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# Methicillin-resistant *Staphylococcus pseudintermedius* Clonal Groups Isolated from Canine Pyoderma in Brazil

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**ABSTRACT**

***Background*:** Since the early reports of *mecA*-positive *Staphylococcus* (*S.*) *pseudintermedius* isolates in the United States and in Europe were published, the frequency of methicillin-resistant *S. pseudintermedius* (MRSP) has increased among skin disease cases in dogs in many countries. Moreover, MRSP isolates frequently present a multi-drug resistant profile, which include most drugs used for the skin disease treatment. The distribution of multi- drug resistant MRSP clonal groups in turn varies according to geographic region. Despite the large dog population in Brazil, no data on the MRSP resistance profile or clonal groups have been reported. The aim of this study was to assess the antimicrobial resistance phenotypes and clonal relationships of MRSP isolates originating from dogs affected by recurrent skin diseases.

***Material, Methods & Results*:** Twenty-one epidemiologically unrelated isolates originating from dogs inflicted with a recurrent skin disease, which were treated at the Veterinary Hospital (HCV) of the Federal University of Rio Grande do Sul (UFRGS) in Porto Alegre, were included in this study. The isolates suspected of being MRSP were subjected to PCR analysis to confirm their identity. Identifications were made using PCR analysis that targeted the *mecA* gene and PCR-RFLP that targeted the *pta* gene. Isolates were further assessed by a disc diffusion test for resistance to 13 antimicrobials. Clonal groups were determined according to *spa* typing and SmaI fingerprinting (Pulsed-field Gel Electrophoresis-PFGE) profiles. All 21 isolates were confirmed to be MRSP and displayed a multiple resistance profile. In total, 4 different *spa* types were identified, and the most prevalent was a novel *spa* type (tyA) described in this study. SmaI-macrorestriction analysis demonstrated that the MRSP isolates presented between seven and twelve fragments and were distributed among 15 PFGE profiles. One major clonal group belonging to the new *spa* type (tyA) and to a common PFGE cluster was identified. This clonal group displayed resistance to fluoroquinolones, macrolides, lincosamide, aminoglycosides, sulfonamides, tetracycline and rifampicin.

***Discussion*:** A major multi-drug resistant clonal group of *S. pseudintermedius* was identified, which was shown to cause recurrent canine pyoderma and which might be widespread among dogs in the region. Among the 13 new *spa* type tyA isolates, 11 were also grouped in a common PFGE cluster (A), indicating that recurrent skin disease in dogs treated at the HCV/UFRGS was often associated with one major MRSP clonal group (A/tyA). Additionally, this clonal group displayed resistance to most of the therapeutically important antimicrobial agents tested as fluoroquinolones, macrolides, lincosamide, aminoglycosides, sulfonamides, tetracycline and rifampicin. The clonal group was also resistant to ciprofloxacin, a fluoroquinolone not intended for animal treatment but highly valuable in human medicine. All drugs considered as a first-line choice, as well as most of the second-line drugs typically used for the treatment of skin diseases showed to be ineffective against the MRSP A/tyA group. Among the second-line drugs tested, only gentamicin showed a susceptibility profile among the isolates and might represent a therapeutic option. These data highlight the importance of culture and antimicrobial susceptibility testing as part of the routine diagnosis of skin diseases, as well as the need for hygiene and disinfection measures at small animal clinics to avoid the dissemination of multi-drug resistant MRSP clonal groups.

**Keywords:** MRSP, *spa* typing, PFGE, antimicrobial resistance.

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## INTRODUCTION

*Staphylococcus* (*S.*) *intermedius* group (SIG) encompasses the three related species *S. pseudintermedius*, *S. delphini* and *S. intermedius* [1,23]. Among them, *S. pseuditermedius* is considered the leading cause of canine pyoderma, and it can occasionally cause human infections [28]. The methicillin resistance in *S. pseudintermedius* (MRSP) is mediated by the *mecA* gene, which encodes a low-affinity penicillin binding protein (PBP2a) [30]. Since the first reports of *mecA*-positive isolates in the United States [12] and in Europe [15], MRSP lineages have been isolated with an increasing frequency [30].

