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Short Communication

Frequency, antimicrobial susceptibility and clonal distribution of methicillin-resistant *Staphylococcus pseudintermedius* in canine clinical samples submitted to a veterinary diagnostic laboratory in Italy: A 3-year retrospective investigation

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ABSTRACT

In the last decade there has been a rapid global spread of methicillin-resistant *Staphylococcus pseudintermedius* (MRSP) clones displaying multidrug resistance in dogs. We investigated prevalence, antimicrobial susceptibility and clonal distribution of MRSP isolated from clinical canine samples between during 2011–2014. Following species identification by *nuc* PCR, MRSP were confirmed by the presence of *mec*A and characterized by antimicrobial susceptibility testing, Pulsed Field Gel Electrophoresis (PFGE), *SCCmec* typing, and Multi-Locus Sequence Typing (MLST) of a few isolates having distinct PFGE profiles. Both the MRSP isolation frequency in the 175 samples tested (12%) and the prevalence of methicillin resistance amongst the 63 *S. pseudintermedius* isolates (33%) were high compared to a previous study in Italy. Sequence type (ST)71 carrying SCC*mec* type II–III, described as the epidemic European MRSP clone, accounted for approximately half of the isolates. The remaining isolates belonged to ST410-SCC*mec* type II–III, ST258-SCC*mec* type IV and other three clones associated with SCC*mec* type IV (ST261, ST290 and ST477). MRSP were consistently resistant to potentiated sulfonamides, and more frequently to clindamycin, ciprofloxacin and doxycycline than methicillin-susceptible isolates. Gentamicin was the only antibiotic showing good *in vitro* activity on all MRSP with 20 of the 21 isolates being susceptible. Results confirm a high prevalence of MRSP amongst clinical samples in Italy, revealing the emergence of new clones other than ST71, such as ST258, ST410, ST261, ST290 and ST477, here describe for the first time. Implementation of antimicrobial stewardship and surveillance programmes are required to prevent the emergence of new MRSP clones and reducing transmission in small animal practice.

1. Introduction

Staphylococcus pseudintermedius is an opportunistic pathogen belonging to the *Staphylococcus intermedius* group (SIG), a frequent cause of canine skin and ear infections, and a sporadic zoonotic pathogen in humans (Bannoehr and Guardabassi, 2012). Methicillin-resistant *S. pseudintermedius* (MRSP) have been first reported in the US in 1999 (Gortel et al., 1999) and in Europe in 2007 (Loeffler et al., 2007). Infections caused by MRSP are difficult to manage due to their characteristic multidrug resistance profiles (Frank and Loeffler, 2012). Five successful MRSP clonal complexes (CC) with specific resistance traits and geographical distribution have spread globally, with CC71 and C-C258 being predominant in Europe (dos Santos et al., 2016). In this study, prevalence, antimicrobial susceptibility and clonal distribution of MRSP were investigated in canine diagnostic and necropsy samples submitted to a diagnostic laboratory in Italy over a period of three years.

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2. Methods, techniques

2.1. Sampling

A total of 175 clinical samples from dogs were submitted for bacteriological diagnosis to the Department of Veterinary Medicine (DVM) of the University of Bari (Italy) from December 2011 to July 2014. Patients with dermatitis, pyoderma, otitis, cystitis, skin lesions or injury to surgical wounds were included. The samples originated from skin (n = 101), ear (n = 33), urinary tract (n = 12), respiratory tract (n = 11), genital tract (n = 6), organs from dead puppies (n = 13) and other body sites (n = 26). Samples were collected from Apulia (n = 161), Piedmont (n = 13), and Sicily (n = 1). The number of males (n = 84) and females (n = 91) in the study population was similar and the average age was 4 years (range 4–13 years). All the samples were independent and no epidemiological relationships were observed. Up to two presumptive *S. pseudintermedius* colonies were selected from each sample based on colony morphology on Mannitol Salt Agar (MSA), i.e. differences in colony size and pigmentation.

2.2. Isolation and identification

Samples were cultured on Columbia blood agar, McConkey agar (McK), Mannitol salt agar (MSA) and Tryptic Soy Broth (TSB) (Liofilchem, Teramo, Italy) and incubated at 37 °C overnight. Initial bacterial species identification were performed by analytical profile index (API®) system (Biomérieux, Marcy-l'Étoile, France). DNA was extracted from presumptive *S. pseudintermedius* mannitol negative colonies growing on MSA using the QIAampcador Pathogen Mini Kit (QIAGEN, Hilden, Germany). *S. pseudintermedius* was identified by *nuc* PCR (Sasaki et al., 2010) and methicillin resistance confirmed by the presence of *mec*A (Murakami et al., 1991).

2.3. Antimicrobial susceptibility testing

All *S. pseudintermedius* confirmed by *nuc* PCR were tested by disc diffusion to evaluate their antimicrobial susceptibility to the following antibiotics (disc concentration in brackets): ciprofloxacin (CIP, 5 μg), clindamycin (CLI, 2 μg), doxycycline (DOX, 30 μg), gentamicin (GEN, 10 μg), co-trimoxazole (SXT, 23.75 μg of sulfamethoxazole + 1.25 μg of trimethoprim). Methicillin-susceptible (MSSP) isolates were additionally tested with amoxicillin + clavulanic acid (AMX, 20 μg + 10 μg), cephalexin (LEX, 30 μg), cefuroxime (CXM, 30 μg), and ceftazidime (CAZ, 30 μg), (Liofilchem, Teramo, Italy). Clinical and Laboratory Standards Institute (CLSI) breakpoints (CLSI, 2017) for bacteria associated with infections in humans were used for the interpretation of disk diffusion results for cefuroxime and ceftazidime, whereas veterinary CLSI breakpoints (CLSI, 2013) were used for the interpretation of the remaining antimicrobials. As stated in the VET05-R document (CLSI, 2011), isolates were considered multidrug resistant if they exhibited resistance to three or more different classes of antimicrobial agents.

