Characterization of meticillin-resistant and meticillin-susceptible isolates of *Staphylococcus pseudintermedius* from cases of canine pyoderma in Australia

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INTRODUCTION

Meticillin-resistant *Staphylococcus pseudintermedius* (MRSP) is an emerging pathogen in veterinary companion-animal practice, affecting dogs, cats and horses (Morris et al., 2010; Bannoehr & Guardabassi, 2012). Furthermore, there is evidence of MRSP transmission to owners and veterinary personnel caring for infected pets (Morris et al., 2010; Paul et al., 2011; van Duijkeren et al., 2011), leading to concerns that MRSP could adapt to become a resident commensal organism in humans, with subsequent horizontal transmission between individuals (Weese, 2012).

Meticillin-resistant *Staphylococcus pseudintermedius* (MRSP) has recently emerged as a worldwide cause of canine pyoderma. In this study, we characterized 22 *S. pseudintermedius* isolates cultured from 19 dogs with pyoderma that attended a veterinary dermatology referral clinic in Australia in 2011 and 2012. Twelve isolates were identified as MRSP by *mecA* real-time PCR and phenotypic resistance to oxacillin. In addition to *β*-lactam resistance, MRSP isolates were resistant to erythromycin (91.6%), gentamicin (83.3%), ciprofloxacin (83.3%), chloramphenicol (75%), clindamycin (66%), oxytetracycline (66%) and tetracycline (50%), as shown by disc-diffusion susceptibility testing. Meticillin-susceptible *S. pseudintermedius* isolates only showed resistance to penicillin/ampicillin (90%) and tetracycline (10%). PFGE using the *Sma*I restriction enzyme was unable to type nine of the 12 MRSP isolates. However the nine isolates provided the same PFGE pulsotype using the *Cfr*91 restriction enzyme. Application of the *mec*-associated direct repeat unit (*dru*) typing method identified the nine *Sma*I PFGE-untypable isolates as dt11cb, a *dru* type that has only previously been associated with MRSP sequence type (ST)45 isolates that possess a unique SCC*mec* element. The dt11cb isolates shared a similar multidrug-resistant antibiogram phenotype profile, whereas the other MRSP isolates, dt11a, dt11af (dt11a-associated) and dt10h, were resistant to fewer antibiotic classes and had distinct PFGE profiles. This is the first report of MRSP causing pyoderma in dogs from Australia. The rapid intercontinental emergence and spread of multidrug-resistant MRSP strains confirms the urgent need for new treatment modalities for recurrent canine pyoderma in veterinary practice.
Despite being contained within a mobile genetic element, sequence analysis of the mec-associated direct repeat unit (dru typing) has been recently described as a simple, rapid and cost-effective technique for subtyping meticillin-resistant staphylococci (Goering et al., 2008). In addition, MRSP dru typing has recently shown that MRSP ST71 and ST68 are predominantly associated with dru clusters 9a and 11a, respectively (Weese et al., 2013).

Although several studies have confirmed the presence of meticillin-resistant Staphylococcus aureus in dogs and cats (Malik et al., 2006), veterinary personnel (Jordan et al., 2011) and horses (Axon et al., 2011) in Australia, MRSP has not been described previously in Australian companion animals. However, as Australia does not have a coordinated antimicrobial-resistance surveillance programme focused on either companion-animal or livestock isolates, identification of MRSP strains, including those with a multidrug-resistant phenotype, may be under-reported.

In this study, we report the isolation of MRSP strains from dogs with pyoderma referred to a veterinary dermatology clinic in Perth, Western Australia, and their preliminary characterization.

METHODS

Bacterial strains and identification. Pyoderma samples were aseptically collected from 19 dogs of various ages, sexes and dermatologic conditions that attended the Animal Dermatology Clinic, Perth, Western Australia, between February 2011 and November 2012. The dogs were diagnosed with either superficial or deep bacterial pyoderma that had not responded to empirically selected systemic antibiotics (White, 1996). A diagnosis of pyoderma was made if the dog had consistent clinical signs including papules, pustules, crusted papules, epidermal collarettes, nodules or draining tracts, and/or cytological evidence of bacteria. Overall, 171 aseptically collected skin samples (skin biopsy, swab or fine-needle aspirate) were sent to a private diagnostic laboratory for culture and susceptibility testing.

