Genetic Characterization of Methicillin Resistant Staphylococcus pseudintermedius in Dogs and Cats in Cyprus: Comparison of MRSP and MRSA Results

Hazel Tamakan* and Huban Gocmen

Department of Microbiology, Faculty of Veterinary Medicine, Near East University, Nicosia, 99138, Northern Cyprus, Mersin-10, Turkey.

ABSTRACT

The aim of this research was to investigate the existence and frequency of Staphylococcus pseudintermedius in cats and dogs with pyoderma and otitis externa by conventional culture methods, automated identification system and PCR-RFLP. This is the first study to research the presence of MRSP in cats and dogs in Cyprus. The PCR-RFLP method based on the pta gene was confirmed to S. pseudintermedius. Methicillin resistance results were revealed with VITEK 2 and mecA gene detection was performed by PCR. A total of 100 samples including 50 cats and 50 dogs were examined from 6 veterinary clinics.

INTRODUCTION

Staphylococcus pseudintermedius was first described as a new species in 2005 by using 16S rRNA gene sequence analysis from samples taken from cats, dogs, horses and parrots (Devriese et al., 2005). Recent studies have revealed that isolates phenotypically recognized as S. intermedius can be classified into three species: S. intermedius, S. pseudintermedius, and S. delphini (groups A and B), together known as the Staphylococcus intermedius group (SIG) (Sasaki et al., 2007). Staphylococcus cornubiensis, which was isolated from human skin in 2018, is also included in this group (Murray et al., 2018).

Staphylococcus pseudintermedius colonizes the skin, hair, and mucocutaneous sites in healthy dogs and cats as part of the normal cutaneous flora (Rubin et al., 2011). S. pseudintermedius may cause opportunistic infections such as pyoderma, otitis externa, urinary tract infections, wound and surgical infections, and abscesses (Scherer et al., 2018; Loeffler and Lloyd, 2018; Diribe et al., 2015; Wettstein et al., 2008). An underlying immunosuppressive condition or break in host barriers is present in the majority of infections (Chrobak-Chniew et al., 2018). S. pseudintermedius is an adapted species to the Canidae family, which includes dogs and foxes, according to epidemiological research (Bannoehr et al., 2009). Both wild and domesticated cats had lower carriage rates than dogs, implying that cats are not a natural host of S. pseudintermedius (Hanselman et al., 2009; Hariharan et al., 2011). However, S. pseudintermedius can be clinically isolated from feline cases with pyoderma, particularly from inflammatory skin lesions (Abraham et al., 2007; Loeffler et al., 2007; Chandak et al., 2019). As in S. pseudintermedius, S. aureus has been reported to cause pyoderma and otitis in cats and dogs (Faires, 2008). In humans, S. pseudintermedius has been found in sporadic diseases such as pyoderma, sinonasal infections, and wounds from dog bites (Chandak et al., 2019; Ference et al., 2019; Börjesson et al., 2015).

Methicillin resistance is a serious worldwide problem in S. pseudintermedius strains isolated from both diseased and healthy cats and dogs (Walther et al., 2017). Methicillin-resistant Staphylococcus pseudintermedius (MRSP) was first reported in Europe in 2006 (Moodley et al., 2013).
Many *S. pseudintermedius* isolates are not only resistant to methicillin, but also to many classes of antibiotics (MDR) (Wegener et al., 2018). In recent years, the rate of methicillin and multidrug-resistant *S. pseudintermedius* (MDR-SP) has increased in clinical samples of pet animals. It has been demonstrated that MRSP is a multi-drug resistant pathogen against almost all antimicrobial agents applicable in veterinary medicine. Methicillin-resistant *Staphylococcus aureus* (MRSA) in pets, like MRSP, has the potential to have serious consequences for both veterinary and human medicine (Worthing et al., 2018; Somayaji et al., 2016; Van Duijkeren et al., 2004; Loeffler et al., 2005).

The aim of this research was to use bacteriological and molecular techniques to determine the presence and frequency of *S. pseudintermedius* in cats and dogs with pyoderma and otitis externa. However, this is the first study to investigate the *mecA* gene and MRSP in cats and dogs in Cyprus in order to establish which strains are resistant to methicillin.

