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Antibiotic resistance profiles of *Staphylococcus pseudintermedius* isolated from dogs and cats

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ABSTRACT

Aims: Antibiotic resistance in *Staphylococcus pseudintermedius* is increasing gradually towards those antibiotics that are frequently used leading to limited therapeutic options due to multidrug resistance. The objectives of the study were to investigate the antibiotic resistance profiles of *S. pseudintermedius* isolates from pet and stray dogs and cats in Selangor, Malaysia and to detect the resistance genes (*mecA* and *BlaZ*) within the isolates.
Methodology and results: A total of 200 stray and pet dogs and cats were sampled. The samples were cultured onto Mannitol Salt agar and all the presumptive colonies were subcultured, then identified using biochemical tests and confirmed by PCR assay targeting the *nuc* gene. The isolates were subjected to antibiotic susceptibility test against 12 antibiotics. Twenty three isolates (11.5%) were positive to *S. pseudintermedius* (stray cats, 11/50; stray dogs, 9/50; pet dogs, 3/50 and pet cats, 0/50). One hundred percent (100%) of the *S. pseudintermedius* isolates were found to be resistant to penicillin, erythromycin and tetracycline while they showed 100% susceptible to oxacillin, amoxicillin-clavulanic acid, gentamicin, chloramphenicol, vancomycin, ciprofloxacin, enrofloxacin, cephalexin and rifampicin. The *blaZ* gene which codes for β-lactamases production was found in all of the isolates that were resistant to penicillin but not to methicillin.
Conclusion, significance and impact of study: A high number of *S. pseudintermedius* from dogs and cats developed antibiotic resistance which is a public health concern**.**

Keywords: Antibiotic resistance, *blaZ, mecA*, polymerase chain reaction, *Staphylococcus pseudintermedius*

INTRODUCTION

Dogs and cats have become an integral part of modern society in the developed world and attention is given to their care and welfare. At some stage of their lives, many cats and dogs may suffer from skin and other superficial staphylococcal infections such as pyoderma, otitis externa and other infections which require treatment with antibiotics. Such infections caused by staphylococci in dogs and cats are usually treated with a wide range of antibiotics. Antibiotics used in cat and dog therapy include penicillin, cephalosporin, macrolides, lincosamides, fusidic acid, tetracyclines, chloramphenicol, potentiated sulphonamide, aminoglycosides and fluoroquinolones (Watson and Rosin, 2000). However, there is lack of reports on monitoring of antibiotics usage in cat and dog therapy (Heuer *et al.*, 2005). Today, there is growing evidence of the occurrence of antibiotic resistant organisms in dogs and cats, often associated with antibiotic usage (Guardabassi *et al.*, 2004; Rantala *et al.*, 2004). The development of antibiotic resistance in microbial pathogens and commensals represents a major threat to animal and public health.

Relatively few studies have addressed the possibility of direct transfer of resistant microorganisms between companion animals and humans, despite their close physical contact in home environments and the use of the same antibacterial agents in human and veterinary practices. *Staphylococcus pseudintermedius* is generally susceptible to penicillinase-stable β-lactam antibiotics in

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the past (Hogan *et al.*, 1986). However, since 2006, methicillin resistant *S. pseudintermedius* (MRSP) has emerged as a significant animal health problem in veterinary medicine (Weese and van Duijkeren, 2010) because they are found to be resistant to most of the antibiotics such as β-lactams, aminoglycosides, and macrolides resulting in limited treatment options to treat these infections.

Resistance to penicillin is caused by the production of β-lactamases which inactivate penicillin by hydrolysis of its β-lactam ring; it is also associated with penicillin-binding protein 2a (PBP2a), encoded by *mecA* (Zapun *et al.*, 2008). Another gene involved in penicillin resistance in staphylococci is *blaZ* which encodes β-lactamase (Zapun *et al.*, 2008). Awareness and monitoring of antibiotic resistance in veterinary staphylococcal isolates is of great importance as the development of resistance in animal pathogens may pose a zoonotic risk to their owners and can result in treatment failure if the owners are infected. According to Loeffler *et al.* (2007), increase in resistance to antibiotics that are important in human medicine may therefore result in limited antibiotic agents for therapeutic use. For treatment of staphylococcal infections, accurate and rapid diagnosis of antibiotic resistance is important to prevent the spread of infections. The objectives of this study were to investigate the antibiotic resistance profiles of *S. pseudintermedius* isolates from dogs and cats and to detect the resistance genes (*mecA* and *blaZ*) in the isolates.

