCC*mec* VII-241 (class A *mec* complex, *ccrA3/B5*) is a newly described element[8] that is not related to SCC*mec* VII from *Staphylococcus aureus*.[13] SCC*mec* II–III (class A, *ccrA3/B3*) consists of a combination of SCC*mec* II from *Staphylococcus epidermidis* and of SCC*mec* III from *S. aureus* and lacks the cadmium resistance operon.[8] SCC*mec* V is largely homologous to SCC*mec* type V (5C2&5), previously named V_T or VII from *S. aureus*.[12,14,15] For identification of SCC*mec* elements in MRSP, the typing method of Kondo *et al.*[16] has been adapted in the present study. In addition to resistance to β-lactams, MRSP isolates display resistance to many classes of antimicrobial agents such as the aminoglycosides, macrolides, lincosamides, tetracyclines, trimethoprim, chloramphenicol and fluoroquinolones.[7,8] MRSP colonizes healthy animals[17] and people working with animals,[18,19] who may then act as a reservoir for MRSP and thereby contribute to the dissemination of MRSP among the animal and human populations.

Previous studies have shown that specific MRSP genotypes are predominantly isolated from infection sites in dogs and cats.[7,8,12,20] An association between lineages and geographical origin has been previously proposed based on the analysis of small numbers of isolates.[4,20] Joint efforts have been combined to conduct an international multicentre study with the objective of characterizing MRSP involved in infections or colonizing dogs in several European countries and North America. Antimicrobial susceptibility testing, detection of antibiotic resistance genes, characterization of SCC*mec* elements and various genotyping approaches were employed to determine whether specific MRSP clones are spreading throughout these regions of the world. It is anticipated that the study will serve as a basis for future molecular characterization of MRSP isolates in animals and humans.

Materials and methods

Sample collection and identification of S. pseudintermedius

Bacterial isolates were obtained from diseased and healthy dogs in veterinary diagnostic laboratories of different countries (Table 1) and were cultivated on tryptone soy agar containing 5% sheep blood (TSA-SB) (Oxoid Ltd, Basingstoke, UK) at 37°C for 18 h. All isolates had previously been identified as SIG by means of either a PCR assay[21] or the ID 32 STAPH system (bioMérieux, Marcy l'Étoile, France). *S. pseudintermedius* isolates were identified using a previously described PCR–restriction fragment length polymorphism (RFLP) assay based on the MboI digestion pattern of a PCR-amplified 320 bp internal fragment of the *pta* gene. MboI restriction of this PCR product generates two restriction fragments of 213 bp and 107 bp only in *S. pseudintermedius* since the MboI restriction site is absent in the other species of the SIG and in *S. aureus*.[22]

Multilocus sequence typing (MLST), SmaI-PFGE and spa typing

Genetic diversity of *S. pseudintermedius* was determined by MLST of five genes (16S rDNA, *tuf*, *cpn60*, *pta* and *agrD*), by SmaI-PFGE and by *spa* typing as described previously.[4,20,23] PFGE was run for 24 h at 5.6 V/cm and with pulse time ramping from 2 to 5 s.[24] MLST sequences were compared with allele sequences present in the NCBI nucleotide database in order to determine the allele number. Sequence type (ST) numbers were assigned using the key table for MLST typing of SIG isolates.[4] New ST numbers are currently assigned by the curator Vincent Perreten (vincent.perreten@vbi.unibe.ch). The *spa* typing was done as previously described,[19] but using an additional nested PCR to allow typing of isolates that were non-typeable with the original protocol. Primers SPspa1F (5′-CCGCTCTATTTTTAGGTTAATC-3′) and SIspaFlkR1 (5′-CGTAACAACTCAATGCTACATA-3′) were used in the first PCR step to amplify the entire *spa* gene. The internal primers SIspaF (5′-AACCTGCGCCAAGTTTCGATGAAG-3′) and SIspaR (5′-CGTGG TTTGCTTTAGCTTCTTGGC-3′) were then used as recently described[19] to amplify and sequence the variable, tandem repeat region (X-region) of the *spa* gene. New types were assigned by the curator of the *spa* database, Arshnee Moodley (asm@life.ku.dk).

Determination of antibiotic resistance profile

MICs of most of the antimicrobial agents tested were determined by broth microdilution according to the recommendations of the CLSI[25] using microdilution panels (VetMIC™; National Veterinary Institute, Uppsala, Sweden) with dried and stabilized antimicrobial agents. Determination of the MICs of rifampicin, mupirocin and the combination quinupristin/dalfopristin was conducted using gradient diffusion strips according to the instructions of the producer (Etest®; AB Biodisk, Solna, Sweden). Inducible clindamycin resistance was tested using the *D*-test.[26] *S. aureus* ATCC 29213 and *S. aureus* ATCC 25923 served as quality control strains. PBP2a was detected with the penicillin binding protein PBP2a latex agglutination test (Oxoid Ltd) in accordance with the supplier's instructions.