The identification of MRSP clonal groups has been accomplished using typing methods such as Pulsed-field Gel Electrophoresis (PFGE) [1,24] and multilocus sequence typing (MLST) [3]. Another approach based on the sequence analysis of the putative staphylococcal protein A (encoded by the *spa* gene), has been shown to be a useful tool in tracing MRSP lineages as well, and it displays a discriminatory power comparable to that of PFGE. Moreover, *spa* typing proved to be less time-consuming and yielded comparable results among laboratories [17].

The dog population of Brazil is estimated in 55.5 million, and 28.9 million households have at least one dog [8]. This fact stresses their importance as pets and their close contact with humans. In Brazil, pyoderma is among the most common diseases in dogs and the occurrence of *S. pseudintermedius* has been previously reported [14,19,21], however there are no reports describing clonal groups of MRSP. Thus, the aim of this study was to assess the antimicrobial resistance and clonal relationship of MRSP isolates originating from dogs affected with recurrent pyoderma.

## MATERIALS AND METHODS

### *Isolates*

A collection of 21 haemolytic coagulase-positive *Staphylococcus* isolates resistant to oxacillin (halo < 17 mm on the disk diffusion test) was included in this study. The isolates originated from dogs with recurrent cases of community-acquired pyoderma that were treated at the Veterinary Hospital of the Federal University of Rio Grande do Sul (UFRGS) in Porto Alegre, Brazil. The dogs had different owners and were attended to at the hospital's dermatology service unit over a two-year period. Other than the fact that they lived in different areas within Porto Alegre (an area approximately 10.2 km$^2$) and were attended to at the same clinic, they had no apparent epidemiological link.

### *Detection of the mecA gene and species identification*

The oxacillin-resistant isolates were screened for the presence of the methicillin resistance-encoding *mecA* gene. Detection was performed by PCR analysis as described previously [3].

The identification of *S. pseudintermedius* was accomplished by a PCR-RFLP test that targeted the *pta* gene followed by MboI[1] restriction analysis as previously described [2]. The *S. pseudintermedius* amplicon contains a single MboI site, resulting in two restriction fragments of 213 bp and 107 bp. In contrast, the *pta* gene in the other SIG species (*S. delphini*, *S. intermedius* and *S. schleiferi*) has no MboI restriction sites. *Staphylococcus aureus* isolates contain a unique MboI site, resulting in restriction fragments of 156 bp and 164 bp that appear as a single band during agarose gel electrophoresis.

### *Antimicrobial resistance profile*

Antimicrobial resistance of MRSP isolates was determined by an agar disc diffusion test according to the guidelines given in the documents M31-A3 and M100-S21 of the Clinical and Laboratory Standards Institute [9,10]. Thirteen antimicrobial discs[2] were used: ciprofloxacin (5 µg), clindamycin (2 µg), erythromycin (10 µg), florfenicol (30 µg), gentamicin (10 µg), kanamycin (30 µg), marbofloxacin (5 µg), nalidixic acid (30 µg), rifampicin (5 µg), tetracycline (30 µg), tobramycin (10 µg), trimethoprim/sulfamethoxazole (1.25/23.75 µg) and vancomycin (30 µg). *Staphylococcus aureus* ATCC® 2592[3] was used as a control for the test. Isolates were defined as multi-drug resistant if they displayed resistance to three or more antimicrobial classes.