MATERIALS AND METHODS

A total of 200 animals consisting of 100 pet and stray dogs and 100 pet and stray cats were sampled. The stray animals were from a municipality council animal pound and an animal shelter. Pet dogs and cats were from a university veterinary hospital and private clinics. For pet animals, owners were individually approached with a consent form to take samples from their pets. In each animal, samples were taken from the skin, nasal cavity, buccal cavity and rectum using individual sterile cotton swabs moistened with normal saline for each of the four sites on/in the animal. Each swab was placed in a bottle containing Tryptone Soya broth (Oxoid) as an enrichment medium and kept cool during transportation to the laboratory. All the enrichment broth containing the swabs were incubated at 37 °C overnight.

Bacterial isolation and phenotypic identification

Ten microliter (10 µL) of enriched culture was streaked onto Mannitol Salt agar plate (Oxoid) and incubated overnight at 37 °C for 24 h. The suspected colonies of *Staphylococcus* on the agar plates were examined by observing the colonial morphology and color (yellow colonies). Presumptive *Staphylococcus* colonies were sub-cultured onto Columbia Blood agar (Oxoid) using 5% horse blood and were incubated at 37 °C for 24 h. Suspected *S. pseudintermedius* isolates were identified on the basis of colony characteristics, pigment production, Gram-stained cellular appearance, and hemolysis. All suspected *S. pseudintermedius* isolates were identified using catalase, coagulase, DNase, o-nitrophenyl-beta-D-galactopyranoside (ONPG), arginine dihydrolase (ADH) tests and acetoin production tests. All the identified isolates were stored in Skim Milk broth (Oxoid) at -80 °C until used.

Genotypic confirmation of isolates

DNA extraction

Frozen isolates were thawed and each was streaked onto Columbia Blood agar (CBA) plate with 5% horse blood added and incubated for 24 h at 37 °C. DNA was extracted by using the boiling method. In brief, 1 mL of sterile distilled water was transferred to a sterile 1.5 mL Eppendorf tube and a loopful of the isolate was picked from a CBA plate and transferred into the Eppendorf tube. The mixture was vortexed for 1 min and then heated in a dry bath at 96 °C for 10 min. The mixture was cooled down for 5-10 min to room temperature and centrifuged at 13000 ×*g* for 3 min. The supernatant was used as DNA template for PCR analysis.

PCR assay procedure

The PCR assay was performed as described by Sasaki *et al.* (2010). The reaction mixture for PCR consisted of 5 µL DNA extract, 25 µL MyTaq Red Reaction Buffer (Bioline), 18 µL deionized water and 2 µL of the primers (forward and reverse) (10 µM) in a total reaction volume of 50 µL. The following PCR conditions were used: Initial denaturation at 95 °C for 120 sec, followed by 30 cycles of denaturation at 95 °C for 30 sec, annealing at 56 °C for 35 sec, extension at 72 °C for 60 sec, and a final extension at 72 °C for 120 sec. The primers used were with the following sequences: NucA1, 5'-TRGGCAGTAGGATTCGTTAA-3' and NucA2, 5'-CTTTTGTGCTYCMTTTTGG-3'. The reference strain *S. pseudintermedius* (CCUG 49543) was used as a positive control. In addition, 5 µL of sterile deionized distilled water was used as a negative control. DNA fragments were analysed by electrophoresis in 1×Tris-acetate-EDTA on a 1 % agarose gel stained with ethidium bromide.

Antibiotic susceptibility test

The antibiotic susceptibility test on the isolates were performed using the disc diffusion method as recommended by Clinical Laboratory and Standard Institute (CLSI, 2013) and commercial antibiotic discs in (Oxoid) were used. The isolates were tested against 12 antibiotics namely, oxacillin (1 µg), penicillin (10 IU), tetracycline (30 µg), ciprofloxacin (5 µg), gentamicin (10 µg), erythromycin (15 µg), enrofloxacin (5 µg), vancomycin (30 µg), rifampicin (5 µg), cephalexin (30 µg), amoxicillin-clavulanic acid (30 µg) and chloramphenicol (30 µg). A suspension of each *S. pseudintermedius* isolate was prepared and the turbidity was adjusted to the

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equivalent of a 0.5 McFarland (1.5×10^8 CFU/mL). Then using a sterile cotton swab, the bacterial suspension was spread gently in three different directions over the surface of Mueller-Hinton agar (Oxoid) plate supplemented with 5% defibrinated horse blood. The plate was left to dry for 3-5 min before antibiotic discs were placed onto the agar. To place 12 antibiotics, two Mueller-Hinton agar plates were needed for each isolate and six antibiotic discs were placed onto each inoculated plate using an automatic disc dispenser. The plates were incubated at 37 °C for 24 h, upon completion of the incubation, the diameters of inhibition zones were measured. The isolates were classified as sensitive or resistant according to breakpoints of CLSI (2013) and to estimate the susceptibility to cephalexin, cephalothin (30 mg) breakpoint was used as indicated in CLSI (2012).