**MATERIALS AND METHODS**

**Ethics statement**

This study was approved by the Ethics Committee of Near East University (No: 2019/87; 27.09.2019).

**Animal sampling**

A total of 100 cats and dogs were randomly selected from 6 veterinary clinics. The samples were obtained from animals brought to Near East Animal Hospital and 5 veterinary clinics in Morphou, Famagusta, Nicosia and Kyrenia districts of Cyprus. Skin and ear examinations of cats and dogs of different ages, races and genders were performed, and skin and ear swabs were taken from animals thought to have pyoderma and otitis externa. A total of 100 animals, including 50 (25 ear and 25 skin samples) cats and 50 (25 ear and 25 skin samples) dogs were sampled. The samples were collected with swabs containing liquid Amies medium (Copan-493CE03, MRSA system).

**Identification of Staphylococcus species**

Columbia blood agar with 5% sheep blood (Biomerieux, 43041), MacConkey agar (Merck, 105465), and Baird Parker agar (Merck, 105406) were used to culture the materials. Under aerobic conditions, all agar plates were incubated for 24 h at 37°C. After incubation, the isolates were determined by colony morphology, microscopic morphology, and growth parameters. The Gram staining (Biomerieux, 55542), colony morphology, haemolysis, and tests for catalase (Biomerieux, 55561), clumping factor (Merck, 113306), tube coagulase (Merck, 113306), and DNase test (Merck, 110449) were all used to identify staphylococci. A VITEK 2 Compact automated system device (Biomerieux, France) was used to perform confirmatory phenotypic identification.

**Antibiotic susceptibility testing**

VITEK GP-AST80 cards (Biomerieux, 421826) and a VITEK 2 Compact system device were used to determine antibiotic susceptibility. VITEK 2 Compact analyzes and interprets MIC values for a total of 14 antibiotics using Clinical Laboratory Standards Institute (CLSI) guidelines (CLSI, 2017). These antibiotics are cefoxitin screening, gentamicin, kanamycin, marbofloxacin, pradofloxacin, erythromycin, enrofloxacin, neomycin, doxycycline, clindamycin, tetracycline, nitrofurantoin, chloramphenicol and trimethoprim/sulfamethazole. The GP-AST card also includes cefoxitin screening to determine methicillin resistance and inducible clindamycin resistance test. The isolates identified as *S. pseudintermedius* and *S. aureus* as a result of VITEK 2 were inoculated on ChromID MRSA SMART agar (Biomerieux, 413050) and incubated at 37°C for 24 h. The isolates that formed pink/red colonies at the conclusion of the incubation period were considered positive.

**PCR-RFLP of *S. pseudintermedius***

The boiling method (Kocatepe, 2015) was used for DNA extraction of the isolated *S. pseudintermedius* strains. A loopful of pure colonies was suspended with deionized water in a 500 µl DNase-RNase free Eppendorf tube and then boiled at 100°C for 10 min. The supernatant (300 µl) was kept at -20°C to be used as target DNA in PCR amplification. The *pta* gene, which encodes the phosphoacetyl-transferase enzyme, was detected in the *S. pseudintermedius* isolates using PCR-RFLP (Banmoehr et al., 2009). A 320-bp fragment of the *pta* gene was amplified by PCR in a 25 µl volume that included 12.5 µl PCR master mix (2X) (Thermo Scientific, K0171), 1.25 µl of each oligonucleotide primer (forward: 5’-GGA GCT TGT GAG TCA GC-3’, reverse: 5’-GCA TAA ACA AGC ATT GTA CCG-3’), 6 µl of nuclease-free water, and 4 µl of template DNA. The amplification process was as follows: initial denaturation at 95°C for 2 min followed by 30 cycles of denaturation at 95°C for 1 min, annealing 53°C for 1 min, extension at 72°C for 1 min and a final extension at 72°C for 7 min.

To detect two fragments of 213 bp and 107 bp of the *pta* gene specified for *S. pseudintermedius*, 10 µl of amplified product was digested with a total volume of 20 µl FastDigest MboI (Thermo Scientific, FD0814), 10X FastDigest Green buffer (Thermo Scientific, B72) and nuclease-free water for 5 min at 37°C. The enzyme was then digested.
In all phenotypic and genotypic analyses, the positive control was the *Staphylococcus pseudintermedius* ED99 (GenBank Accession NC_017568.1) strain obtained from Ross Fitzgerald (The Roslin Institute of the Edinburgh University, Scotland).