Isolates were identified as S. pseudintermedius on the basis of exhibiting double-zoned haemolysis on sheep blood agar, growth on mannitol salt agar, a positive reaction to the tube coagulase and pyrrolidonyl arylamidase tests and a negative reaction for the production of acetoin on the Voges–Proskauer test. Identification was confirmed by Vitek mass spectrometry (matrix-assisted laser desorption/ionization time of flight).

Antibiogram phenotyping. Antimicrobial susceptibility profiles were determined by disk diffusion according to Clinical Laboratory Standards Institute criteria (CLSI, 2008, 2013). The following antimicrobials were included: penicillin (10 units), ampicillin (10 μg), amoxicillin (30 μg), oxacillin (1 μg), cephalothin (20 μg), cefotetan (30 μg), erythromycin (15 μg), clindamycin (2 μg), gentamicin (10 μg), chloramphenicol (10 μg), tetracycline (30 μg), oxytetracycline (30 μg), ciprofloxacin (5 μg), moxifloxacin (5 μg) and rifampicin (5 μg). Oxacillin MICs were determined using Etest strips (bioMérieux). Resistance scores were calculated for each isolate as the cumulative number of resistance phenotypes for the nine tested non-β-lactam antimicrobials.

Screening for mecA and PFGE. The mecA gene was detected by real-time PCR, as described previously (Costa et al., 2005). Genetic relatedness of the isolates was determined by PFGE using SmaI (Roche) and CfrI (Thermo Scientific) restriction enzymes, as described previously (Perreten et al., 2013). The pulse times were 5–40 s over 18 h and 20–25 s over 5 h. Chromosomal patterns were examined visually, scanned with a Quantity One device (Bio-Rad Laboratories) and digitally analysed using FPQuest (Applied Maths). The Dice coefficient and the unweighted pair group method with arithmetic mean were used with settings for tolerance and optimization of 1.25 and 0.5%, respectively. Isolates with 80% or greater similarity were considered to be the same pulsotype.

dru typing. Sequence analysis of the mec-associated dru region was performed on all mecA-positive isolates, as described previously (Goering et al., 2008). Cluster analysis of dru sequences was performed using the polymorphic variable number tandem repeat plug-in tool of the BioNumerics software program (version 6.6; Applied Maths). A minimum spanning tree was generated from the similarity matrix using BioNumerics with the root node assigned to the ST with the greatest number of related types. Distance intervals were created using a bin distance of 1.0%. dru types separated by a multispace ST distance of less than 1 (>98% similarity) were considered closely related and assigned to the same cluster.

RESULTS

Antimicrobial-resistance phenotypes and mecA status

Twelve Staphylococcus isolates (7% of total swabs or tissue biopsies submitted for culture and susceptibility during the study period; Table 1) harboured the mecA gene and had oxacillin MICs ranging from 1.5 to >256 μg ml⁻¹, confirming their identification as MRSP. Between August 2012 and November 2012, the first 10 S. pseudintermedius isolates showing in vitro susceptibility to two of three β-lactam antibiotics (cephalexin, amoxicillin or amoxicillin–clavulanic acid) were selected for comparison with the MRSP isolates. These 10 isolates were confirmed as meticillin-susceptible S. pseudintermedius (MSSP), based on the oxacillin MIC (0.125–0.25 μg ml⁻¹) and a negative mecA PCR result. Thus, a total of 22 S. pseudintermedius isolates (16 swabs, six skin biopsies) from 19 dogs were further characterized. Signalment, site of collection, sample type and the clinical history of each isolate are shown in Table 1.