### *spa typing*

PCR was performed to amplify the polymorphic X-region of the protein A gene (*spa*) in *S. pseudintermedius*, as previously described [17]. The primers SIspa F (5'-AAC CTG CGC CAA GTT TCG ATG AAG-3') and SIspa R (5'-CGT GGT TTG CTT TAG CTT CTT GGC-3') were used to amplify a 1,389 bp segment of the *spa* gene. Thermal cycling reactions consisted of an initial denaturation (10 min at 95°C), followed by 30 cycles of denaturation (30 s of at 95°C), annealing (30 s at 58°C) and extension (60 s at 72°C) with a final extension step (10 min at 72°C). PCR products were purified using a NucleoSpin Extract II Kit[3]. DNA sequencing was

performed at the Unidade de Análises Moleculares e de Proteínas (Centro de Pesquisa Experimental, HCPA, UFRGS) using an ABI 3500 Genetic Analyzer[4] with 50 cm capillaries and POP7 polymer. PCR products were labelled with 5.0 pmol of the primer 5'-NNN NNN NNN NNN N-3' and 1 µL of BigDye Terminator v3.1 Cycle Sequencing Kit[4] in a final volume of 10 µL. Labelling reactions were performed in a Veriti® 96-Well Thermal Cycler thermocycler[4] with an initial denaturing step of 96ºC for 1 min followed by 35 cycles of 96ºC for 15 s, 50ºC for 15 s and 60ºC for 4 min. Labelled samples were purified using a BigDye X Terminator Purification Kit4 and electro-injected into the automatic sequencer. Sequence analyses and comparisons were carried out with the BLAST® programs BLASTN and BLASTP (http://blast.ncbi.nlm.nih.gov/Blast.cgi). A numeric code was given to each unique repeat sequence, and spa types were designated numerically depending on the composition and order of repeats.

*Macrorestriction analysis (PGFE)*

To determine the genetic relatedness of the isolates, macrorestriction using the enzyme SmaI[1] was conducted according to the protocol described previously [16]. PFGE was performed using a contour-clamped homogeneous electric field apparatus, the CHEF DR-II system[5]. The SmaI fragment patterns were resolved using the following running parameters: 6 V.cm$^{-1}$ and 14°C, with an initial switch of 5 s and a final switch of 40 s, for 23 h. After the electrophoresis run was completed, the gel was stained in a 1 µg.mL$^{-1}$ ethidium bromide solution for 20 min and destained in distilled water for 45 min. Fragment patterns were analysed using the GelCompar[6] software package. Similarities between profiles were calculated using the Dice coefficient, with 1.5% optimization sets and a tolerance of 1.5%. The patterns were clustered using the unweighted pair group method with arithmetic averages (UPGMA). A similarity cut-off of 80% was used to define a cluster.