The MIC breakpoints for the classification of the isolates as resistant were those recommended in CLSI documents M100-S19 and M31-A3.[26,27] The resistance breakpoint of ≥0.5 mg/L for oxacillin was used as recommended by Bemis *et al.*[28] and has recently been approved by the CLSI subcommittee on Veterinary Antimicrobial Susceptibility Testing (http://data.memberclicks.com/site/aavld/Letter to the Editor.pdf). Despite the lack of CLSI-approved interpretive criteria for streptomycin, isolates for which the MIC was ≥32 mg/L were tentatively considered as streptomycin resistant.

Antibiotic resistance genes were detected using a microarray capable of detecting >90 resistance genes known to occur in Gram-positive bacteria.[29] The custom ArrayTubes were manufactured by CLONDIAG Chip Technologies (Jena, Germany) and are distributed by Identibac (Weybridge, UK). The presence of the bifunctional gene *aac(6′)-Ie–aph(2′)-Ia* was demonstrated by PCR using one forward primer specific to *aac(6′)-Ie* and one reverse primer specific to *aph(2′)-Ia*.[30]

Characterization of SCCmec

The presence of SCC*mec* types I–VI was determined by multiplex PCR assays.[16] In multiplex PCR 1, the presence of *mecA* was confirmed and the *ccr* gene complex was determined. The PCR products for the internal part of the *ccrC* gene were sequenced to identify the *ccrC* type. In multiplex PCR 2, the type of *mec* complex was assigned. For detection of the novel recombinase gene complex *ccrA3/B5* present in SCC*mec* type VII-241 cassettes,[9] primers 5′-GCCAAAATTTCTTTCGAGACC-3′ and 5′-TAC GTGCGAGTCGATTTGTT-3′ were used. The presence of SCC*mec* II–III was demonstrated by the absence of the cadmium resistance operon (present in SCC*mec* III and absent in SCC*mec* II and SCC*mec* II–III)[8] using a multiplex PCR containing primers sccmecIII-F4 (5′-AACAGC CATGACAAGCAC-3′) and sccmecIII-R3 (5′-TAATGCCCATCATTTCAC-3′) that anneal in the flanking regions of the cadmium resistance operon and

Table 1. Origin, year of isolation and clinical condition of dogs infected with MRSP of different MLST groups

Clinical condition	*N*	Year of isolation	*n*	Region (*n*)	MLST (*n*)
Healthy[a]	22	2004	4	ON (4)	ST58 (3), ST113
		2006	1	S (1)	ST69
		2007	9	D (9)	ST71 (9)
		2008	8	D (8)	ST71 (8)
Pyoderma	25	2004	1	D (1)	ST5
		2006	10	TN (10)	ST68 (10)
		2007	8	NL (5), CH (2), I (1)	ST71 (6), ST106, ST114
		2008	6	CA (3), D (2), DK (1)	ST71 (3), ST68, ST112, ST116
Wound infections after surgery	22	2005	1	CH (1)	ST71
		2006	4	CH (3), S (1)	ST71 (4)
		2007	12	CH (9), S (1), NL (2)	ST71 (12)
		2008	5	CH (4), NC (1)	ST71 (4), ST68
Wound infections	5	2007	2	I (1), NL (1)	ST71 (2)
		2008	2	CH (1), D (1)	ST71 (2)
		2009	1	DK (1)	ST71
Otitis externa	8	2004	1	CH (1)	ST73
		2006	1	CH (1)	ST71
		2007	2	CH (2)	ST71 (2)
		2008	4	D (1), DK (1), CA (1), ON (1)	ST71 (2), ST111, ST106
Urinary tract infection	6	2006	2	I (2)	ST71 (2)
		2008	4	CH (1), ON (3)	ST71 (3), ST68
Arthritis/synovitis	5	2007	5	NL (3), CH (1), I (1)	ST71 (5)
Respiratory tract infection	2	2005	1	D (1)	ST71
		2006	1	I (1)	ST71
Encephalitis	1	2006	1	I (1)	ST71
Gingivitis	1	2006	1	CH (1)	ST71
Hepatitis	1	2007	1	I (1)	ST71
Peritonitis	1	2007	1	NL (1)	ST71
Septicaemia	1	2007	1	I (1)	ST71
Unknown	3	2008	3	D (2), CA (1)	ST71 (2), ST115
Total per year	103	2004	6	CH (1), D (1), ON (4)	ST58 (3), ST5, ST73, ST113
		2005	2	CH (1), D (1)	ST71 (2)
		2006	21	CH (5), I (4), TN (10), S (2)	ST68 (10), ST71 (10), ST69
		2007	41	CH (14), D (9), NL (12), I (5), S (1)	ST71 (39), ST106, ST114
		2008	32	CH (6), D (14), CA (5), ON (4), DK (2), NC (1)	ST71 (24), ST68 (3), ST106, ST111, ST112, ST115, ST116
		2009	1	DK (1)	ST71
Total	103	2004–09	103	CH (27), D (25), I (9), DK (3), CA (5), NC (1), NL (12), ON (8), S (3), TN (10)	ST71 (76), ST68 (13), ST58 (3), ST106 (2), ST5, ST69, ST73, ST111, ST112, ST113, ST114, ST115, ST116

