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Clonal Complexes and Antimicrobial Susceptibility Profiles of *Staphylococcus pseudintermedius* Isolates from Dogs in the United States

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Staphylococcus pseudintermedius is the primary cause of canine pyoderma and has been associated with diseases in other animals, including human beings. A high prevalence of methicillin and multidrug resistance has been reported in this bacterium in some geographic regions of the United States. Multilocus sequence type (MLST) 68 was implicated, initially, as the major clonal genotype based on a limited number of samples. The objectives of this study were to determine the population genetics of *S. pseudintermedius* isolated from a cross-section of the United States using a seven-locus multilocus sequence typing method, to identify clonal complexes (CCs), and to correlate sequence types with antimicrobial susceptibility profiles. A total of 190 *S. pseudintermedius* with 86 different MLSTs were detected and the constituents of three major CCs of methicillin-resistant *S. pseudintermedius* (MRSP), CC68, CC71, and CC84, were identified. Different patterns of resistance were associated with each CC. CC71 from the United States had notable differences with CC71 studied on other continents with chloramphenicol, tetracycline, and trimethoprim/sulfamethoxazole resistance. Some isolates with resistance to the broadest range of drugs tested, including that to chloramphenicol, had STs unrelated to the major CCs, suggesting the potential for the emergence of new clonal populations of MRSP that are resistant to most therapeutically useful antimicrobials.

Keywords: *Staphylococcus pseudintermedius*, methicillin resistance, MLST, clonal complex, antimicrobial, United States

Introduction

Staphylococcus pseudintermedius, a coagulase-positive *Staphylococcus*, is a canine commensal and opportunistic pathogen.[1,2] It is recognized as the most frequent cause of skin and postoperative infections in dogs and cats and is also associated with wound infections, pneumonia, and urinary tract infections.[3] Infections from this organism may be underdiagnosed due to misidentification in humans who can acquire transient subclinical infections or, more rarely, become clinically infected.[4,5]

Methicillin resistance in *S. pseudintermedius* isolates is predominantly due to the *mecA* gene, which encodes penicillin binding protein 2a (PBP2a). This protein has relatively low affinity for beta-lactam antibiotics.[6] *MecA* is contained within a variable genomic island referred to as the staphylococcal cassette chromosome mec (*SSCmec*).[7,8] PBP2a causes resistance to all beta-lactams, and methicillin resistance is often associated with resistance to unrelated classes of antimicrobials (multidrug resistance). The extent of this relationship has not been determined in isolates collected in the United States and little is known about how this differs between clonal populations of *S. pseudintermedius*.[8,9] The prevalence of methicillin-resistant *S. pseudintermedius* (MRSP) has increased rapidly in some European countries.[10] Anecdotally, it has reached 40% in some regions of the United States; however, a countrywide survey has not been conducted. Vertical transmission and clonal spread of MRSP strains may be responsible for the increased prevalence of methicillin resistance.[3,8,11–14]

In 2009, Black *et al.* reported that 37 out of 38 methicillin-resistant isolates submitted to one veterinary clinical laboratory in the United States belonged to the same sequence type, ST68.[11] Perreten *et al.* found that two strains with different sequence types, ST71 and ST68, are frequently isolated in Europe and in North America, respectively.[8] This previous study of MRSP in the United States characterized a limited number of samples, including 10 from Tennessee, 5 from California, and 1 from North Carolina, using the typing scheme of Bannoehr and Guardabassi[2] The samples in the Perreten study were collected from 2004 through 2009. A recent systematic review of fifty-eight *S. pseudintermedius*

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studies published over the past 10 years identified CC68 as the most prevalent clonal complex (CC) in North America.[15] Sixty-seven percent of CC68 consisted of isolates from North America and only 4.4% (CC112), 3.9% (CC45), 1% (CC258), and .8% (CC71) of the other major CCs identified worldwide contained isolates from North America. However, only 6.2% of the isolates in this study were obtained from North America, highlighting the dearth of samples from this region.