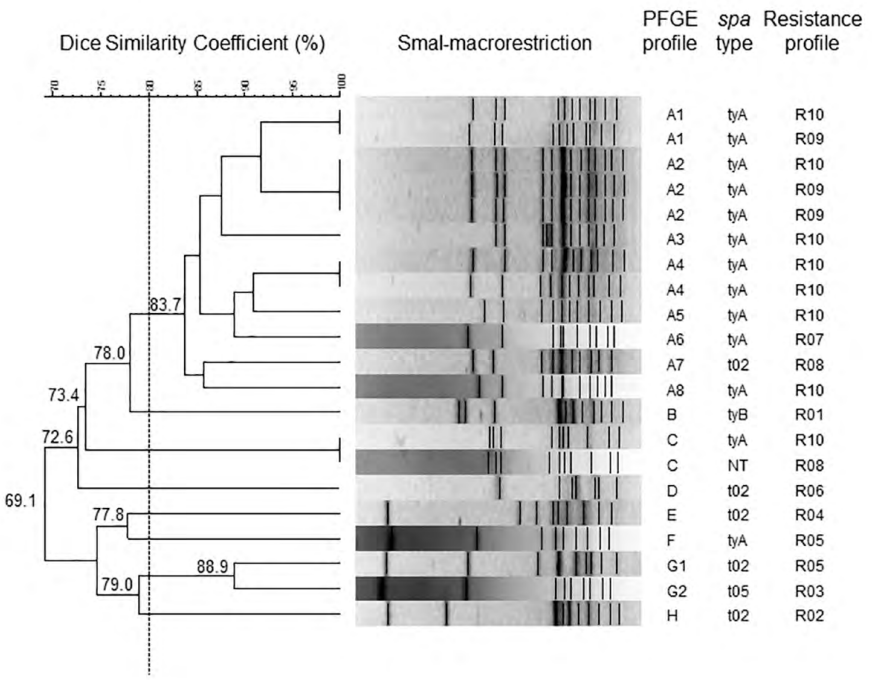


**Figure 1.** Dendrogram of MRSP isolates based on SmaI-macrorestriction (PFGE) patterns. Similarity analysis was performed using the Dice coefficient and UPGMA method (tolerance, 1.5%).

## RESULTS

All 21 coagulase-positive and oxacillin-resistant *Staphylococcus* isolates were confirmed to be *S. pseudintermedius* by PCR-RFLP targeting the *pta* gene, and all carried the *mecA* gene. The MRSP isolates also displayed resistance to nalidixic acid (100%), trimethoprim/sulfamethoxazole (95.2%), kanamycin (95.2%), erythromycin (90.5%), ciprofloxacin (90.5%), enrofloxacin (90.5%), marbofloxacin (85.7%), clindamycin (81.0%), tetracycline (76.1%), rifampicin (47.6%) and tobramycin (42.8%). All the isolates were susceptible to vancomycin, gentamicin and florfenicol. The 21 MRSP isolates were all resistant to multiple antimicrobials, and the most prevalent phenotypes (R8, R9, and R10) were resistant to at least

11 antimicrobial agents (Table 1). Thirteen (61.9%) of the MRSP isolates displayed one of these resistance profiles.

Twenty MRSP isolates were able to be *spa* typed (20/21; 95.2%), and these isolates displayed between five and eight tandem repeats (Table 2). In total, 4 different *spa* types were identified, and the most prevalent was a novel *spa* type (tyA) described in this study. An additional new *spa* type (tyB) was also found, which presented a new repeat sequence. SmaI-macrorestriction analysis demonstrated that the MRSP isolates presented between seven and twelve fragments and were distributed among 15 PFGE profiles (Figure 1). Twelve MRSP isolates (57.1%) were grouped into a single major cluster (A) with ≥ 80% similarity, while the remaining nine isolates were distributed among seven other clusters (B to H). All except one of the isolates belonging to the major PFGE-cluster presented the same *spa* type (tyA). Among them, seven presented indistinguishable PFGE-profiles (A1, A2 or A4), indicating that they are identical strains. The entire major clonal group (A/tyA) was highly resistant to antimicrobials, displaying resistance to at least 10 of the tested drugs.

**Table 1.** Multi-drug resistance profiles for 21 methicillin-resistant *Staphylococcus pseudintermedius* isolates taken from dogs in southern Brazil

| Resistance profile | Resistance phenotype* | Isolates (n) |
|---|---|---|
| R1 | ERY-KAN-NA-OX-STX | 1 |
| R2 | KAN-NA-OX-STX-TET | 1 |
| R3 | CLI-ERY-KAN-NA-OX-STX | 1 |
| R4 | CIP-ENR-MAR-NA-OX-STX-TET | 1 |
| R5 | CIP-ENR-ERY-KAN-MAR-NA-OX-STX | 2 |
| R6 | CIP-CLI-ENR-ERY-KAN-MAR-NA-OX-STX-TOB | 1 |
| R7 | CIP-CLI-ENR-ERY-KAN-MAR-NA-OX-STX-TET | 1 |
| R8 | CIP-CLI-ENR-ERY-KAN-MAR-NA-OX-RIF-STX-TET | 2 |
| R9 | CIP-CLI-ENR-ERY-KAN-MAR-NA-OX-STX-TET-TOB | 3 |
| R10 | CIP-CLI-ENR-ERY-KAN-MAR-NA-OX-RIF-STX-TET-TOB | 8 |

*CIP: Ciprofloxacin; CLI: Clindamycin; ERY: Erythromycin; ENR: Enrofloxacin; KAN: Kanamycin; MAR: Marbofloxacin; NA: Nalidixic Acid; OX: Oxacillin; RIF: Rifampicin; STX: Trimethoprim/sulfamethoxazole; TET: Tetracycline; TOB: Tobramycin.