mecA and blaZ genes detection and confirmation

In the initial stage, resistance to methicillin and penicillin in *S. pseudintermedius* was determined by using oxacillin (5 mg) and penicillin (6 mg) disks on Mueller-Hinton agar plates according to Coyle, (2005). The outcomes were recorded either as sensitive or resistant after 24 h incubation at 37 °C. The suspected isolates which showed resistance to methicillin and penicillin were further analyzed by multiplex PCR (mPCR) assay as described by El Zubeir *et al.* (2007) to detect the presence of *mecA* and *blaZ* genes using primers as shown in Table 1. The amplification was done using the following parameters: initial denaturation at 94 °C for 240 sec, followed by 40 cycles of denaturation at 94 °C for 30 sec, annealing at 55 °C for 30 sec, 72 °C for 60 sec and elongation at 72 °C for 300 sec. The amplified mPCR products were resolved by electrophoresis with 1% agarose gel, stained with ethidium bromide and visualized using UV-gel documentation system (BIO-RAD) (Figure 1).

Table 1: Oligonucleotide sequence used for the amplification of *mec*A and *bla*Z genes.

Primers	Sequence	Size of bp	Reference
*mecA*1	5'-AAAATCGATGGTAAAGGTTGGC-3'	532	Strommenger *et al*. (2006)
*mecA*2	5'-AGTTCTGCAGTACCGGATTTGC-3'		
*balZ*1	5'-ACTTCAACACCTGCTGCTTTC-3'	173	Martineau *et al.* (2000)
*blaZ*2	5'-TGACCACTTTTATCAGCAACC-3'		

RESULTS

Twenty three animals (11.5%) tested positive for the presence of *S. pseudintermedius.* All 23 isolates contained *nuc* gene by producing 926 bp amplicon (Figure 1) confirming the identity of isolates as *S. pseudintermedius.*

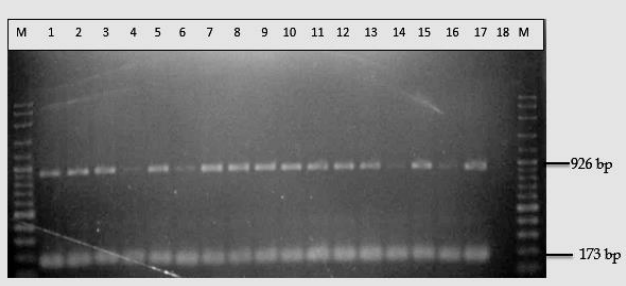

Figure 1: Electropherogram showing mPCR amplification of 926 bp fragment of thermo-stable nuclease gene (*nuc*) and 173 bp *bla*Z gene of *S. pseudintermedius.* Lane M: marker 100 bp DNA ladder (Qiagen), Lanes 1 - 16: *S. pseudintermedius* isolates, Lane 17: *S. pseudintermedius* CCUG 49543 as positive control; Lane 18: negative control.

Of these 23 *S. pseudintermedius* positive isolates, 11 (22%) were from stray cats, 9 (18%) were from stray dogs and three (6%) were from pet dogs. *S. pseudintermedius* was not isolated from any pet cats (Table 2).

Table 2: Distribution of animals positive for *S. pseudintermedius*.

Animal	Number of animals	No. (%) of animals positive to *S. pseudintermedius*
Stray dogs	50	9(18)
Stray cats	50	11(22)
Pet dogs	50	3(6)
Pet cats	50	0(0)
Total	200	23(11.5%)

All the 23 isolates (100%) from both stray and pet dogs were resistant to three antibiotics namely penicillin, erythromycin and tetracycline and two isolates (7%) from stray dogs were resistant to gentamicin (Figure 2). In stray cats, the isolates were resistant to three antibiotics namely penicillin, tetracycline and erythromycin as shown in Figure 3. Results of susceptibility of *S. pseudintermedius* against methicillin and penicillin showed that all the isolates were susceptible to oxacillin but resistant to penicillin. Upon mPCR assay to detect *mecA* and *blaZ* genes, it was found that all the isolates were positive for *blaZ*, the gene responsible for penicillin resistance (Figure 1); however, the *mecA* gene was absent. Thus, none of the isolates were methicillin resistant *S. pseudintermedius* (MRSP). However, the isolates were resistant to penicillin due to presence of β-lactamase.