The objectives of this study were to determine the population genetics of *S. pseudintermedius* isolated from a cross-section of the United States using a seven-locus multilocus sequence typing method,[12] to identify CCs, and to correlate sequence types with antimicrobial susceptibility profiles. This information will improve our understanding of the prevalence and patterns of antimicrobial resistance in the United States and provide a basis of comparison for global analysis of *S. pseudintermedius* epidemiology. In addition, identification of conserved traits in clonally diverse populations of *S. pseudintermedius* is important for the development of new strategies to treat and control antimicrobial resistant *S. pseudintermedius*. For example, this information will be useful for the identification of antigens and virulence factors common to all major CCs for vaccine development. The importance of determining antigen conservation among predominant strains has long been recognized as a key toward the development of broadly effective vaccines.[16]

Materials and Methods

Bacterial isolates

Nonduplicate, clinical isolates from dogs were obtained as convenience samples from private, state, and university-associated veterinary diagnostic laboratories in the United States. Participants were requested to provide recently obtained MRSP and methicillin-susceptible *S. pseudintermedius* (MSSP). Samples were received over a 3-year period (2008–2010). To facilitate collection of regionally diverse samples, states were grouped using the United States Health and Human Services designated geographical regions. The distribution of states and territories among each of the regions was as follows: region I (CT, ME, MA, NH, RI, and VT), region II (NJ, NY, Puerto Rico, and US Virgin Islands), region III (DE, DC, MD, PA, VA, and WV), region IV (AL, FL, GA, KY, MS, NC, SC, and TN), region V (IL, IN, MI, MN, OH, and WI), region VI (AR, LA, NM, OK, and TX), region VII (IA, KS, MO, and NE), region VIII (CO, MT, ND, SD, UT, and WY), region IX (AZ, CA, Guam, HI, and NV), and region X (AK, ID, OR, and WA). A minimum of eight isolates were obtained from each region (Table 1 and Supplementary Table S1; Supplementary Data are available online at www.liebertpub.com/mdr).

Species identification

S. pseudintermedius isolates were inoculated onto Trypticase Soy Agar plates containing 5% sheep blood (BD Diagnostics) and incubated overnight at 37°C. Three to four bacterial colonies were suspended in 3 ml of sterile Tryptic Soy Broth, incubated at 37°C overnight, and a commercial kit (UltraClean® Microbial DNA isolation Kit; Mo Bio Laboratories, Inc.) was used for DNA extraction. Identification of isolates by polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) based on a single *Mbo*I restriction site in the *pta* gene of *S. pseudintermedius* was performed as previously described.[13]

Antimicrobial susceptibility

Antimicrobial susceptibility tests were performed on all isolates that were confirmed as *S. pseudintermedius*. A disk diffusion method was performed and interpreted as recommended by the Clinical and Laboratory Standards Institute (CLSI) 2008 guideline, the most current version available at the time of this study.[17] Mueller Hinton agar plates and antimicrobial disks were obtained commercially (BD Diagnostic Systems). Antimicrobials included amoxicillin–clavulanic acid, penicillin, cefpodoxime, cefoxitin, cefalotin, oxacillin, clindamycin, erythromycin, tetracycline, gentamicin, trimethoprim/sulfamethoxazole, marbofloxacin, and chloramphenicol. Erythromycin and clindamycin disks were placed approximately 15 mm apart; so interpretation of inducible clindamycin resistance could be made for isolates that were resistant to erythromycin, but otherwise susceptible to clindamycin. Isolates in the intermediate category were classified as resistant for the purpose of analysis. Detection of the *mecA* gene was performed by conventional PCR using the following primers: *mecA* fwd 5′-CATATCGTG AGCAATGAACTGA-3′ and *mecA* rev 5′-AGCAACCAT