N, number of animals; *n*, number of MRSP isolates.
European countries and North American regions (USA and Canada): CA, California; CH, Switzerland; D, Germany; DK, Denmark; I, Italy; NC, North Carolina; NL, the Netherlands; ON, Ontario; S, Sweden; TN, Tennessee.
[a]Nose, mouth or perineum colonization.

Table 2. Genetic characteristics and distribution of antibiotic resistance in MRSP from different countries

Sequence type and PFGE profile			Antibiotic resistance properties and genes															
MLST	*spa*	PFGE	OXA	PEN	GEN/KAN	KAN	STR	STH	ML	TMP	LIN	TET	CHL	CIP	RIF	SCC*mec* type	Origin (no. of isolates)	Total no. of isolates
ST71	t02	J	*mecA*	*blaZ*	*aac(6′)-Ie-aph(2′)-Ia*	*aph(3′)-III*	*ant(6′)-Ia*	*sat4*	*erm*(B)	*dfr*(G)		*tet*(K)	cat_{pC221}	+	+	II-III	I (1)	1
ST71	t02	J (34), K (1), P (1)	*mecA*	*blaZ*	*aac(6′)-Ie-aph(2′)-Ia*	*aph(3′)-III*	*ant(6′)-Ia*	*sat4*	*erm*(B)	*dfr*(G)		*tet*(K)	cat_{pC221}	+		II-III	CH (9), D (17), I (8), NL (1), ON (1)	36
ST71	t05	J	*mecA*	*blaZ*	*aac(6′)-Ie-aph(2′)-Ia*	*aph(3′)-III*	*ant(6′)-Ia*	*sat4*	*erm*(B)	*dfr*(G)		*tet*(K)	cat_{pC221}	+		II-III	D (1)	1
ST71	t06	M	*mecA*	*blaZ*	*aac(6′)-Ie-aph(2′)-Ia*	*aph(3′)-III*	*ant(6′)-Ia*	*sat4*	*erm*(B)	*dfr*(G)		*tet*(K)	cat_{pC221}	+	+	II-III	NL (1)	1
ST71	t06	J	*mecA*	*blaZ*	*aac(6′)-Ie-aph(2′)-Ia*	*aph(3′)-III*	*ant(6′)-Ia*	*sat4*	*erm*(B)	*dfr*(G)		*tet*(K)	cat_{pC221}	+		II-III	CH (1)	1
ST71	t02	J (10), K(1)	*mecA*	*blaZ*	*aac(6′)-Ie-aph(2′)-Ia*	*aph(3′)-III*	*ant(6′)-Ia*	*sat4*	*erm*(B)	*dfr*(G)		*tet*(K)		+		II-III	CH (7), D (2), NL (2)	11
ST71	t02	J (9), G (2), H (1), L (1), M (1)	*mecA*	*blaZ*	*aac(6′)-Ie-aph(2′)-Ia*	*aph(3′)-III*	*ant(6′)-Ia*	*sat4*	*erm*(B)	*dfr*(G)			cat_{pC221}	+		II-III	CH (9), S (1), NL (4)	14
ST71	t03	J	*mecA*	*blaZ*	*aac(6′)-Ie-aph(2′)-Ia*	*aph(3′)-III*	*ant(6′)-Ia*	*sat4*	*erm*(B)	*dfr*(G)			cat_{pC221}	+		II-III	NL (1)	1
ST71	t02	J (2), G (1), R (1)	*mecA*	*blaZ*	*aac(6′)-Ie-aph(2′)-Ia*	*aph(3′)-III*	*ant(6′)-Ia*	*sat4*	*erm*(B)	*dfr*(G)				+		II-III	D (1), CA (2), NL (1)	4
ST71	t02	H	*mecA*	*blaZ*	*aac(6′)-Ie-aph(2′)-Ia*	*aph(3′)-III*	*ant(6′)-Ia*	*sat4*	*erm*(B)	*dfr*(G)				+		IV	DK (1)	1
ST71	t03	J	*mecA*	*blaZ*	*aac(6′)-Ie-aph(2′)-Ia*	*aph(3′)-III*	*ant(6′)-Ia*	*sat4*	*erm*(B)	*dfr*(G)				+		II-III	D (1)	1
ST71	t06	J (2)	*mecA*	*blaZ*	*aac(6′)-Ie-aph(2′)-Ia*	*aph(3′)-III*	*ant(6′)-Ia*	*sat4*	*erm*(B)	*dfr*(G)				+		II-III	ON (2)	2