The MRSP isolates were resistant to erythromycin (91.7%), gentamicin (83.3%), ciprofloxacin (83.3%), chloramphenicol (75%), clindamycin (66.7%), oxytetracycline (66.7%) and tetracycline (50%) (Table 2). By contrast, the MSSP isolates were primarily resistant to only penicillin and ampicillin (both 90%), with only one isolate resistant to tetracycline.
<table>
<thead>
<tr>
<th>Isolate</th>
<th>Date of isolation</th>
<th>Age</th>
<th>Sex</th>
<th>Breed</th>
<th>Site of collection</th>
<th>Collection technique</th>
<th>Underlying primary disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP1</td>
<td>17 February 2011</td>
<td>3 years, 4 months</td>
<td>MN</td>
<td>Mastiff cross</td>
<td>Trunk</td>
<td>Swab</td>
<td>Atopic dermatitis</td>
</tr>
<tr>
<td>SP2*</td>
<td>22 February 2011</td>
<td>14 years, 5 months</td>
<td>FS</td>
<td>Shar pei cross</td>
<td>Foot</td>
<td>Tissue biopsy</td>
<td>Atopic dermatitis, fibroadnexal dysplasia</td>
</tr>
<tr>
<td>SP3</td>
<td>3 August 2011</td>
<td>7 years, 11 months</td>
<td>MN</td>
<td>Cavalier King Charles spaniel</td>
<td>Foot</td>
<td>Swab</td>
<td>Atopic dermatitis</td>
</tr>
<tr>
<td>SP4</td>
<td>3 August 2011</td>
<td>10 years, 1 month</td>
<td>MN</td>
<td>Shar pei cross</td>
<td>Trunk</td>
<td>Swab</td>
<td>Atopic dermatitis, adverse food reaction</td>
</tr>
<tr>
<td>SP5</td>
<td>25 August 2011</td>
<td>10 years, 3 months</td>
<td>MN</td>
<td>Miniature dachshund</td>
<td>Foot</td>
<td>Swab</td>
<td>Atopic dermatitis, adverse food reactions</td>
</tr>
<tr>
<td>SP6‡</td>
<td>27 August 2011</td>
<td>8 months</td>
<td>MN</td>
<td>British bulldog</td>
<td>Trunk</td>
<td>Swab</td>
<td>Pemphigus foliaceus</td>
</tr>
<tr>
<td>SP7‡</td>
<td>17 October 2012</td>
<td>6 months</td>
<td>MN</td>
<td>British bulldog</td>
<td>Right external ear canal</td>
<td>Tissue biopsy</td>
<td>Atopic dermatitis, polycystic ovaries</td>
</tr>
<tr>
<td>SP8‡</td>
<td>17 October 2012</td>
<td>11 months</td>
<td>FS</td>
<td>Akita</td>
<td>Trunk</td>
<td>Swab</td>
<td>Atopic dermatitis, polycystic ovaries</td>
</tr>
<tr>
<td>SP9‡</td>
<td>17 October 2012</td>
<td>11 years, 6 months</td>
<td>FS</td>
<td>Great dane</td>
<td>Trunk</td>
<td>Swab</td>
<td>Adverse food reaction, cutaneous papillomatosis</td>
</tr>
<tr>
<td>SP10</td>
<td>3 October 2012</td>
<td>8 months</td>
<td>MN</td>
<td>Bull terrier</td>
<td>Trunk</td>
<td>Tissue biopsy</td>
<td>Atopic dermatitis, cutaneous mast-cell tumour</td>
</tr>
<tr>
<td>SP11</td>
<td>11 October 2012</td>
<td>2 years, 3 months</td>
<td>MN</td>
<td>Labrador retriever</td>
<td>Trunk</td>
<td>Swab</td>
<td>Atopic dermatitis, cutaneous mast-cell tumour</td>
</tr>
<tr>
<td>SP12</td>
<td>20 November 2012</td>
<td>9 months</td>
<td>FE</td>
<td>Labrador retriever</td>
<td>Trunk</td>
<td>Swab</td>
<td>Atopic dermatitis, cutaneous mast-cell tumour</td>
</tr>
<tr>
<td>SP13</td>
<td>24 August 2012</td>
<td>7 years, 1 month</td>
<td>MN</td>
<td>Labrador retriever</td>
<td>Trunk</td>
<td>Tissue biopsy</td>
<td>Atopic dermatitis, cutaneous mast-cell tumour</td>
</tr>
<tr>
<td>SP14</td>
<td>12 October 2012</td>
<td>6 years</td>
<td>FS</td>
<td>Shih tzu cross</td>
<td>Trunk</td>
<td>Tissue biopsy</td>
<td>Pemphigus foliaceus</td>
</tr>
<tr>
<td>SP15</td>
<td>16 October 2012</td>
<td>4 year, 1 month</td>
<td>MN</td>
<td>Cavalier King Charles spaniel</td>
<td>Right external ear canal</td>
<td>Tissue biopsy</td>
<td>Atopic dermatitis</td>
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<tr>
<td>SP16</td>
<td>1 November 2012</td>
<td>14 years</td>
<td>MN</td>
<td>Maltese</td>
<td>Trunk</td>
<td>Swab</td>
<td>Atopic dermatitis, cutaneous mast-cell tumour</td>
</tr>
<tr>
<td>SP17</td>
<td>1 November 2012</td>
<td>7 years</td>
<td>MN</td>
<td>Labrador retriever</td>
<td>Right external ear canal</td>
<td>Swab</td>
<td>Aural inflammatory polyp</td>
</tr>
<tr>
<td>SP18</td>
<td>1 November 2012</td>
<td>12 years, 5 months</td>
<td>MN</td>
<td>Maltese</td>
<td>Trunk</td>
<td>Tissue biopsy</td>
<td>Atopic dermatitis, adverse food reactions, cutaneous squamous-cell carcinoma</td>
</tr>
<tr>
<td>SP19</td>
<td>2 November 2012</td>
<td>9 years, 9 months</td>
<td>FS</td>
<td>Jack Russell terrier</td>
<td>Trunk</td>
<td>Tissue biopsy</td>
<td>Atopic dermatitis, adverse food reaction</td>
</tr>
<tr>
<td>SP20</td>
<td>2 November 2012</td>
<td>1 year, 3 months</td>
<td>FS</td>
<td>Fox terrier</td>
<td>Trunk</td>
<td>Swab</td>
<td>Atopic dermatitis</td>
</tr>
<tr>
<td>SP21</td>
<td>8 November 2012</td>
<td>11 year, 4 months</td>
<td>ME</td>
<td>Labrador retriever</td>
<td>Foot</td>
<td>Swab</td>
<td>Atopic dermatitis</td>
</tr>
<tr>
<td>SP22*</td>
<td>28 November 2012</td>
<td>15 years, 4 months</td>
<td>FS</td>
<td>Shar pei cross</td>
<td>Trunk</td>
<td>Swab</td>
<td>Atopic dermatitis, fibroadnexal dysplasia</td>
</tr>
</tbody>
</table>

