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ORIGINAL ARTICLE

Characterization of methicillin-resistant *Staphylococcus pseudintermedius* isolated from dogs and cats

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ABSTRACT

The aim of this study was to explore the presence of methicillin-resistant *Staphylococcus pseudintermedius* (MRSP) in a collection of *S. pseudintermedius* strains isolated from dogs and cats with dermatitis in Japan and to compare their genotypic and phenotypic characteristics. Clonal relationships were determined by pulse field gel electrophoresis (PFGE), staphylococcal chromosomal cassette *mec* (SCC*mec*) typing, and multilocus sequence typing (MLST). Biofilm formation assay was performed using safranin staining in microplates. Three virulence genes coding for *S. intermedius* exfoliative toxin and Panton-Valentine leukocidin (*siet*, *lukS-PV* and *lukF-PV*) were searched for in a collection of strains. Antimicrobial resistance against 15 antibiotics was studied by a disc diffusion method. Twenty-seven MRSP were isolated. According to PFGE results the isolates were not closely related except for a few strains. MLST showed that the strains belonged to five groups, ST71 and ST26 being the two most prevalent. Three types of SCC*mec* (II, II–III and V) were identified. All isolates were *siet*-positive but *PVL*-negative. Most strains (except for two) produced strong biofilm in tryptic soy broth with glucose. Seventy-eight percent of the isolates were resistant or intermediate to twelve or more antibiotics. Our study demonstrates that the ST71 lineage is widespread in Japan and that ST26 could represent an emerging lineage. Moreover, most of our strains are capable of forming strong biofilm and possess *siet* gene, two virulence characteristics that probably help the bacteria to persist and spread. Finally, our MRSP strains show a strong resistance profile to antibiotics commonly used in veterinary medicine.

Key words Japan, methicillin-resistant *Staphylococcus pseudintermedius*, pets.

In 2005, Devriese *et al*. first described *S. pseudintermedius* as a new species mainly found in cats and dogs (1). This bacterium is commonly confused with *S. intermedius* in routine diagnostic practice and, rather than *S. intermedius*, is probably the major cause of canine pyoderma (2). This commensal bacterium lives in many different animal species and can be an opportunistic pathogen that is mainly responsible for skin infections, such as pyoderma, in dogs and cats (3). *S. pseudintermedius* rarely causes infections in humans;

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List of Abbreviations: Luk-PV, Panton–Valentine leukocidin; MLST, multilocus sequence typing; MRSP, methicillin-resistant *Staphylococcus pseudintermedius*; OD, optical density; PFGE, pulsed field gel electrophoresis; RFLP, restriction fragment length polymorphism; *S. pseudintermedius*, *Staphylococcus pseudintermedius*; SCC*mec*, staphylococcal cassette chromosome *mec*; *siet*, *Staphylococcus intermedius* exfoliative toxin; ST, sequence type; TC, tissue culture; TSB, tryptic soy broth; TSB_{glc}, TSB containing 0.25% glucose; UV, ultraviolet.

when it does, they are usually wound infections after animal contact (4, 5).

Methicillin-resistant *S. pseudintermedius* have recently emerged as significant nosocomial pathogens in companion animals (6–8). Because the therapeutic options are limited, both for animals and humans, their increasing incidence is an alarming problem. Moreover, in addition to having been isolated from cats and dogs, MRSP have also been isolated from humans, highlighting a public health issue for veterinarians and pet owners (9, 10).

The aim of this study was to explore the presence of MRSP in a collection of *S. pseudintermedius* isolated from dogs and cats in Japan and to compare the following genotypic and phenotypic characteristics: SCC*mec*-typing, PFGE, MLST, virulence-associated factor-encoding genes (*siet*, *lukS-PV* and *luk-F-PV*), biofilm formation and resistance profiles to 15 frequently used antibiotics.