ST71	t02	J	*mecA*	*blaZ*	*aac(6′)-Ie-aph(2′)-Ia*	*aph(3′)-III*	*ant(6′)-Ia*	*sat4*		*dfr*(G)		*tet*(K)	cat_{pC221}	+		II-III	D (1)	1
ST71	t02	J	*mecA*	*blaZ*	*aac(6′)-Ie-aph(2′)-Ia*					*dfr*(G)			cat_{pC221}	+		II-III	S (1)	1
ST68	t06	C (6)	*mecA*	*blaZ*	*aac(6′)-Ie-aph(2′)-Ia*	*aph(3′)-III*	*ant(6′)-Ia*	*sat4*	*erm*(B)	*dfr*(G)		*tet*(M)		+		V	TN (6)	6
ST68	t06	C (6)	*mecA*	*blaZ*	*aac(6′)-Ie-aph(2′)-Ia*	*aph(3′)-III*	*ant(6′)-Ia*	*sat4*	*erm*(B)	*dfr*(G)	*lnu*(A)	*tet*(M)		+		V	TN (3), CA (1), NC (1), ON (1)	6
ST68	t06	C	*mecA*	*blaZ*	*aac(6′)-Ie-aph(2′)-Ia*							*tet*(M)		+		V	TN (1)	1
ST5	t05	S	*mecA*	*blaZ*	*aac(6′)-Ie-aph(2′)-Ia*					*dfr*(G)		*tet*(K)	*tet*(M)			A1[a]	D (1)	1
ST58	t06	F (3)	*mecA*	*blaZ*												VII-241	ON (3)	3
ST69	t07	A	*mecA*	*blaZ*		*aph(3′)-III*	*ant(6′)-Ia*	*sat4*	*erm*(B)				cat_{pC221}			B2[a]	S (1)	1
ST73	t24	S	*mecA*										*tet*(M)	±		VII-241	CH (1)	1
ST106	t02	U	*mecA*	*blaZ*		*aph(3′)-III*	*ant(6′)-Ia*	*sat4*	*erm*(B)	*dfr*(G)			*tet*(M)			IV	NL (1)	1
ST106	t02	ND	*mecA*	*blaZ*						*dfr*(G)			*tet*(M)			III	DK (1)	1
ST111	t05	U	*mecA*	*blaZ*		*aph(3′)-III*	*ant(6′)-Ia*	*sat4*	*erm*(B)	*dfr*(G)			*tet*(M)			IV	CA (1)	1
ST112	t25	Q	*mecA*	*blaZ*												IV	CA (1)	1
ST113	t06	D	*mecA*	*blaZ*		*aph(3′)-III*	*ant(6′)-Ia*	*sat4*	*erm*(B)							IV	ON (1)	1
ST114	t06	V	*mecA*	*blaZ*									*tet*(M)			III	NL (1)	1
ST115	t21	E	*mecA*	*blaZ*	*aac(6′)-Ie-aph(2′)-Ia*	*aph(3′)-III*	*ant(6′)-Ia*	*sat4*	*erm*(B)	*dfr*(G)		*tet*(K)	*tet*(M) cat_{pC221}	+		V	D (1)	1
ST116	t02	W	*mecA*	*blaZ*		*aph(3′)-III*	*ant(6′)-Ia*	*sat4*	*erm*(B)							IV	DK (1)	1
Total			103	102	91	93	93	93	92	93	6	54	20	59	90	2			103
Percentage			100	99	88.3	90.3	90.3	90.3	89.3	90.3	5.8	52.4	19.4	57.3	87.4	1.9			

ND, not determined.

Antibiotics: OXA, oxacillin; PEN, penicillin; GEN, gentamicin; STR, streptomycin; KAN, kanamycin; CHL, chloramphenicol; TMP, trimethoprim; ML, macrolides/lincosamides; LIN, lincosamides; TET, tetracycline; STH, streptothricin; CIP, ciprofloxacin; RIF, rifampicin.

European countries and North American regions (USA and Canada): CA, California; CH, Switzerland; D, Germany; DK, Denmark; I, Italy; NC, North Carolina; NL, the Netherlands; ON, Ontario; S, Sweden; TN, Tennessee.

+, resistant to the tested drug, but the resistance mechanism was not determined; ±, intermediate resistance; blank spaces indicate no resistance.