Table 1. Geographical Distribution of *Staphylococcus pseudintermedius* Clonal Complexes and Founders

	Geographical region of isolation										
Isolates	*I*	*II*	*III*	*IV*	*V*	*VI*	*VII*	*VIII*	*IX*	*X*	*Total*
All isolates	11	11	10	30	17	28	16	8	33	26	190
All MSSP	2	1	0	16	4	11	0	1	6	8	49
All MRSP	9	10	10	14	13	17	16	7	27	18	141
CC68	4	5	4	8	8	7	5	3	12	13	69
ST68	3	5	4	6	8	3	4	1	7	13	54
CC71	1	1	2	2	0	1	2	4	6	0	19
ST71	1	1	2	2	0	1	2	3	5	0	17
CC84	2	1	2	3	3	7	5	1	6	3	33
ST84	2	0	0	0	3	3	2	0	0	0	10
Nonclonal MRSP	2	3	2	2	2	2	4	0	4	2	23

MRSP, methicillin-resistant *Staphylococcus pseudintermedius*; MSSP, methicillin-susceptible *S. pseudintermedius.*

CGTTACGGATT-3′. Multidrug resistance was defined as resistance to representatives of three or more non-beta-lactam drug classes.

Genetic relatedness

The sequence type (ST) of each isolate was determined by multilocus sequence typing of seven genes as previously described.[12] Sequences from the targeted genes were analyzed using commercial software (Lasergene, DNAStar) and compared with allele sequences using the *S. pseudintermedius* PubMLST database (http://pubmlst.org/speudintermedius). New numeric designations, when needed, were assigned to alleles and ST by the curator Vincent Perreten (vincent.perreten@vetsuisse.unibe.ch). eBURST analysis was performed using eBURST v3 hosted by the Imperial College of London (http://eburst.mlst.net/). Each CC was composed of STs that differ by only a single locus variant and shared at least six identical alleles. The founder was identified as the ST with the greatest number of single locus variants.[18]

Results

Isolate collection

Based on RFLP of the *pta* gene, 190 isolates were confirmed as *S. pseudintermedius* and were distributed geographically as shown in Table 1.

Multilocus sequence typing and genetic relatedness

Eighty-six different STs were identified (Supplementary Table S1), 67 of which had not been previously assigned. The 141 MRSP isolates had 42 different STs and the 49 MSSP isolates were distributed among 45 different STs. ST68 occurred most frequently (38% of MRSP) and other predominant STs were ST71 (12% of MRSP) and ST84 (7% of MRSP) (Table 1). No other sequence type had more than five representatives (3% of MRSP). ST68 isolates were obtained from all 10 geographic regions, ST84 isolates were from four regions, and ST71 isolates were obtained from eight regions. ST71 contained 16 MRSP and two MSSP. The allelic profile for each ST is available in the *S. pseudintermedius* PubMLST database (http://pubmlst.org/speudintermedius). eBURST analysis identified three clonal populations or clusters (Fig. 1). They were categorized by their putative founders as CC84, CC71, and CC68. The geographical distribution of the CCs is shown in Table 1. CC84 contained 15 STs, of which 14 were MRSP. CC68 contained 10 STs, all of which were MRSP, and CC71 consisted of 3 MRSP STs, although ST71 contained both MRSP and MSSP. CC84 and CC68 isolates were obtained from every geographical region. CC71 isolates, which included ST71, ST123, and ST169, were obtained from all regions except VI and X.

Antimicrobial resistance

Antimicrobial resistance data are presented for individual isolates in Supplementary Table S1 and summarized in Table 2. Among MRSP, the most common antibiogram profile was resistance to all antimicrobials except chloramphenicol, which occurred in 47.5% of MRSP isolates. Resistance to all antimicrobials except chloramphenicol and tetracycline occurred in 7.8% of MRSP isolates. There were seven isolates (5.0% of MRSP) resistant to all antimicrobials, including chloramphenicol. Among all isolates, a total of 11 (5.8%) were resistant to chloramphenicol, including 9 MRSP and 2 MSSP. Eleven MSSP isolates (22.4% of MSSP) were not resistant to any antimicrobials and 11 (22.4% of MSSP) were only resistant to penicillin. Of the isolates analyzed in this study, 138 (71.1%) were resistant to at least three antimicrobials of different classes other than beta-lactams. The percentage of multidrug resistance was 85.6% among MRSP and 27.1% among MSSP.

