Veterinary Microbiology 213 (2018) 58–65

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Clonal diversity and geographic distribution of methicillin-resistant *Staphylococcus pseudintermedius* from Australian animals: Discovery of novel sequence types

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ARTICLE INFO

Keywords:
Antimicrobial resistance
Staphylococcal infections
MRSP

ABSTRACT

Methicillin-resistant *Staphylococcus pseudintermedius* (MRSP) is an increasingly prevalent pathogen in veterinary medicine. This study examined the molecular epidemiology of clinical MRSP isolated from Australian animals. Clinical staphylococci submitted to all Australian veterinary diagnostic laboratories were collected during 2013 and identified using traditional phenotypic tests and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). Phenotypic antimicrobial resistance was determined using broth microdilution and disk diffusion. MRSP isolates were characterized by whole genome sequencing which included identification of the *mecA* gene. Phylogenetic relationships were inferred by comparison of single nucleotide polymorphisms. Of the 669 *S. pseudintermedius* isolates collected from dogs, cats and cattle, 77 (11.5%) were MRSP. Nineteen multilocus sequence types (STs) were identified, with most isolates belonging to one of five STs (ST71, ST497, ST316, ST496 and ST45). Phylogenetic analysis revealed that Australian ST71 appears closely related to ST71 from overseas. ST497 and ST496 represented novel sequence types, not previously reported outside Australia. Most other STs were novel and only distantly related to each other. Geographical clustering of STs was observed. All isolates belonging to the five main STs were multi- to extensively- drug resistant while isolates from singleton STs generally had lower levels of antimicrobial resistance. The frequency of ciprofloxacin, trimethoprim-sulfamethoxazole, gentamicin, chloramphenicol and tetracycline resistance varied significantly between STs ($p < 0.01$). Australian MRSP isolates are phylogenetically diverse, with a mix of previously unreported and well known international MRSP clones that demonstrate geographic clustering and exhibit both multidrug-resistant and extensively drug-resistant phenotypes.

1. Introduction

Staphylococcus pseudintermedius is a major veterinary pathogen, most commonly associated with dogs. Analogous to *Staphylococcus aureus* in humans, *S. pseudintermedius* in dogs can range from being a commensal (Cox et al., 1988) to causing a wide range of infections (Weese and Duijkeren, 2010). Methicillin-resistance in *S. pseudintermedius* (Gortel et al., 1999) is a more recent phenomenon than in *S. aureus* (Jevons et al., 1963) but its frequency has increased remarkably, ranging from 8.7% to 28% of *S. pseudintermedius* infections in dogs (Bardiau et al., 2013; Couto et al., 2016; Haenni et al., 2014; Kadlec et al., 2016; Lucia et al., 2011) and up to 60% of canine *S. pseudintermedius* pyoderma cases (Kawakami et al., 2010). Many methicillin-resistant *S. pseudintermedius* (MRSP) are also multidrug-resistant (dos Santos et al., 2016), leaving veterinary clinicians with few treatment options. MRSP can be carried by pet owners (Duijkeren et al., 2011) and veterinarians (Paul et al., 2011) and is an occasional zoonotic pathogen (Starlander et al., 2014). MRSP appears able to acquire and maintain multidrug resistance (McCarthy et al., 2015) (resistant to three or more antimicrobial categories) and extensive drug resistance

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https://doi.org/10.1016/j.vetmic.2017.11.018
Received 15 August 2017; Received in revised form 27 September 2017; Accepted 17 November 2017

(susceptibility to two or less antimicrobial categories) (Magiorakos et al., 2012). Its propensity for horizontal transfer of genetic resistance determinants makes it a potential threat to public health (dos Santos et al., 2016; McCarthy et al., 2015). Molecular typing has identified several dominant MRSP lineages around the world, including ST71 (Bardiau et al., 2013; Couto et al., 2016; Damborg et al., 2016; Haenni et al., 2014; Perreten et al., 2010), ST68 (dos Santos et al., 2016; Perreten et al., 2010) and ST45 (dos Santos et al., 2016; Perreten et al., 2013). Although early anecdotal reports of MRSP in Australia coincided with reports from other countries, MRSP was not reported in Australia in the scientific literature until 2014 (Ravens et al., 2014; Siak et al., 2014). A recent study by our research team examined 888 clinical *Staphylococcus* spp. isolates collected from Australian companion animals in 2013 and found that 11.8% of *S. pseudintermedius* submissions were MRSP (Saputra et al., 2017). However, the molecular epidemiology of Australian MRSP has not yet been examined. Consequently, our study aimed to determine the molecular epidemiology of 77 MRSP isolates collected from Australian animals in 2013 using whole genome sequencing.

2. Methods

2.1. Sample acquisition, speciation and antimicrobial susceptibility testing

S. pseudintermedius were collected in 2013 during the first Australian survey into antimicrobial resistance in veterinary clinical isolates and involved all 22 Australian veterinary diagnostic laboratories (VDLs) (Saputra et al., 2017). VDLs were located in all Australian states and mainland territories. Preliminary staphylococcal speciation was determined by traditional phenotypic tests and confirmed by the BD™ Bruker MALDI Biotyper™. Briefly, a small amount of a single colony of each pure culture was transferred to a FlexiMass™ target well, overlaid with 0.5 µL of 2,5-dihydroxybenzoic acid matrix solution and air-dehydrated within 1–2 min at 24–27 °C before being placed in the spectrometer. All isolates were run in duplicate and results with a confidence score above 1.8 were deemed acceptable for speciation. While our previous study focused on the phenotypic antimicrobial resistance profiles of 629 *S. pseudintermedius* from dogs and cats only (Saputra et al., 2017), here we screened a larger collection of 669 *S. pseudintermedius* isolates collected from companion animals and food-producing animals.