[a]A1 and B2 designations were used for non-typeable SCC*mec*: A1 belongs to *mec* class A with *ccrA1/B1*; and B2 belongs to *mec* class B with *ccrA2/B2* and *ccrA4/B4*.

primer scc241-F6 (5′-AAGACTTAGCAGGAAAACGC-3′) that anneals within the *cadB* gene. This multiplex PCR generated either a 1118 bp fragment in the presence of the cadmium resistance operon or a 831 bp fragment in the absence of the operon. PCRs were performed using an annealing temperature of 54°C and an extension time of 1 min.

Results

Identification of and clonal relationship between MRSP

Eighty-one isolates were from animals with clinical symptoms and 22 isolates colonized healthy animals (Table 1). A *pta* PCR product of 320 bp was amplified from all isolates examined, and all PCR products contained a single MboI site (data not shown), indicative of *S. pseudintermedius*.[22]

The 103 MRSP isolates were assigned to 13 different STs, 18 PFGE types and 8 *spa* types (Table 2). The majority of the investigated MRSP isolates belonged to the two STs ST71 ($n=76$, 73.8%) and ST68 ($n=13$, 12.6%), the two PFGE types J ($n=64$, 62.1%) and C ($n=13$, 12.6%) and the two *spa* types t02 ($n=72$; 69.9%) and t06 ($n=22$; 21.4%) (Table 2). All isolates assigned to ST68 displayed the same PFGE profile C and had *spa* type t06 [ST68(MLST)-C(PFGE)-t06(*spa*)]. Sixty-four (84.2%) of the 76 isolates representing ST71 displayed the same PFGE pattern J. They belonged mainly to *spa* type t02 (58/76) (ST71-J-t02) and rarely to *spa* types t06 (3/76), t03 (2/76) and t05 (1/76). Among the remaining 12 ST71 isolates (15.8%), 7 unique PFGE profiles were observed. Eleven of these isolates belonged to *spa* type t02, while the remaining isolate belonged to t06 (Table 2). Moreover, *spa* type t02 was also detected among MRSP isolates that were assigned to ST106 and ST116. These two STs were not closely related to ST71 and clustered in two separate branches of the eBURST diagram (Figure 1). Three isolates (2.9%) belonged to ST58-F-t06 (Table 2). Other genotypes, including ST5, ST69, ST73 and ST111–ST116, were represented by single isolates; they also had distinct PFGE profiles, except ST5 and ST73 that share the same profile (Table 2).

SCCmec distribution in MRSP

SCC*mec* typing revealed the presence of five SCC*mec* types (II–III, III, IV, V and VII-241) as well as two non-typeable cassettes: one harboured *ccrA1/B1* with class A *mec* complex (A1); and the other harboured the recombinase genes *ccrA2/B2* and *ccrA4/B4* with class B *mec* complex (B2) (Table 2). SCC*mec* II–III was most frequently seen (75/103) and mainly associated with MRSP isolates of ST71. SCC*mec* III was detected in two isolates assigned to ST106 and ST114, respectively. SCC*mec* V was found in all ST68 isolates and ST115. SCC*mec* IV was associated with six different STs (71, 106, 111, 112, 113 and 116), while SCC*mec* VII-241 was identified in ST58 and ST73 (Table 2). All isolates of the clonal lineage ST68 and the ST115 isolate harboured *ccrC2* and *ccrC8* and thus very likely belonged to type V (5C2&5). The two non-typeable SCC*mec* elements were detected in two isolates that belonged to ST5 and ST69, respectively (Table 2).

Antibiotic resistance profile

All isolates contained *mecA* and 102 carried the β-lactamase gene *blaZ* (Tables 2 and 3). All the isolates were considered to be resistant to oxacillin using the recently revised CLSI breakpoints applicable to *S. pseudintermedius*. Tetracycline resistance was attributed to either *tet*(M) or *tet*(K), or both genes in two isolates. Resistance to macrolides and lincosamides was due to the methylase gene *erm*(B). Two isolates containing *erm*(B) displayed inducible resistance to clindamycin, otherwise clindamycin resistance was constitutively expressed. The lincosamide nucleotidyltransferase gene *lnu*(A) that confers resistance only to lincosamides, but not to macrolides, was only detected in six isolates that also contained *erm*(B). The chloramphenicol acetyltransferase gene cat_{pC221} was detected in chloramphenicol-resistant isolates and the dihydrofolate reductase gene *dfr*(G) was found in the trimethoprim-resistant isolates. The streptothricin acetyltransferase gene *sat4* was detected in 93 (90.3%) isolates. Resistance to aminoglycosides was associated with the adenyltransferase gene *ant(6′)-Ia* (streptomycin resistance), the phosphotransferase gene *aph(3′)-III* (kanamycin resistance) or the bifunctional acetyltransferase/phosphotransferase gene *aac(6′)-Ie–aph(2′)-Ia* (gentamicin and kanamycin resistance).[31,32] Nineteen isolates containing the *aac(6′)-Ie–aph(2′)-Ia* gene displayed MICs of gentamicin of either 4 or 8 mg/L that classify the respective isolates as either borderline susceptible or intermediate according to the currently available CLSI breakpoints for staphylococci.[27]