MATERIALS AND METHODS

Bacterial isolates

Two hundred *S. pseudintermedius* isolates (22 isolated from cats and 178 from dogs) were collected from animals with dermatitis across south Japan (Kyushu area) between 2008 and 2010 by the Laboratory of Veterinary Public Health (Faculty of Agriculture, University of Miyazaki, Japan; import permit nr 604654). Identification of the species was confirmed by PCR assay based on amplification of the thermonuclease (*nuc*) gene of staphylococcal species as previously described (11). All isolates were tested for methicillin resistance by streaking onto chromogenic chromID MRSA agar (bioMérieux, Marcy-l'étoile, France). After 24 hrs incubation at 37° C, blue green colonies, considered as positive methicillin-resistant colonies, were stored at −80°C in Luria–Bertani broth with 50% glycerol until further characterization.

Genotypic characterization

DNA extraction was carried out using the ChargeSwith gDNA Mini Bacteria Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions for staphylococci. After purification, DNA was stored at −20°C.

Genotypic characterization on methicillin-resistant isolates was performed by PCR in two steps: (i) detection of *mecA* gene, coding for the penicillin-binding protein 2a; and (ii) detection on *mecA*-positive isolates of *siet*, *lukS-PV*, and *lukF-PV* genes coding respectively for *S. intermedius* exfoliative toxin and Panton-Valentine leukocidin (LukF-PV and LukS-PV). PCRs were performed on 2 μL of DNA using *Taq* DNA polymerase (New England BioLabs, USA) and 1 μM of each primer as previously described (12, 13). All PCR products were separated by electrophoresis in 1.5% agarose gels. Gels were stained with ethidium bromide and photographed under UV light.

Staphylococcal cassette chromosome *mec*-typing

Typing of SCC*mec* was performed using multiplex PCR assays as previously described (14). PCR products were separated by electrophoresis in 3% agarose gels. Gels were stained with ethidium bromide and photographed under UV light. Amplification patterns were compared to the patterns of prototype strains used as positive controls.

Pulsed field gel electrophoresis

All isolates were compared by PFGE. Briefly, lysostaphin pre-treated bacterial cells were embedded in 0.9% certified low-melt agarose (Bio-Rad, Hercules, CA, USA), lysed in a 0.5 M EDTA (pH 8) buffer containing 10% *N*-lauroylsarcosine and 2 mg/mL lysozyme, and treated with 1 mg/mL proteinase K (Sigma, St Louis, MO, USA). Genomic DNA was digested with 20 U of *Sma*I (Sigma). Restricted fragments were separated using CHEF Mapper (Bio-Rad) with 1% pulsed field certified agarose (Bio-Rad) gel in 0.5% Tris/borate/EDTA buffer at 6 V/cm for 20 hrs with pulse times ranging from 5 to 60 s (angle of 120° and linear ramp factor). The size of each DNA band was estimated by Biogene (Vilber Lourmat, Marne-la-Vallée, France). A dendrogram was prepared by the unweighted-pair group method using an arithmetic average algorithm, dice coefficient and optimization and position tolerance of 2%.

Multilocus sequence typing

Multilocus sequence typing based on the sequence of five housekeeping genes (*pta*, *cpn60*, *tuf*, 16SrRNA, and *agrD*) was performed on all methicillin-resistant strains as previously described (6). Briefly, all five genes were amplified and sequenced, and allele number and ST assigned according to the scheme proposed by Bannoehr *et al.*(6).

Biofilm production assay

Biofilm formation assay was performed using safranin staining in microplates. Briefly, isolates were incubated in TSB overnight at 37°C. Cultures were then diluted 1:100 in TSB_{glc} and cell suspensions inoculated into wells of sterile 96-well polystyrene TC plates in triplicate for each tested isolate. TSB_{glc} without bacteria served as negative control. The TC plates were incubated at 37°C for 24 hrs. Each well was then carefully washed twice with sterile PBS, dried and stained with safranin 0.1% (w/v) for 10 min. The TC plates were further washed twice with

distilled water and dried again at 37°C before adding a mixture of 50% ethanol–50% acetic acid to each well. Finally, absorbance (OD) of adherent biofilm was measured at 490 nm using a microplate reader. Results are reported according to published recommendations based on average OD values and on the cut-off value, named ODc (ODc = average OD of negative control + [3 × SD of negative control]) (15). Strains were divided into the following four categories: not a biofilm producer (OD ≤ ODc); weak biofilm producer (ODc < OD ≤ 2 × ODc); moderate biofilm producer (2 × ODc < OD ≤ 4 × ODc); and strong biofilm producer (4 × ODc < OD). Biofilm formation assays were performed twice.