FE, Female entire; FS, female spayed; ME, male entire; MN, male neutered.
*Samples were taken from the same animal, 21 months apart.
†Samples were taken from different sites in the same animal.
‡Samples were taken from the same animal, 4 months apart.
Resistance scores ranged from 1 to 7 (mean 5.2, median 6) for the MRSP isolates and were 0 or 1 for the MSSP isolates.

**Molecular characterization**

Using the *Sma*I restriction enzyme, all 10 of the MSSP and three of the 12 MRSP isolates could be classified into 11 PFGE pulsotypes. MRSP isolate SP11 and MSSP isolate SP19 were assigned to the same pulsotype (pulsotype D). The nine MRSP isolates that could not be typed using *Sma*I had the same PFGE pulsotype (pulsotype K) using the *Cfr*91 restriction enzyme.

All 12 MRSP isolates were typable by *dru* typing (Table 2). Four different *dru* types were identified. The nine pulstype K isolates belonged to *dru* type dt11cb. Single isolates of *dru* types dt11af (dt11a-associated), dt11a and dt10h were also identified. Apart from dt10h, all of the *dru* types had been grouped into the 11a *dru* cluster in previous studies (Table 2). The dt11cb isolates were resistant to four to seven non-β-lactam antimicrobials (mean 6.2; median 6), whereas the three remaining MRSP isolates were resistant to one to four non-β-lactam antimicrobials (Table 2).

**DISCUSSION**

We report the first isolation of MRSP from dogs with recurrent pyoderma in Australia, with the first isolates obtained in February 2011. While it is entirely possible that MRSP isolates were present in Australia prior to this date, they were probably not recognized as, at the time, veterinary diagnostic laboratories were not routinely screening *Staphylococcus* isolates for oxacillin resistance. As reported by other groups (Sasaki et al., 2007; Ruscher et al., 2009; Perreten et al., 2010), the majority of MRSP isolates in this study were found to be resistant to multiple antibiotic classes.