Using the human breakpoints for resistance to ciprofloxacin and veterinary-specific breakpoints for resistance to enrofloxacin (both at ≥4 mg/L),[26,27] 90 isolates were classified as resistant to ciprofloxacin and 87 isolates as resistant to enrofloxacin. While only a single isolate for which the MIC was 2 mg/L was classified as intermediate to ciprofloxacin, five isolates for which the MIC was 1 or 2 mg/L were classified as intermediate to enrofloxacin (Table 3). Two isolates displayed high MICs of ≥64 mg/L rifampicin. The resistance mechanism to fluoroquinolones or rifampicin was not determined in the present study.

Geographical and clinical distribution of MRSP clones

The multiresistant clonal lineage ST71(MLST)-J(PFGE)-t02(*spa*)-II–III(SCC*mec*) was widespread in several European countries, but was detected only sporadically among dogs from North America, namely in California and Ontario. In North America, ST68-C-t06-V was the predominant lineage found in several regions (Tennessee, California, North Carolina and Ontario). This North American clonal lineage contained virtually the same resistance genes as the European clonal lineage ST71, with the exception of *tet*(M), which replaced *tet*(K), the absence of cat_{pC221} and an additional lincosamide resistance gene *lnu*(A) in some ST68 isolates. MRSP isolates belonging to unique STs, such as ST69, ST113, ST114 and ST116, originated from different countries (Table 2 and Figure 1). They also harboured less antibiotic resistance genes than isolates belonging to the predominant ST71 and ST68 lineages (Table 2). Genetic diversity was more frequent among isolates from skin infection sites than among isolates from surgical infection sites, which all belonged to ST71. ST71 was also the predominant sequence type (77.3%) among MRSP isolates from healthy dogs (Table 1).

Discussion

Two major clonal lineages characterized by resistance to nine therapeutically important classes of antimicrobial agents were