Antimicrobial susceptibility testing

Susceptibility tests were carried out on MSRP isolates by the disc diffusion method of Bauer *et al.* (16) on Mueller-Hinton agar (Oxoid, Cambridge, UK). Inhibition zones were measured (in mm) after overnight incubation at 37° C and were interpreted according to the 2010 recommendations of the Comité de l'Antibiogramme de la Société Française de Microbiologie. Fifteen antibiotics were tested, namely: penicillins (penicillin 10 IU, ampicillin 10 μg), cephems (cephalexin 30 μg, cefoperazone 30 μg, ceftiofur 30 μg), aminoglycosides (gentamicin 15 μg, kanamycin 30 IU, streptomycin 10 IU), macrolide (erythromycin 15 IU), tetracycline (tetracycline 30 IU), fluoroquinolone (ciprofloxacin 5 μg), lincosamide (clindamycin 2 IU), folate pathway inhibitor (trimethoprim-sulfamethoxazole 1.25 − 23.75 μg), aminocyclitol (spectinomycin 100 μg) and phenicol (chloramphenicol 30 μg; (ceftiofur, kanamycin, ciprofloxacin; Becton Dickinson, Sparks, MD, USA; The remaining antibiotics listed; I2A, Perols, France).

RESULTS

Methicillin-resistant *Staphylococcus pseudintermedius* identification

Of the 200 *S. pseudintermedius* isolates collected from cats and dogs with dermatitis, 27 (13.5%) were characterized as MRSP after growth on chromID MRSA agar plates. The *mecA* gene presence was confirmed by PCR for all MRSP isolates. Most of the identified MRSP were isolated from dogs ($n = 25$).

Clonal relationship

After *Sma*I digestion of total DNA, 22 different pulsotypes were obtained for the 26 isolates (Fig. 1). DNA from one isolate could not be digested with *Sma*I. Using a cut-off of 80% similarity, only a few isolates could be grouped together, they formed four distinct clusters. The first (A),