There were notable differences in resistance to chloramphenicol, marbofloxacin, tetracycline, and trimethoprim/

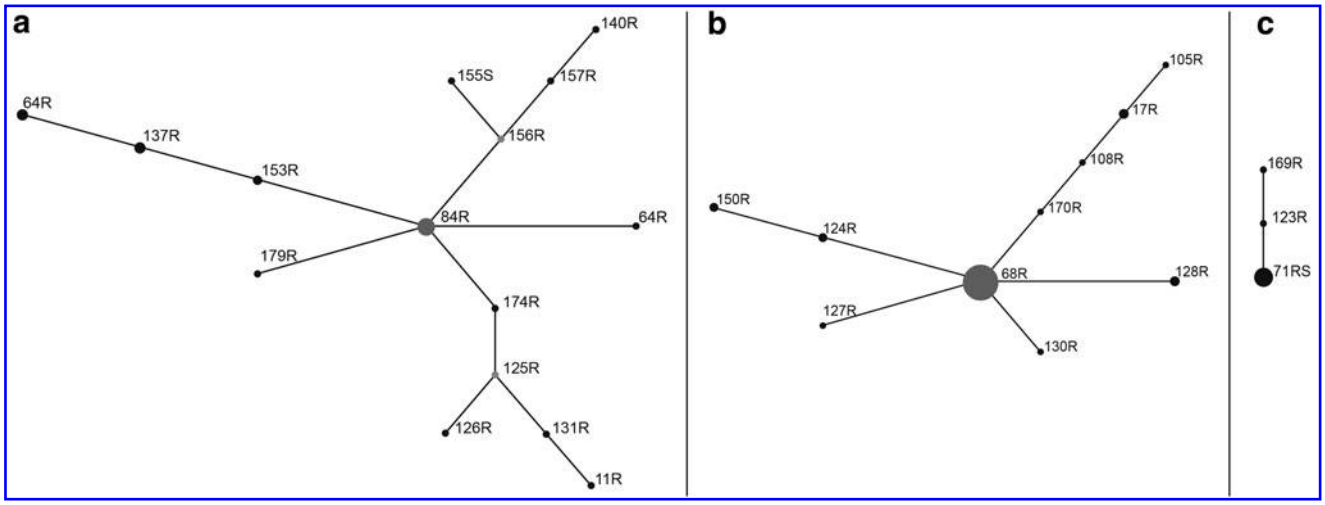

FIG. 1. Schematic diagram showing the three clonal complexes as determined by eBURST analysis. The CC84, CC68, and CC71 clonal complexes are depicted in **(a–c),** respectively. The numbers represent STs. STs containing entirely methicillin-resistant isolates have the letter ''R'' suffix, and sequence types containing entirely methicillin-susceptible isolates have the letter ''S'' suffix. ST71 contained both methicillin-resistant and methicillin-susceptible isolates and is labeled with ''RS.'' ST, sequence type.