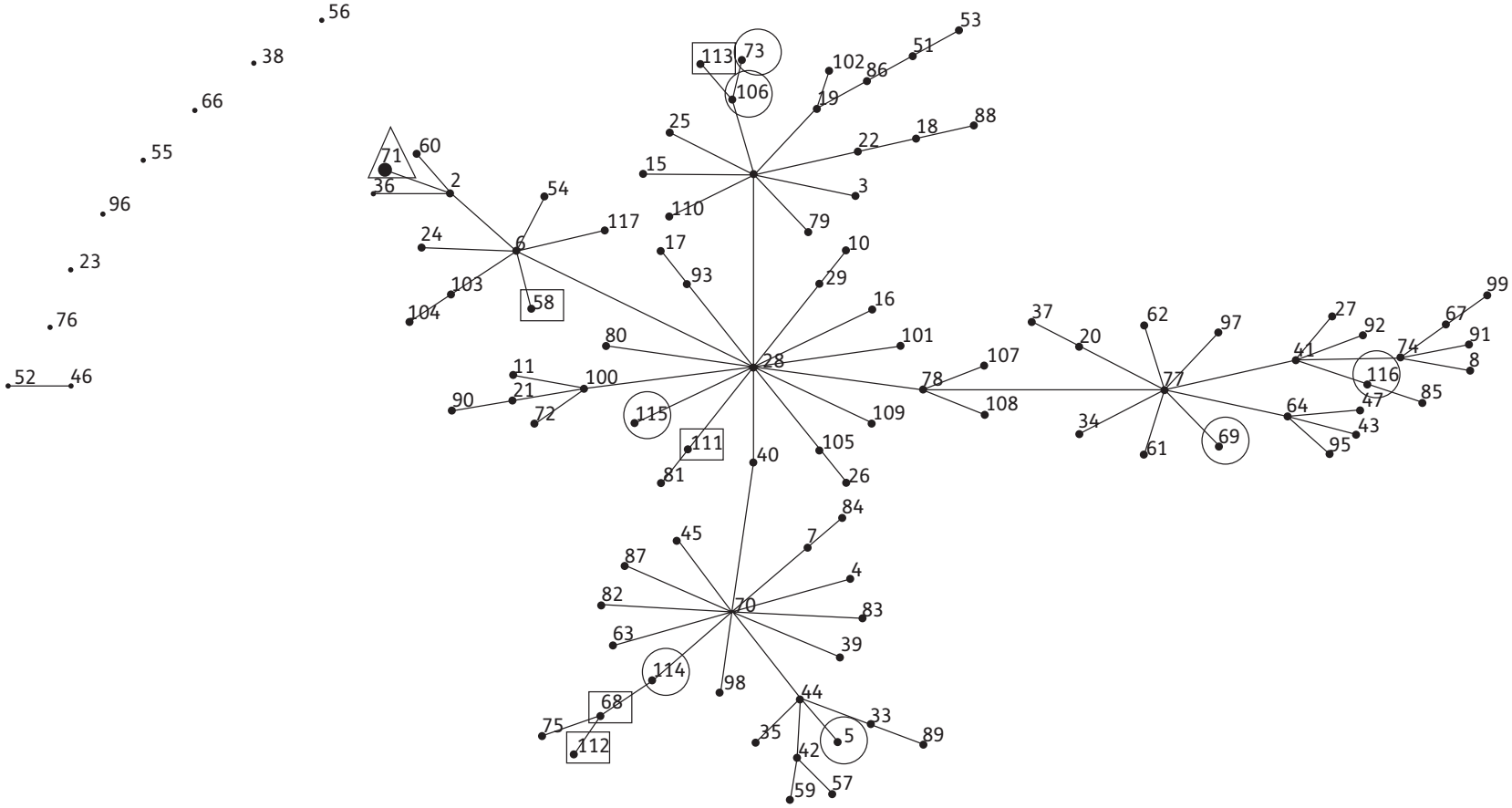

Figure 1. Population snapshot of *S. pseudintermedius*. Clusters of related STs within the entire *S. pseudintermedius* MLST database are displayed as a single eBURST diagram by setting the group definition to zero of five shared alleles. Clusters of linked isolates correspond to clonal complexes. The STs found in the present study are marked by geometric symbols that also indicate geographical origin: open circles, exclusive to Europe; open squares, exclusive to North America; and open triangles, found in Europe and North America.

Table 3. MICs of 21 antimicrobial agents for 103 MRSP and distribution of antibiotic resistance genes

			MIC (mg/L)																	
Antimicrobial	Resistance breakpoint (mg/L)	Range tested	<0.03	0.03	0.06	0.12	0.25	0.5	1	2	4	≥8	≥16	≥32	≥64	≥128	≥256	Number of resistant isolates (%)	Resistance genes (*n*)	Percentage of isolates with resistance genes
Cefoxitin	NA	0.12–16					1	4	13	18	30	23	9	5				—	*mecA* (103)	100
Cefalotin	≥32	0.06–8				1	5	7	3	2	4	5	76					—	*mecA* (103)	100
Oxacillin with 2% NaCl	≥0.5	0.12–16							1	4	5	2	2	89				103 (100)	*mecA* (103)	100
Penicillin	≥0.25	0.06–4									1	102						103 (100)	*mecA* (103); *blaZ* (102)	100; 99.0
Chloramphenicol	≥32	4–64									8	36		6	51	2		59 (57.3)	cat_{pC221} (59)	57.3
Ciprofloxacin	≥4	0.06–4			1	8		3		1		90						90 (87.4)	ND	ND
Clindamycin	≥4	0.25–32					9	3		1					90			92[a] (89.3)	*erm*(B) (92); *lnu*(A) (6)	89.3; 5.8
Enrofloxacin	≥4	0.12–16				6	3	2	3	2	3	24	18	42				87 (84.5)	ND	ND
Erythromycin	≥8	0.25–32						11							92			92 (89.3)	*erm*(B) (92)	89.3
Fusidic acid	NA	0.06–8			3	38	43	11	6	1	1									
Gentamicin	≥16	0.5–64						11	1		8	11	9	27	32	4		72 (69.9)	*aac(6′)-Ie–aph(2′)-Ia* (91)	88.3
Kanamycin	≥64	0.25–32							4	2			1		96			96 (93.2)	*aph(3′)-III* (93)	90.3
Linezolid	NA	0.12–16						15	84	4								—		
Mupirocin	NA	0.06–256			77	24	1	1										—		
Quinupristin/dalfopristin	≥4	0.06–32			1	9	41	52										0 (0)		
Rifampicin	≥4	0.03–32	101												2			2 (1.9)	ND	ND
Streptomycin	≥32	2–128								1	8	1					93	93 (90.3)	*ant(6′)-Ia* (93)	90.3
Streptothricin	NA	ND																ND	*sat4* (93)	90.3
Tetracycline	≥16	0.5–64						31						36	35	1		72 (69.9)	*tet*(M) (18); *tet*(K) (52); *tet*(M) and *tet*(K) (2) (total: 72)	17.5; 50.5; 1.9 (total: 69.9)
Trimethoprim	≥16	0.5–32									6	4			93			93 (90.3)	*dfr*(G) (93)	90.3
Vancomycin	≥32	0.12–16						3	93	7								0 (0)		

NA, not available; ND, not determined.