**Table 2.** *spa* types found among methicillin-resistant *Staphylococcus pseudintermedius* isolates taken from dogs in southern Brazil.

| *spa* type | Repeat sequences | Isolates (n) | Reference |
|---|---|---|---|
| t02 | r01 r02 r03 r03 r03 r06 r05 | 5 | Moodley *et al.* [17] |
| t05 | r01 r02 r03 r03 r03 r03 r06 r05 | 1 | Moodley *et al.* [17] |
| tyA | r03 r03 r03 r06 r05 | 13 | This study |
| tyB | r01 r02 rxx* r02 r06 r05 | 1 | This study |

*rxx: AAAGAAGACAAAGCTGAAGACAAAGGCAAC.

## DISCUSSION

In this study, the first report of MRSP *spa* typing in Brazil, the presence of closely related MRSP strains associated with recurrent pyoderma was detected, and clonal groups based on *spa* typing and PFGE profiles were identified. A novel *spa* type (tyA) was identified and shown to be the most prevalent (13/21) among the isolates. tyA members lack the two initial tandem repeats (r01 and r02), which are present in the most prevalent *spa* types (t02, t03, t04, t05 and t06) in Europe, USA and China [17,20,11]. The remaining 7 typeable isolates were distributed among the two closely related European types (t02 and t05), as well as another novel *spa* type (tyB), which was represented by a single isolate. New

*spa* types result from duplications or deletions of entire repeats or point mutations within repeats in the variable region of the *spa* gene [17,26]. It was demonstrated that the genetic diversification in this region is relatively low [17]; therefore, different *spa* types are considered epidemiologically unrelated, and new *spa* types are thought to have divergent evolutionary origins.From a global perspective, MLST has been the more widely applied method for detecting major clonal lineages and their distributions [26]. In Europe and America, the MLST-identified MRSP clonal lineages ST71 and ST68 predominate, respectively [20,22], while other sequence types, such as ST4 and ST5, are predominately found in China [11]. In Brazil, the lineage ST71 was found colonizing the nasal mucosa of a healthy dog [21]. Among the predominant sequence types, several *spa* types and PFGE profiles have been identified [17,20,26]. In fact, *spa* typing and PFGE-profiling have been considered more suitable for the analysis of the local short-term epidemiology than other methods [26]. In our study, among the 13 tyA isolates, 11 were also grouped in a common PFGE cluster (A), indicating that recurrent skin disease in dogs from Porto Alegre is often associated with one major MRSP clonal group (A/tyA), which has been circulating over time. Additionally, this major MRSP clonal group displayed resistance to most of the therapeutically important antimicrobial agents tested.

MRSP isolates are characterized by the presence of the *mecA* gene, which is part of a mobile genetic island called "staphylococcal cassette chromosome *mec*" (SCC*mec*) and confers resistance to all β-lactam antimicrobials [20]. Therefore, among first-line antibiotics, which are considered the initial choice for empiric treatment of canine pyoderma [5], only clindamycin and lincomycin remain as options for the treatment of skin diseases caused by MRSP. However, in addition to *mecA*, SCC*mec* usually carries several other resistance genes, and therefore, MRSP isolates can display resistance to many classes of antimicrobial agents [6,7]. In fact, all MRSP isolates tested in the present study were resistant to at least five antimicrobial classes, and the major clonal group (A/tyA) displayed resistance to fluoroquinolones, macrolides, lincosamide, aminoglycosides, sulfonamides, tetracycline and rifampicin. Therefore, all drugs considered as a first-line choice, as well as most of the second-line drugs typically used for the treatment of skin diseases [5,13], are ineffective against the MRSP A/tyA group. Additionally, this clonal group was also resistant to ciprofloxacin, a fluoroquinolone not intended for animal treatment but highly valuable in human medicine. Among the second-line drugs tested, only gentamicin showed a susceptibility profile among the isolates and might represent another therapeutic option. Moreover, all MRSP isolates were susceptible to vancomycin; however, considering its importance in the treatment of MRSA in humans, vancomycin administration to animals has been strongly discouraged [5,13].