Table 2. Antimicrobial Resistance of Clonal and Nonclonal Isolates

	MSSP n = *49*	*MRSP* n = *141*	*CC68* n = *69*	*CC71* n = *19*	*CC84* n = *33*	*Nonclonal MRSP* n = *23*
	Percent resistant					
Cephalosporin	0.0	100.0	100.0	100.0	100.0	100.0
Chloramphenicol	4.1	6.4	1.4	15.8	3.0	17.4
Clindamycin	6.1	82.3	92.7	89.5	66.7	60.8
Erythromycin	6.1	80.8	91.3	89.5	63.6	60.8
Gentamicin	8.2	76.6	96.6	89.5	51.5	43.5
Marbofloxacin	4.1	70.9	98.6	94.7	9.1	60.8
Penicillin	73.5	100.0	100.0	100.0	100.0	100.0
Tetracycline	28.6	77.3	98.6	26.3	60.6	73.9
Trimethoprim/ sulfamethoxazole	38.8	87.9	98.6	94.7	60.6	91.3

sulfamethoxazole among isolates in different CCs (Table 2). Resistance to tetracycline occurred in 98.6% of CC68, whereas it occurred in only 60.6% of CC84, 26.3% of CC71, 73.9% of nonclonal MRSP, and 28.6% of MSSP isolates. Chloramphenicol resistance occurred in 1.4% of CC68, 15.8% of CC71, 3.0% of CC84, 17.4% of nonclonal MRSP, and 4.1% of MSSP. Fluoroquinolone resistance (determined with marbofloxacin) occurred in 98.6% of CC68, 98.6% of CC71, 9.1% of CC84, 60.8% of nonclonal MRSP, and 4.1% of MSSP. Trimethoprim/sulfamethoxazole resistance occurred in 98.6% of CC68, 94.7% of CC71, 60.6% of CC84, 91.3% of nonclonal MRSP, and 38.8% of MSSP.

Based on *mecA* PCR, 48 isolates were MSSP and 142 were MRSP. Among the CC71, two ST71 isolates were susceptible to all beta-lactams, including oxacillin. These included NA91, which was PCR negative for *mecA*, and NA221, which was PCR positive. The only other member of a clonal group that was methicillin susceptible was NA107, which was an ST155 in CC84. This isolate was multidrug resistant, but susceptible to all beta-lactams.

Discussion

Due to the sources and nature of the sample request, which emphasized MRSP, the proportion of MRSP to MSSP isolates included in this study are not representative of the distribution of *S. pseudintermedius* in the dog population within the United States. However, a mix of MRSP and MSSP was obtained from almost every region of the United States, and this study is similar to previous studies summarized by Pires Dos Santos *et al.*,[15] in which MRSP represented 76% of the isolates studied on average in 58 publications compared to 74% in this study. Furthermore, there was no evidence for clonal expansion of any MSSP ST. This lack of clonality among MSSP is consistent with previous findings.[11] In recent years there has been an increase in the prevalence of MRSP.[9,19,20] This is of particular concern because methicillin resistance has been associated with multidrug resistance, a finding supported by this study in which greater than threefold more multidrug resistance was found in MRSP compared to MSSP. Previous studies associated ST68 with methicillin resistance in the United States.[8,15] In this study, ST68, ST71, and ST84 and their CCs were identified as the predominant types of MRSP. Ninety-eight percent of the isolates within the CCs were methicillin-resistant, indicating efficient vertical transmission and stable integration of SCC*mec*. Furthermore, the maintenance of methicillin resistance in evolutionary progeny of putative founder organisms suggests long-term stability of the methicillin resistance cassettes. The presence of a limited number of widespread CCs may result from a selective advantage conferred by characteristics such as efficient host-to-host transmission, biofilm formation, or other virulence factors, in addition to antimicrobial selection potentially coupled with the widespread use of these drugs.[2,8,15] However, the emergence of a large number of nonclonal (singleton) MRSP isolates, as found in previous studies,[10,15] suggests that *S. pseudintermedius* may be more permissive for acquisition and expression of *mecA* than *S.aureus*, for which successful acquisition of *mecA* is believed to be a rare event associated with its genetic background.[21]