The MIC breakpoints determining resistance were those recommended for staphylococci in CLSI documents M100-S19[26] and M31-A3.[27] The resistance breakpoint of ≥0.5 mg/L for oxacillin was used as approved by the CLSI subcommittee on Veterinary Antimicrobial Susceptibility Testing. The resistance breakpoint of ≥32 mg/L was tentatively used for streptomycin.

[a]Two isolates harbouring *erm*(B) displayed inducible resistance to clindamycin.

found to be prevalent in Europe (ST71-J-t02-II–III) and North America (ST68-C-t06-V) (Table 2). Different antibiotic resistance gene profiles were present among isolates belonging to ST71 suggesting independent acquisition or loss of resistance genes, such as *tet*(K), cat_{pC221} or *erm*(B). Borderline susceptibility to gentamicin was observed in some MRSP isolates that contained the bifunctional aminoglycoside resistance gene *aac(6′)-Ie–aph(2′)-Ia*. Similarly, lower MICs have already been observed for staphylococci from horses that expressed *aac(6′)-Ie–aph(2′)-Ia*.[33] In this regard, it should be noted that clinical breakpoints may reflect more than just the presence or absence of resistance genes and that the observed MICs may also depend on the expression level and/or the copy number of a resistance gene present.[34] Although inducible clindamycin resistance appeared to be more frequent in methicillin-resistant *Staphylococcus aureus* (MRSA) than in MRSP,[35] two MRSP isolates were found to have the resistance gene *erm*(B) and inducible resistance to clindamycin. Since *erm*(B) gene prevalence is very high in MRSP, it is recommended that the *D*-test be performed for all erythromycin-resistant isolates that are not simultaneously resistant to clindamycin. All but one of the isolates containing the aminoglycoside resistance genes *aph(3′)-III* and *ant(6′)-Ia*, as well as the streptothricin resistance gene *sat4*, also harboured the *erm*(B) gene, suggesting the presence of Tn*5405*-like elements that have already been reported to be present in *S. pseudintermedius* from dogs.[36] In these elements, *erm*(B) was linked to the resistance gene cluster *ant(6′)-Ia–sat4–aph(3′)-III*.[36]

MLST followed by eBURST clustering has been shown to be an excellent tool to study bacterial population structures and global epidemiology. To investigate the evolution of MRSP, eBURST clustering of all *S. pseudintermedius* STs revealed one large clonal complex, with ST28 as the founder and 15 sub-founders (Figure 1). This clustering pattern might suggest a high recombination rate over point mutations within this species;[37,38] however, recombination and mutation rates have not yet been studied in *S. pseudintermedius*. In addition, the current *S. pseudintermedius* MLST scheme needs optimization. Most MLST schemes have seven or more loci and none includes 16S rDNA.

Initially, only 93 (90.3%) of the 103 isolates were *spa* typeable using the method described by Moodley *et al.*[20] The *spa* products of non-typeable isolates could not be sequenced due to the presence of multiple bands. This was later found to be related to the presence of two adjacent *spa* genes, with 79% nucleotide sequence identity (data not shown). This second *spa* gene has three IgG-binding domains and a partial deletion of the X-region. The biological significance of this gene duplication in MRSP is unclear, but by using the nested PCR approach described in the present study, only the *spa* gene containing the complete X-region is amplified. The results of the present study indicated that the same *spa* type can be found in unrelated STs and PFGE types. This has been reported in MRSA and hypothesized to be a result of convergent evolution or genetic recombination.[39,40] PFGE showed good correlation with MLST and was more discriminatory than *spa* typing for isolates belonging to the same ST lineage. Nevertheless, 58 (56.3%) of all MRSP isolates shared the same ST71-J-t02-II–III profile and the same antibiotic resistance genes indicating that a predominant MRSP clone is spreading in the dog population. MRSP ST71 isolates containing SCC*mec* type II–III have also been detected in dogs in Canada and the USA and have been recently reported in Hong Kong,[19] emphasizing the global spread of MRSP ST71. Further surveillance and genetic characterization of the newly emerging STs of MRSP will facilitate comparison of their pathogenic potential with the established clonal lineages ST68 and ST71.

It is clear that MRSP is a nosocomial pathogen in veterinary settings in a similar fashion to hospital-acquired MRSA lineages in human medicine.[41] The dissemination of such multidrug-resistant staphylococci among dogs is of concern as the options for therapy are limited. People working with animals have become carriers of MRSP.[18,19] Thus, veterinary personnel could transfer the pathogen from animal to animal, which further emphasizes its veterinary nosocomial potential. As cases of human infections with *S. pseudintermedius* have been reported,[5,6] including MRSP infections,[42,43] the appropriateness of veterinary use of 'antibiotics of last resort', like vancomycin, linezolid and the combination quinupristin/dalfopristin, that are used for the treatment of methicillin-resistant staphylococci in human medicine, is questionable.

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None to declare.

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