Statistical analysis was performed using the Chi-square Test with Yates correction to compare MRSP and MSSP antibiotic resistance profile, considering a p value < 0.05 as statistically significant.

2.4. Molecular characterization of MRSP

MRSP were typed by pulsed field gel electrophoresis (PFGE) according to Harmony protocol (Murchan et al., 2003) with few modifications (Paul et al., 2012). Gel images were analyzed by Gel Compar II (Applied Maths, Belgium) and the cluster analysis was performed by UPGMA using a similarity coefficient with optimization set at 0.5% and position tolerance at 1.5%. Strains were considered identical or closely related when the PFGE band patterns were 93% similar. From each PFGE cluster, one strain was selected and further analyzed by multilocus sequence typing (MLST) (Solyman et al., 2013). Alleles and STs were identified using the scheme available on PubMLST.org and new STs were assigned by the curator of the MLST database. eBURST analysis on the entire *S. pseudintermedius* MLST database was used to determine the clonal relationships between STs described in this study (Feil et al., 2004). SCC*mec* types (I–VI) were identified by multiplex PCR (Kondo et al., 2007) and the occurrence of SCC*mec* type II–III was determined based on absence of the cadmium resistance operon (Perreten et al., 2010).

3. Results

During the three-year period, 151/175 clinical canine samples were culture positive, from which 187 bacterial isolates were identified. The remaining samples (24/175) were considered negative, based on the presence of microorganisms belonging to *Bacillus* spp. The most common bacterial species was *S. pseudintermedius* (n = 63), followed by *S. aureus* (n = 9), other *Staphylococcus* spp. (n = 52), *Proteus* spp. (n = 20), *Streptococcus* spp. (n = 19), *Pseudomonas aeruginosa* (n = 12), *Escherichia coli* (n = 10), *Bordetella bronchiseptica* (n = 1), and *Serratia liquefaciens* (n = 1). Most *S. pseudintermedius* isolates originated from skin infections (73%), and 21/63 isolates were additionally methicillin resistant (33%). The overall MRSP isolation frequency amongst the samples tested was 12% (21/175). Two samples, originating from pyoderma and skin lesion, respectively, were positive for both MSSP and MRSP. The statistical analysis revealed that MRSP were significantly more resistant to non β-lactam antibiotics than MSSP. In particular, the difference was significant for SXT ($p = 0.001$), CLI and CIP ($p < 0.001$), and for DOX ($p = 0.047$) (Fig. 1). All MRSP isolates were STX-resistant, and all except one were susceptible to GEN. More variation was observed in susceptibility to CLI (2/21), DOX (10/21) and CIP (7/21) (Table 1). Amongst MSSP, 29% of the isolates were susceptible to all five antibiotics tested. In general, a low prevalence (9.5%) of resistance was detected for at least two antibiotics except SXT. The prevalence of multidrug resistance among MRSP (76%) was higher than MSSP (9.5%) ($p < 0.001$).

Nine PFGE clusters were observed amongst the 21 MRSP. Combined PFGE and MLST analysis revealed a dominance of ST71, which accounted for 48% the isolates (10/21). The remaining isolates were assigned to ST410 (n = 4), which is a single locus variant (SLV) of ST71, ST258 (n = 3) and the related SLV ST261 (n = 2), and double locus variant (DLV), ST290 (n = 1). One strain exhibited a new allelic profile ST477, which was unrelated to ST71 and ST258 (Fig. 2). SCC*mec* typing revealed the presence of two SCC*mec* types: 67% of the isolates harboured SCC*mec* type II–III (14/21), confirmed by the absence of the cadmium resistance operon, and the remaining 33% of isolates were found to carry SCC*mec* type IV (7/21). Isolates assigned to ST71 and ST410 carried cassette type II–III, whereas ST258 and other STs were

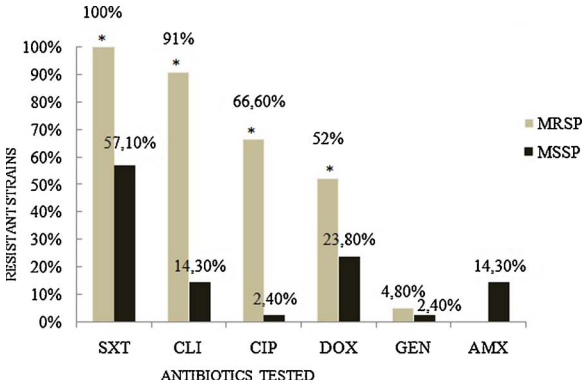

Fig. 1. Percentages of MSSP and MRSP isolates resistant to different antimicrobial drugs. MRSP isolates were significantly more resistant to SXT, CLI, CIP and DOX than MSSP. Susceptibility to AMX was only tested amongst MSSP isolates.