**Detection of mecA gene of Staphylococcus spp.**

In order to determine methicillin resistance of isolates defined as *S. pseudintermedius* and *S. aureus*, the mecA gene was investigated using the method previously used by (Choi et al., 2003). A 314-bp fragment of the mecA gene was amplified by PCR in a 25 µl volume that included 12.5 µl PCR master mix (2X) (Thermo Scientific, K0171), 1.25 µl of each oligonucleotide primer (forward: 5'-CCT AGT AAA GCT CCG GAA-3', reverse: 5'-CTA GTC CAT TCG GTC CA-3'), 6 µl of nuclelease-free water, and 4 µl of template DNA. The amplification process as follows: initial denaturation at 95°C for 2 min followed by 30 cycles of denaturation at 95°C for 1 min, annealing 54°C for 1 min, extension at 72°C for 1 min and a final extension at 72°C for 7 min. The amplicons were separated on a 2.5% agarose gel, stained with ethidium bromide, and visualised using UV light. *Staphylococcus aureus* 33591 (mecA positive) strain obtained from the Bursa Uludag University (Department of Microbiology, Faculty of Veterinary Medicine) was used as the positive control of the mecA gene.

**Statistical analysis**

Statistical tests were used to compare the data in the tables: following cross tabulation, statistical significance was determined using Pearson’s chi-square test. The IBM SPSS Statistics 18 program was used to conduct the analyses. P values less than 0.05 were considered statistically significant. Statistical data are indicated in the Tables I, II and III.

**RESULTS**

**Bacteriological identifications**

From 50 (25 ear and 25 skin samples) cat samples of 100 samples collected from veterinary clinics, 6 (12%) coagulase positive staphylococci (CoPS) and 19 (38%) coagulase negative staphylococci (CoNS) were isolated. Of the CoPS, 2 isolates (4%) were identified as *S. pseudintermedius* and 4 isolates (8%) as *S. aureus* (Table I). There was no growth in 25 (50%) cat samples. From 50 (25 ear and 25 skin samples) dog samples of the 100 samples collected from veterinary clinics, 32 (64%) CoPS and 3 (6%) CoNS were isolated. Of the CoPS, 28 (56%) were identified as *S. pseudintermedius*, 3 (6%) as *S. aureus* and 1 (2%) as *S. schleiferi* (Table I). There was no growth in 15 (30%) dog samples. The difference between bacterial species isolated according to animal species was statistically significant ($\chi^2=31.926$, p=0.0001). According to the sample type, 11 (22%) CoPS and 17 (34%) CoNS were isolated from ear swabs, whereas 27 (54%) CoPS and 5 (10%) CoNS were isolated from skin swabs. There was no growth in 22 (44%) ear swabs and 18 (36%) skin swabs. The difference between bacterial species isolated according to the sample types was statistically significant ($\chi^2=13.682$, p=0.001).

*Staphylococcus pseudintermedius* was isolated from 2 (4%) cat and 28 (56%) dog samples. A comparison of the sample types (Table I) reveals that *S. pseudintermedius* was isolated from 21 (42%) pyoderma and 9 (18%) otitis externa cases (Table II). As a result of the Pearson chi-square test, the difference between the *S. pseudintermedius* identification rate according to sample type ($\chi^2=6.857$, p=0.009) and animal species ($\chi^2=32.190$, p=0.0001) was determined to be statistically significant. Furthermore, 7 (7%) *S. aureus* isolates were identified with the VITEK 2 compact system. The distribution rate of *S. aureus* isolates according to animal species (p=1) and sample type (p=0.436) in the VITEK 2 method was identified to be homogeneous. It was statistically insignificant (Table I).

**PCR-RFLP of S. pseudintermedius**

According to the VITEK 2 system and PCR-RFLP (*pta* gene) analysis, 30 (30%) *S. pseudintermedius* were identified from a total of 100 swab samples. As a result of the Pearson chi-square test, the difference between the PCR-RFLP analysis results of the isolates identified with the VITEK 2 system and *S. pseudintermedius* was determined to be statistically significant ($\chi^2=100.00$, p<0.0001). PCR-RFLP electrophoresis results of *S. pseudintermedius* strains are shown in Figure 1.