In the current study, MRSP isolates were definitively identified using a combination of *in vitro* resistance to oxacillin and detection of the *mecA* gene by real-time PCR.
Using Smal, only three of the MRSP isolates were typable by PFGE, with each belonging to a distinct pulsortype. The nine MRSP isolates not typable by Smal belonged to one unique Cfr91 pulsortype. In contrast, the 10 MSSP isolates belonged to nine different pulsortypes, indicating high heterogeneity. MRSP isolates that could not be resolved by Smal PFGE were first reported in the Netherlands, where they were associated with ST29. These isolates could be typed by PFGE using Cfr91 (Laarhoven et al., 2011). Most recently, a high proportion of atypical MRSP isolates were obtained from dogs and cats in Israel and Thailand, with the majority belonging to ST45 and shown to contain a novel SCCmec (‘YSmec50795’) (Perreten et al., 2013). While the ST45 isolates from Israel were highly clonal and belonged to dru type 11c, the 17 ST45 isolates from Thailand were more diverse and could be further subdivided into four Cfr91 pulsortypes and five dru types. Four isolates were identified as dt11cb. It is possible, therefore, that the nine Australian Smal non-typable MRSP isolates obtained in our study are most closely related to the MRSP ST45 isolates from Thailand. However, a direct comparison using multilocus sequence typing will be required to confirm this hypothesis.

In the present study, dru typing showed that 11 of the 12 MRSP isolates belonged to dru cluster 11a. Although the 11a dru cluster has been previously reported to be associated with the internationally disseminated ST68 clonal lineage (Weese et al., 2013), the recent findings of Perreten et al. (2013) confirm that it is not exclusively associated with this ST. The nine Smal non-typable MRSP isolates that belonged to dru type dt11cb were highly resistant, whereas the three remaining MRSP cases with different dru and PFGE types had lower resistance scores.

The last isolate was typed as dt11cb. It is possible, therefore, that the nine Australian Smal non-typable MRSP isolates obtained in the current study are most closely related to the MRSP ST45 isolates from Thailand. However, a direct comparison using multilocus sequence typing will be required to confirm this hypothesis.

Nosocomial transmission might explain the cluster of five MRSP infections caused by dt11cb strains that occurred in four dogs within a 22-day period in 2011. However, bacterial cultures from samples from other dogs seen during this period by the dermatology clinic, as well as by other departments within the veterinary hospital, yielded only MSSP isolates. In addition, isolates SP6 and SP7 (obtained at the same time from different sites) were collected from a dog that presented for the first time to the clinic from a region about 1538 km from Perth. While the clinic adopts the practice guidelines recommended by the British Small Animal Veterinary Association (2011) for infection control, nosocomial transmission cannot be completely ruled out. However, the cluster of cases might just be a temporal association, given that some of the dogs originated from distinct geographical locations in Western Australia.

Current systemic antibiotics reported to be effective against MRSP are chloramphenicol, amikacin and rifampicin (which should always be given in combination with another class). Each of these antibiotics is frequently associated with undesirable adverse events, potential toxicity and/or expense (Frank & Loeffler, 2012; Papich, 2012). The dt11cb isolates recovered in the current study were resistant to chloramphenicol, thus further limiting treatment options for these cases. All MRSP and MSSP infections in this study resolved after treatment with topical antimicrobials (e.g. 3% chlorhexidine, 2% mupirocin, 2% fusidic acid), with or without concurrent systemic antibiotics (e.g. rifampicin, chloramphenicol) selected on the basis of in vitro antimicrobial susceptibilities. To avoid relapses, the underlying diseases were also managed appropriately. Given the in vitro susceptibility of the isolates to moxifloxacin and resistance to ciprofloxacin, combined therapy with dual-targeting fluoroquinolones such as pradofloxacin (Wetzstein & Hallenbach, 2011) or moxifloxacin and other antimicrobial classes to which MRSP isolates are susceptible could be an appropriate systemic approach for deep pyoderma caused by MRSP, to prevent the rapid emergence of resistance in either class.

Meticillin resistance is conferred by the meca gene, which encodes a modified penicillin-binding protein (PBP2a) with low affinity for all β-lactam antibiotics (penicillins, cephalosporins and carbapenems), rendering them ineffective despite apparent in vitro susceptibility to some β-lactams. It is therefore important that veterinary diagnostic laboratories include screening for oxacillin resistance in their routine susceptibility testing for coagulase-positive Staphylococcus.

In conclusion, we report the isolation of MRSP from dogs with chronic recurrent pyoderma referred to a specialist dermatology practice in Perth, Western Australia. The first nine MRSP isolates, which appeared to be clonally related on the basis of Cfr91 PFGE and dru typing, possessed a multidrug-resistant (resistant to more than three antibiotic classes) phenotype. Similar MRSP isolates with the same dru type and a novel SCCmec element have recently been reported in another country within the Asia-Pacific region. The spread of dt11cb PFGE pulsortype K MRSP isolates in the rest of Australia remains to be determined.

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