The phenotypic antimicrobial resistance profile of each isolate was determined by broth microdilution and disk diffusion according to the Clinical Laboratory Standards Institute guidelines (CLSI, 2013). As previously described (Saputra et al., 2017), when CLSI guidelines were not available, MIC data was interpreted based on European Committee on Antimicrobial Susceptibility Testing guidelines and/or using ECOFFinder. Isolates were tested against 18 antimicrobials from 9 drug categories. Rather than using antimicrobial 'classes' to classify organisms as multi-, extensive- or pan-drug resistant, we used the drug 'category' system previously described (Magiorakos et al., 2012). This method of classification groups antimicrobials into broader 'categories' than the traditional system of antimicrobial 'classes'. Magiorakos et al. (2012) argued that the use of categories is more valid for epidemiological studies than the class system, as pathogens such as MRSA and MRSP are automatically multidrug-resistant when defined by the class system, due to their resistance to almost all β-lactam classes. The following antimicrobial categories (Magiorakos et al., 2012) were tested, with the drugs from each category in parentheses: anti-staphylococcal β-lactams (oxacillin, cefoxitin, amoxicillin-clavulanic acid, cephalothin, cefovecin, ceftriaxone); aminoglycosides (gentamicin, amikacin); ansamycins (rifampicin); fluoroquinolones (enrofloxacin, ciprofloxacin, marbofloxacin, pradofloxacin), folate pathway inhibitors (trimethoprim-sulfamethoxazole); lincosamides (clindamycin); macrolides (erythromycin); phenicols (chloramphenicol) and tetracyclines (tetracycline). Most antimicrobials were tested by both disk diffusion and broth microdilution. However, for the macrolides, erythromycin (ERY) was tested by disk diffusion only. For the aminoglycosides, gentamicin (GEN) was tested by disk diffusion and amikacin (AMK) was tested by broth microdilution. Antimicrobials were obtained from Sigma Aldrich (Australia) and Zoetis (Australia). *S. aureus* ATCC 25923 and ATCC 29213 were used as quality control strains. If a discrepancy between broth microdilution and disk diffusion occurred, assignment was made based on the presence or absence of the relevant resistance genes. As genetic resistance markers were not used for rifampicin, the microdilution result was used to determine the resistance status. Isolates defined as 'intermediate' according to CLSI guidelines were defined as resistant in this study. An isolate was classified as methicillin-resistant if phenotypically oxacillin resistant (oxacillin MIC $\geq$0.5 mg/L and/or disk diffusion diameter $\leq$17 mm) and harbored the *mecA* or *mecC* gene as determined by whole genome sequencing.

2.2. Whole genome sequencing, in silico typing and resistance gene screening

Whole genome sequencing was performed on all phenotypically methicillin-resistant *S. pseudintermedius* using the MiSeq System (Illumina), as previously described (Worthing et al., 2017). De novo assembly, molecular typing and resistance gene detection were performed using the bioinformatics program, CLC Genomics Workbench (CLCbio, Qiagen, USA). For multilocus sequence typing, a local BLAST database was created by downloading the alleles listed in the multilocus sequence type (MLST) databases for *S. pseudintermedius,* (http://pubmlst.org/spseudintermedius/) (Solyman et al., 2013). BLAST analysis was undertaken using downloaded MLST alleles against *de novo* assembled contigs. Isolates were assigned a sequence type (ST) by entering the allelic profiles into the MLST website. New STs of MRSP were submitted to the MLST database curator (vincent.perreten@vetsuisse.vbi.unibe.ch).

spa typing of MRSP isolates was performed using the *spa* repeat codes described for *S. pseudintermedius* (Moodley et al., 2009) and from the *S. pseudintermedius spa* typing website, http://www.pse-spa.org/. *dru* typing was performed using the *dru* repeats listed on the *dru* typing website, http://dru-typing.org/site/ (Goering et al., 2008). As contigs from *de novo* assembly tend to cleave at repeat regions, 250 bp fastq raw read files were analysed. Fastq files were searched for *spa* repeat codes and neighboring regions were searched for subsequent *spa* repeats. *spa* types were assigned according to *spa* repeat sequence profiles described by Moodley and colleagues (Moodley et al., 2009). *dru* typing was similarly performed by searching for *dru* repeats in fastq sequences. *dru* types were assigned by entering the *dru* repeat succession into the *dru* typing server. The nucleotide sequences of antimicrobial resistance genes were downloaded from the open-access bioinformatics website, ResFinder (https://cge.cbs.dtu.dk//services/ResFinder/). A local BLAST database was created and the sequences were blasted against *de novo* contigs. Isolates required 90% homology with the reference nucleotide were deemed to be positive for that gene. For fluoroquinolone resistance, isolates were screened for mutations in the topoisomerase II (*gyrA*) and IV genes (*grlA*). Screening was performed by downloading *gyraA* and *grlA* sequences (Accession numbers: AM262968 and AM262971 respectively), creating an alignment in CLC Genomics Workbench and screening for nucleotide polymorphisms associated with fluoroquinolone resistance (Descloux et al., 2008).

2.3. Phylogenetic analysis of MRSP isolates

A phylogenetic tree was generated to infer evolutionary history, by uploading Illumina paired-end sequencing reads to the Nullarbor bioinformatic pipeline software (https://github.com/tseemann/nullarbor). Single nucleotide polymorphisms (SNPs) in the core genome were identified by comparison with a reference genome (ST71 MRSP 081661, Accession numbers: CP16073.1). SNPs in recombination