**Table I. According to animal species and sample types Staphylococcus species isolation rates.**

<table>
<thead>
<tr>
<th></th>
<th>Coagulase positive staphylococci</th>
<th>Coagulase negative staphylococci</th>
<th>p-value and $\chi^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>S. pseudintermedius</em></td>
<td><em>S. aureus</em></td>
<td><em>S. schleiferi</em></td>
</tr>
<tr>
<td>Cat</td>
<td>2 (4%)</td>
<td>4 (8%)</td>
<td>1 (2%)</td>
</tr>
<tr>
<td>Dog</td>
<td>28 (56%)</td>
<td>3 (6%)</td>
<td>13 (26%)</td>
</tr>
</tbody>
</table>
Table II. *S. pseudintermedius* isolation rates by sample type.

<table>
<thead>
<tr>
<th>Sample type</th>
<th>Positive samples</th>
<th>Negative samples</th>
<th>Total</th>
<th>p-value and χ²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyoderma (skin swap)</td>
<td>21 (42%)</td>
<td>29 (58%)</td>
<td>50 (100%)</td>
<td>p&lt;0.0001</td>
</tr>
<tr>
<td>Otitis externa (ear swap)</td>
<td>9 (18%)</td>
<td>41 (82%)</td>
<td>50 (100%)</td>
<td>χ²=100.00</td>
</tr>
<tr>
<td>Total</td>
<td>30 (30%)</td>
<td>70 (70%)</td>
<td>100 (100%)</td>
<td></td>
</tr>
</tbody>
</table>

Table III. Methicillin resistance rates of *S. pseudintermedius* and *S. aureus* isolates according to VITEK 2, MRSA agar and PCR analyzes.

<table>
<thead>
<tr>
<th>Staphylococcus species</th>
<th>VITEK 2 compact system</th>
<th>p-value and χ²</th>
<th>MRSA agar</th>
<th>p-value and χ²</th>
<th>PCR (mecA gene)</th>
<th>p-value and χ²</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. pseudintermedius</em></td>
<td>2/30 (6.7%)</td>
<td>p&lt;0.0001</td>
<td>3/30 (10%)</td>
<td>χ²=88.556</td>
<td>3/30 (10%)</td>
<td>χ²=90.013</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>2/7 (28.6%)</td>
<td>p&lt;0.0001</td>
<td>3/7 (42.9%)</td>
<td>χ²=17.111</td>
<td>3/7 (42.9%)</td>
<td>χ²=18.568</td>
</tr>
</tbody>
</table>

Methicillin resistance profiles

According to the antibiotic susceptibility test performed with the VITEK 2 system, *S. pseudintermedius* isolates (6.7%) were detected to be resistant to methicillin (χ²=88.556, p<0.0001). After incubation on MRSA agar, methicillin resistance was found in 10% of *S. pseudintermedius* isolates (χ²=90.013, p<0.0001). In 10% of *S. pseudintermedius* isolates, the mecA gene was also detected (χ²=90.013, p<0.0001). The methicillin resistance of these isolates was found to be statistically significant (Table III). Of the MRSA strains, 2 (28.6%) were isolated from cats with pyoderma and 1 (14.3%) from a cat with otitis externa. The mecA gene electrophoresis results of *S. pseudintermedius* strains are shown in Figure 2.