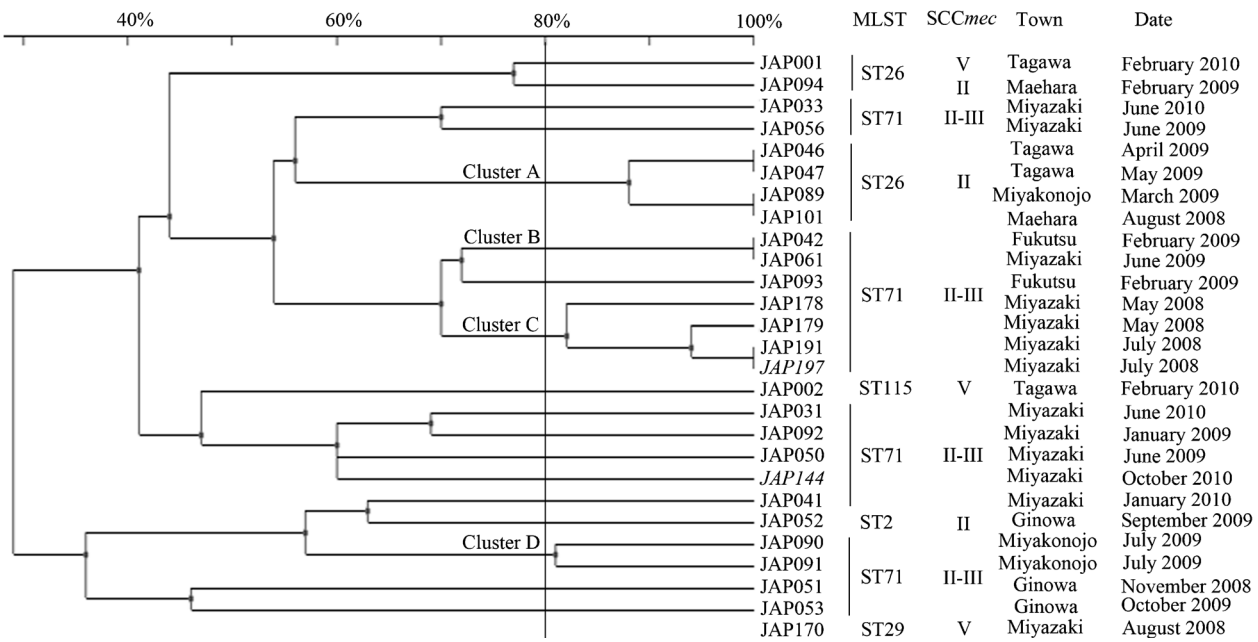

Fig. 1. PFGE dendrogram, MLST and SCC*mec*-typing results for MRSP strains. Strains in italics were isolated from cats. The dendrogram was constructed using UPGMA (Dice coefficient, 2% position tolerance). The strain JAP170 was untypeable by PFGE.

second (B), third (C), and fourth (D) clusters comprised four, two, four and two MRSP isolates, respectively. Interestingly, clusters A and B included isolates collected from different cities and at different times (Fig. 1). According to the PFGE results, the other 14 isolates were not genetically related (Fig. 1).

Multilocus sequence typing showed that the 27 isolates belonged to five different types: ST2, ST26, ST29, ST71 and ST115 (Fig. 1). Most MRSP belonged to ST71 group (18 isolates). Six MRSP isolates belonged to ST26. The three remaining isolates belonged to ST29 (MRSP isolate untypeable by PFGE), ST2 and ST115 types.

Staphylococcal chromosomal cassette *mec*-typing identified three types of SCC*mec* cassette, namely the II, II–III and V types (Fig. 1). A good correlation was observed between the MLST results and SCC*mec*-typing. Indeed, all isolates belonging to ST71 were assigned to type II-III SCC*mec* and all isolates belonging to ST26 were assigned to type II SCC*mec*, except for one isolate (type V SCC*mec*). Isolates ST115, ST29 and ST2 were assigned to types V, V and II SCC*mec*, respectively.

Virulotyping and biofilm formation

All but one isolate from a cat (JAP144) tested positive for the *siet* gene, which codes for the exfoliative toxin specific to *S. intermedius*. Conversely, none of the isolates harbored the *lukS-PV* and *lukF-PV* genes, coding for LUK–PV.

After growth in TSB_{glc}, biofilm production was estimated by spectrophotometry using safranin staining. All but two isolates produced strong biofilm in TSB_{glc}. The remaining two isolates (JAP001 and JAP178) were moderate and weak biofilm producers, respectively.

Antibiotic susceptibility profiles

All isolates were resistant to at least five of the fifteen tested antibiotics (Fig. 2): gentamicin, kanamycin, spectinomycin, erythromycin and trimethoprim-sulfamethoxazole. Moreover, all but one isolate were also resistant to penicillin, clindamycin and ciprofloxacin. By contrast, more than 40% of the isolates were sensitive to cefoperazone, cephalexin and ceftiofur. One isolate was resistant to all tested antibiotics.

DISCUSSION

Staphylococcus pseudintermedius is one of the bacterial species that causes skin infections, primarily in dogs and cats. MRSP emergence could therefore complicate the treatment of pet infections, leading to recurrent disease. Both animal-to-animal and animal-to-human transmission are potential risks that have to be considered. The purpose of this study was to isolate MRSP from cats and dogs with dermatitis in south Japan and to characterize several of their genotypic (PFGE, MLST, SCC*mec*-typing and virulotyping) and phenotypic (biofilm formation, antibiotic resistance) features.

Twenty-seven of the 200 tested isolates (13.5%) were methicillin-resistant. According to PFGE results, only the four MRSP isolates with pulsotype C were closely related and clonal. Not surprisingly, these strains were obtained