The high prevalence of multidrug resistance reported in this and in previous studies[8,10,22,23] reflects the widespread occurrence of antimicrobial resistance in this bacterial species, especially as resistance to non-beta-lactams frequently occurs with methicillin resistance. The rapid emergence of multidrug resistance has been associated with a limited number of mobile genetic elements and the use of numerous classes of antimicrobials.[24] In addition to the obvious impact on dogs, increased occurrence of multidrug resistance among *S. pseudintermedius* populations is a public health concern for the rare cases of human infection that can result from exposure to such organisms, and for the potential for mobile genetic elements conferring resistance in these bacteria to be transferred between staphylococcal species of human and animal origin.[25]

Identification of the genes encoding resistance in the major clonal populations was beyond the scope of this study; however, complete genome sequences from one representative of each of the major clonal populations CC68, CC71, and CC84 have been obtained, deposited in GenBank (CP016072, CP016073, and CP015626), and their microbial resistance genes identified.[26] In addition to the methicillin resistance gene *mecA*, all three representative strains contained *blaZ*, encoding beta-lactamase and the streptomycin and kanamycin resistance genes *ant(6)-Ia* and *aph(3')-III*. Strains 063228 (ST68) and 081661 (ST71) also contain genes for resistance to gentamicin-kanamycin (*aac(6')-Ie-aph(2')-Ia*) and macrolides (*erm* B), and 063228 contains lincosamide and tetracycline resistance genes *lnu*A and *tet*M.

Chloramphenicol was the only antimicrobial to which almost all MRSP (94%) were susceptible. However, the use of this antimicrobial is generally restricted to prevent expansion of resistant bacteria and prolong its efficacy. The occurrence of chloramphenicol resistance in isolates representing nine different sequence types, including all three CCs and five isolates not in CCs, and the fact that it always occurred in multidrug-resistant isolates suggest the potential for spread through clonal expansion, especially as coselection occurs from antimicrobial therapy.

MRSP has been associated worldwide with ST71, ST258, ST 45, ST68, and ST112 and their CCs,[10,15] but has been primarily associated with ST68 and CC68 in North America.[8,11,15] The three main STs identified in this study were ST68, ST71, and ST84 and their CCs. CC71 in the United States warrants attention as they are the most commonly identified MRSP clone spreading worldwide to date, and their rate of expansion in the United States is unknown.[8,15] The two MSSP ST71 isolates in this study, NA91 and NA221, deserve further study as they are the only methicillin-susceptible ST71 isolates that we are aware of to date.

Among the CCs identified in this study, CC71 has been the most studied and has the best basis for comparison with other reports. Most notably, the low percentage of isolates resistant to tetracycline in the United States is about half as high as the number reported from previous studies compiled from six continents.[15] The low incidence of resistance to chloramphenicol in CC71 from the United States (15.8%) is in even sharper contrast to data from the same study, in which about 40% of all CC71 isolates were resistant. Conversely, resistance to trimethoprim/sulfamethoxazole is somewhat higher in isolates from the United States.

To our knowledge, this is the largest and most comprehensive study done to better understand the distribution of clonal patterns among *S. pseudintermedius* isolates from dogs in the United States. The data presented in this study show the presence of MRSP lineages and their clonal expansion. Because the samples were collected several years ago, a new study to identify changes in their distribution and resistance profiles is warranted. This is justified because some rapid changes in MRSP genetic lineages have been observed.[14]

The lineage information obtained in this study should be useful to target the development of new therapeutics and prophylactics toward the most commonly occurring strains of *S. pseudintermedius* in the United States. Development of alternatives to antimicrobial therapy such as vaccines and small molecule inhibitors or protein function requires knowledge of strain prevalence and diversity to identify conserved epitopes and molecular moieties. Furthermore, the findings of multidrug-resistant MRSP and MSSP in STs unrelated to the major clonal populations support the need for continued surveillance to track the emergence of *S. pseudintermedius* clonal populations and the acquisition of antimicrobial resistance, and emergence of new clonal populations.

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Author Disclosure Statement

The authors have no commercial interests that would create a conflict of interest with this study.

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