DISCUSSION

Staphylococci can cause many infections in pet animals (Haag *et al*., 2019; Lynch and Helbig, 2021). In particular, *S. pseudintermedius* and *Malassezia* species, two commensal species of the skin, are known...
as secondary causes of otitis externa (Bugden, 2013). *S. pseudintermedius* is known to be the primary cause of pyoderma in dogs (Loeffler and Lloyd, 2018). The rate of *S. pseudintermedius* in canine pyoderma can reach up to 92% (Bugden, 2013; Szewczuk et al., 2020) and it can vary between 20 to 94.3% in otitis externa (Bugden, 2013; Sim et al., 2019). The prevalence of *S. pseudintermedius* in dogs has been found to be higher than in cats (Rusenova et al., 2020; Göçmen et al., 2020). In a study in Poland, 255 skin and 219 ear samples were collected from dogs with pyoderma and otitis externa. Szewczuk et al. (2020) identified *Staphylococcus* species in 82.4% of pyoderma cases and 44.8% of otitis externa. *S. pseudintermedius*, the most dominant species, and 1 *S. aureus* have been identified as *Staphylococcus* isolates. A total of 24 *S. pseudintermedius* have been isolated from 18 strains of skin and 6 strains of ear swabs and all isolates were resistant to methicillin (Szewczuk et al., 2020). Scherer and colleagues collected 104 ear swabs from both ears of 52 dogs with otitis externa, and 44 *S. pseudintermedius* strains were identified from 31 dogs in their investigation (Scherer et al., 2018). The rate of *S. pseudintermedius* isolation was 60% in dogs with otitis externa. In this study, *S. pseudintermedius* was identified in 21 (42%) pyoderma specimens and 9 (18%) otitis externa specimens. When these two studies were compared, a similarly high rate of *S. pseudintermedius* was identified depending on the number of samples. The rate of *S. pseudintermedius* identification according to sample type was detected to be statistically significant ($\chi^2=6.857$, $p=0.009$).

In the present study, 6 (12%) CoPS and 19 (38%) CoNS were isolated from cats. Of the CoPS, 2 (4%) *S. pseudintermedius* and 4 (8%) *S. aureus* were identified. In a study that investigated *Staphylococcus* species in healthy and sick cats with similar results, the frequency of CoNS was found to be higher. Statistical analysis, unlike this work, confirmed that animals in the sick cat group were more frequently colonized with *S. pseudintermedius* and *S. haemolyticus* (Bierowiec et al., 2019). In another study that described *S. pseudintermedius* by conventional and molecular methods, no *S. pseudintermedius* isolates were detected in cat samples (Rusenova et al., 2020). Contrary to this study, our study revealed that *S. pseudintermedius* and MRSP were also isolated from cat samples. In this study, 32 (64%) CoPS and 3 (6%) CoNS were isolated from dogs. Of the CoPS, 28 (56%) *S. pseudintermedius*, 3 (6%) *S. aureus* and 1 (2%) *S. schleiferi* were identified. Sarreyyüpoğlu and colleagues revealed that the rate of *S. pseudintermedius* detected in dogs with skin infections was 80.4% (Sarreyyüpoğlu et al., 2014). In another study conducted in Turkey, 61 *S. pseudintermedius* strains, 18 of which were MRSP, were isolated from 77 dogs with skin infections (Miştak et al., 2020). Similar to the results of these studies, the most common strain isolated among CoPS in dog samples was *S. pseudintermedius*. Based on the statistical analyses conducted in this study, the difference between bacterial species isolated according to animal species was statistically significant ($\chi^2=31.926$, $p=0.0001$). *S. aureus*, a human and animal pathogen, was detected in 7 (7%) isolates from dog and cat samples. These isolates were from 4 cat samples and 3 dog samples. Cats and dogs are not considered a typical reservoir of *S. aureus*, as confirmed by our data (Haag et al., 2019). Just as *S. pseudintermedius* is transmitted from pets to humans, *S. aureus* can also be transmitted from humans to animals and cause serious infections (Somayaji et al., 2016; Van Duijkeren et al., 2004; Loeffler et al., 2005).

It is difficult to differentiate between bacterial species of SIG by phenotypic tests (Sasaki et al., 2007). In a study investigating the *pta* gene, phosphoacetyl-transferase enzyme, using the PCR-RFLP method, a total of 37 strains, which were from 27 patient and healthy dogs as well as 10 from their owners, were identified as *S. pseudintermedius* (29 strains) (Alcalà et al., 2015). However, both phenotypic and genotypic techniques were used to identify *S. pseudintermedius* in the current study. The VITEK 2 device was primarily used for the identification of *S. pseudintermedius* and these isolates were confirmed by PCR-RFLP. A total of 30 (30%) *S. pseudintermedius* strains were detected with both methods. As a result of the Pearson chi-square test, the difference between the PCR-RFLP analysis and VITEK 2 results identified as *S. pseudintermedius* was revealed to be statistically significant ($\chi^2=100.00$, $p=0.0001$). These results also supported other studies that used VITEK 2 and MALDI-TOF to identify *S. pseudintermedius* (Chandak et al., 2019; Alcalà et al., 2015; Yarbrough et al., 2018).