The wide distribution of the major MRSP clonal group (A/tyA) within the canine population in the studied region can be inferred by the fact that the isolates originated from dogs with no evident epidemiological relationship. Moreover, seven epidemiologically unrelated isolates included in this group were indistinguishable by both *spa* typing and PFGE and were thus considered the same strain. However, the origin of this major clonal group could not be traced in our study because all of our isolates originated from dogs with recurrent pyoderma. The idea that isolates belonging to this group are circulating in the healthy canine population cannot be excluded because MRSP colonization has already been reported in healthy dogs as well as in those animals that were previously treated for pyoderma [18,4]. The wide distribution of strains belonging to this clonal group in turn indicates that once they are established on the skin, they can persist and acquire new resistance genes or undergone successive mutations.

The distribution of MRSP and its antimicrobial resistance are a concern to both animal and human health, and hygiene measures to minimize the spread of epidemic strains are necessary. It is not possible to exclude the idea that the hospital's dermatology service itself may have played a role in transmitting these MRSP isolates because dogs with recurrent pyoderma and associated diseases usually come to the hospital for regular consults. Studies have demonstrated that dogs with MRSP infections have often had previous contact with other MRSP-infected dogs at hospitals, in clinic waiting rooms or elsewhere [27,18]. Separate waiting and consultation areas for the different risk groups within a small animal practice would be ideal, but this clinic design could lead to a patient bottleneck if this configuration was adopted [29].

Another suggested approach has been to diminish the time spent by dogs with recurrent pyoderma in the waiting room [5]. Considering that most clinics do not have the space to have a dedicated consulting room for suspected cases, special attention should be paid to cleaning and disinfecting contact surfaces after attending to MRSP-infected animals [5,29]. In this sense, compliance with personal cleaning and disinfection procedures, as well as the use of a proper cleaning product, is critical [29]. Special attention should be paid by pet owners and those who come in contact with suspected MRSP-infected dogs because humans can become carriers of these strains [27]. Therefore, owners should be informed about hygienic measures such as hand washing and disinfection of fomites and household surfaces that come in contact with the infected dogs [29].

Antimicrobial exposure has been identified as a risk factor for methicillin-resistant staphylococci colonization in animals [25]. Therefore, bacterial culture and antimicrobial susceptibility testing should be part of routine diagnostic procedures for canine pyoderma. Oxacillin should be included as one of the antimicrobials tested by a diagnostic laboratory to detect MRSP isolates. According to the guidelines of the CLSI [9,10], these isolates should be reported to clinicians as resistant to all penicillins, cephems and carbapenems, independent of the test results. The judicious use of antimicrobials, based on susceptibility testing, is of utmost importance because their indiscriminate use can contribute to the selection of *S. pseudintermedius*-resistant clonal groups. The use of antimicrobials only when needed, at optimal doses, for only the required period of time and with as narrow a spectrum as possible is advised [29]. Moreover, the need for full treatment compliance should be stressed to the dogs' owners.

## CONCLUSION

In conclusion, a major clonal group of *S. pseudintermedius* was identified as the causal agent of recurrent pyoderma in dogs treated at HCV/UFRGS. This clonal group showed resistance to first- and second-line antimicrobials typically used in the treatment of skin diseases, and therefore constitute a concern to animal health. These data highlight the importance of culture and antimicrobial susceptibility testing as part of the routine diagnosis of skin diseases and the need for hygiene and disinfection measures at small animal clinics to avoid the spread of multi-drug resistant MRSP.

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