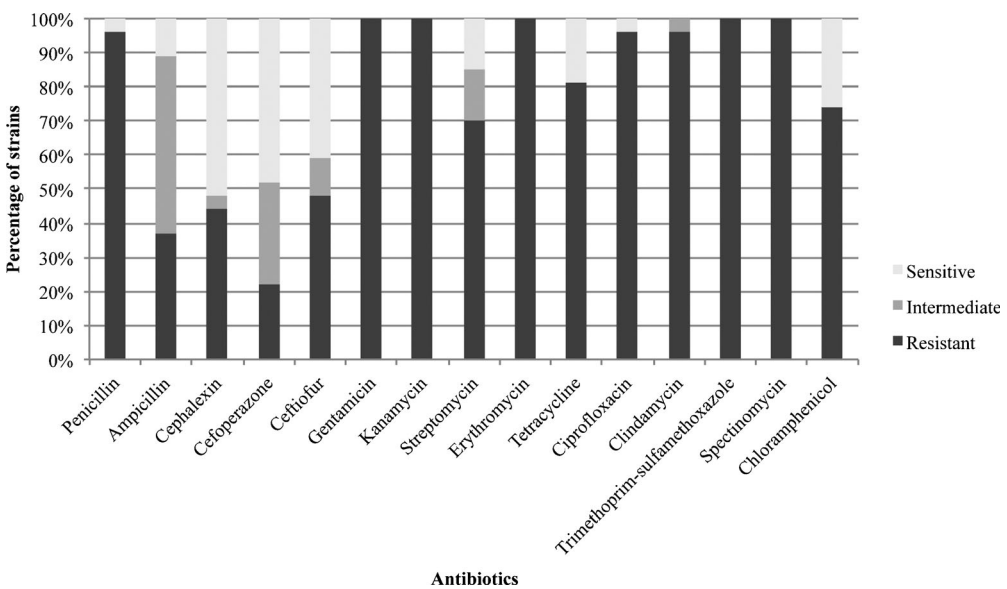

Fig. 2. Antibiotic susceptibility profiles to fifteen antibiotics.

from specimens from the same city (Miyazaki) and over the same period of time (May–July 2008). On the other hand, most other MRSP isolates did not form any homogeneous groups and were not clonal. Nevertheless, because two-third of the strains belonged to ST71 and almost one-third to ST26, the MLST results did not reflect this major clonal variation. To our knowledge, this is the first report of the ST71 lineage in Japan, supporting the contention that this clone is disseminated worldwide (17). The ST71 lineage is highly prevalent in Europe and has also been identified in China, USA and Canada (7, 18–21). Similarly to the findings of other studies (20), the ST71 lineage we isolated harbored the II-III SCC*mec*-type. Such a widespread clone presents a potential risk for pet owners. Indeed, Stegmann *et al.* recently reported one case of human infection associated with the MRSP ST71 strain (4). Contrastingly, the second more prevalent lineage in our study (ST26) is not so much widespread, having been only sporadically identified (4, 18, 20, 21). Most of our ST26 lineage isolates were associated with II SCC*mec*-type (except for one isolate that was associated with V SCC*mec*-type).

The ability of MRSP to form biofilm has rarely been studied (22). In agreement with previously published results on Norwegian MRSP, we found that MRSP are good biofilm producers (22). Moreover, Osland *et al.* showed that MRSP belonging to the ST71 lineage have the ability to stronger produce biofilm than isolates of other MLST lineages. This feature could confer advantages in persistence and spreading on strains of this lineage (22). The other main MLST lineage identified in our study (ST26) also showed a strong biofilm formation. Concerning virulotyping, all strains possessed the *siet* gene. This exfoliative toxin may also help the bacteria to be more virulent and to persist.

Concerning the antibiotic sensitivity tests, all strains but one were multiresistant (resistant to more than eight antibiotics and three antibiotic classes), 78% of multiresistant strains being resistant or intermediate to twelve or more of the fifteen tested antibiotics. On average, strains were resistant to 12 antibiotics (78% of the tested antibiotics). Our study represents one piece of supplemental evidence for the necessity to regulate and control the use of antibiotics in veterinary medicine, not only in farm but also in pet animals, as was recently concluded by the Council of the European Union (23).

In conclusion, this study provides an insight into MRSP by demonstrating that: (i) ST71 is one of the main MLST lineages in Japan; (ii) ST26 MRSP could represent an emerging MLST lineage in Japan; (iii) most MRSP in our study are strong biofilm producers and possess the exfoliative toxin gene (*siet*); and (iv) all strains are resistant to a large number of antibiotics.

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DISCLOSURE

The authors declare that they have no conflict of interest.

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