The resistance developed to methicillin and other antimicrobials by staphylococci has become a global problem in the chemotherapy of staphylococcal infections (Walther et al., 2017). MRSP isolates have been detected at rates of up to 59% in canine pyoderma cases (Yoo et al., 2010; Wang et al., 2012) and in 10 to 48.1% of otitis externa cases (Sim et al., 2019; Chan et al., 2018). In a study in which 771 clinical specimens were collected from a total of 556 dogs, MRSP isolates were identified at a rate of 22% from the skin and 7% from ear swabs (Saab et al., 2018). In these two studies on dogs with pyoderma in China and Japan, the prevalence of MRSP was 48% and 66%, respectively (Kawakami et al., 2010; Feng et al., 2012). On the other hand, in a study conducted in China, MRSP was identified in dogs at a rate of 44% (Wang et al., 2012). Jayalakshmi and colleagues identified 5 (35.7%) MRSP and 14 (56%) *S. pseudintermedius* from 25 swabs collected in China.
from healthy dogs and dogs with otitis externa (Jayalakshmi et al., 2019). When we investigated methicillin resistance with VITEK 2 in S. pseudintermedius strains, 2/30 (6.7%) MRSP strains were detected, but when the mecA gene was examined by PCR, 3/30 (10%) MRSP strains were detected. Of the MRSPs, 2 (6.7%) isolates were identified from pyoderma samples of dogs and 1 (3.3%) isolate was identified from the pyoderma sample of a cat. Contrary to other studies, the rate of MRSP was found to be low in our study and the number of infection cases in the sample was also low. However, this was thought to be due to the relatively infrequent use of beta-lactam antibiotics or the conscious use of antibiotics in cats and dogs. While most of the S. pseudintermedius strains were detected in dog samples in other studies, an MRSP isolate was detected in a cat sample in this study. On the other hand, studies reporting low MRSP rates supporting our results are also available in the literature (Maluping et al., 2014; Dziva et al., 2015). Likewise, the methicillin resistance rate of S. aureus in VITEK 2 was 2/7 (28.6%) and the mecA gene rate was 3/7 (42.9%). Of the MRSA strains in cats, (28.6%) isolates were identified from pyoderma samples and 1 (14.3%) isolate was identified from an otitis externa sample. MRSA was not identified in dogs with pyoderma and otitis externa samples. The differences in the results of PCR with VITEK 2, which was applied to investigate methicillin resistance, once again emphasized the need for genotypic tests.

CONCLUSIONS

In conclusion, this was the first investigation to reveal the existence of S. pseudintermedius and methicillin-resistant Staphylococcus isolates in veterinary clinics in Cyprus. As a result of this, the correct diagnosis of S. pseudintermedius, which is accepted as an important micro-organism in terms of human and animal health, was achieved by genotypic methods. In infections caused by bacteria resistant to antibiotics, the selection of sensitive antibiotics and the implementation of the correct treatment protocols for this purpose, as well as the increase in studies on the patients’ response to treatment, reduction of hospital visits and treatment costs, are important and thought to be beneficial. It is positive for our country that the MRSP ratio obtained did not reach high values. Contrary to the low MRSP rate, the high rate of S. pseudintermedius in dogs and the zoonotic importance of this bacterium are likely to have potential public health effects.

ACKNOWLEDGEMENTS

This work was funded by the Scientific Research Projects Unit of Near East University, Cyprus (Project No, SAG-2019-2-029). This study is doctorate thesis publication of Res. Assist. PhD. student Hazel Tamakan. We gratefully thank Dr. Gulten Tuncel and Assoc. Prof. Dr. Mahmut Cerkez Ergoren for their help.

Statement of conflict of interest

The authors have declared no conflict of interest.

REFERENCES

Kocatepe, D., 2015. Sağlıklık ve otitis externa köpeklerden Staphylococcus pseudintermedius