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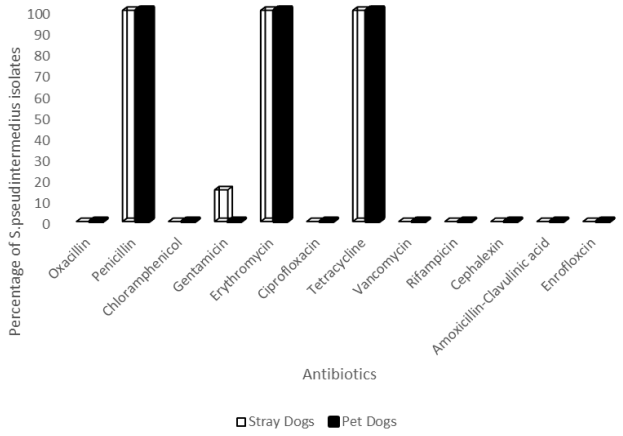

Figure 2: Antibiotic resistance profiles of *S. pseudintermedius* in stray and pet dogs.

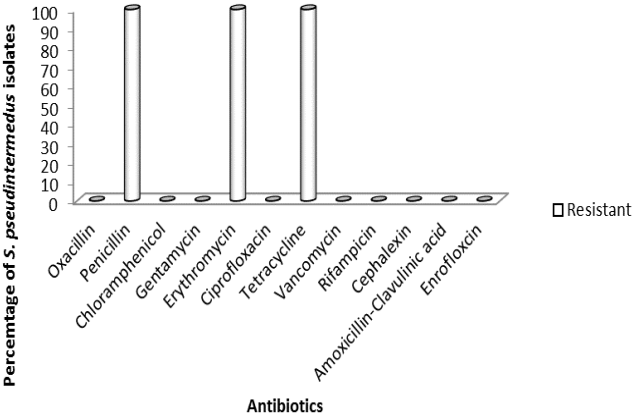

Figure 3: Antibiotic resistance profiles of *S. pseudintermedius* in stray cats.

DISCUSSION

Over the last several decades, antibiotic resistance has been considered as one of the most relevant issues in public health due to the dramatic increase of antibiotic resistance worldwide in not only pathogenic but also commensal bacteria. The relatively scarce availability in recent years of new antimicrobial drugs has also added to the problem (Frimodt-Møller *et al.*, 2004).

In this study, 100% resistance of *S. pseudintermedius* isolates was observed against three antibiotics namely, penicillin, erythromycin and tetracycline. Resistance to beta-lactamase penicillin is widespread in staphylococci of human and animal origin. Penicillin resistance is usually associated with beta-lactamase production and is very common in staphylococcal isolates among companion animals. In this study, the isolates were 100% resistant against penicillin and all penicillin-resistant isolates harbored the *blaZ* gene. This shows that penicillin may not be effective for the treatment of *S. pseudintermedius* infections in dogs and cats in Malaysia. This percentage of resistance is close to the 96% resistance rate which was reported by Won *et al.* (2010) in Korea.

The resistance of *S. pseudintermedius* to erythromycin (100%) in the study was similar to a study reported in a veterinary teaching hospital in Japan (Sasaki *et al.*, 2007). However, a study conducted in Canada showed that erythromycin resistance was only 5% among *S. pseudintermedius* isolated from dogs with canine otitis externa (Hariharan *et al.*, 2006). A low resistance to erythromycin was also reported in isolates from diseased dogs in France (28%) (Ganiere *et al.*, 2005), USA (23%) (Hartmann *et al.*, 2005) and in Denmark (28%) (Pedersen *et al.*, 2007). A 37.7% of erythromycin resistance was reported in Croatia (Matanović *et al.*, 2012).

In this study, the resistance to tetracycline was 100%, which was higher than that in Croatia (38%) (Matanović *et al.*, 2012) and in canine clinical isolates in USA (38%) (Hartmann *et al.*, 2005), Japan (48%) (Onuma *et al.*, 2011), Norway (42%) (Norström *et al.*, 2009) and France (46%) (Ganiere *et al.*, 2005) and also in diseased dogs in Denmark (24%) (Pedersen *et al*., 2007). However, in canine patients in Korea the resistance was high at 92% (Won *et al.*, 2010) with the isolates highly susceptible to gentamicin (93%) and only 7% resistant to the antibiotic. This was similar to that reported in *Staphylococcus intermedius* group (SIG) isolates in Croatia (Matanović *et al.*, 2012). Other studies also showed low resistance to gentamicin ranging from 0% to 19% among staphylococcal isolates (Ganiere *et al.*, 2005; Hartmann *et al.*, 2005; Hariharan *et al.*, 2006; Futagawa-Saito *et al.*, 2007; Norström *et al.*, 2009; Vanni *et al.*, 2009). All the isolates were 100% susceptible to chloramphenicol. This drug is not commonly used in small animals due to the narrow margin of safety in dogs and cats, and the need for frequent administration in dogs to maintain enough concentrations. However, its usage has increased in recent years to treat staphylococcal infections and chloramphenicol resistance rates were reported in different parts of the world such as Croatia (26%) (Matanović *et al.*, 2012), France (30%) (Ganiere *et al.*, 2005), Japan (27%) (Onuma *et al.*, 2011) and Norway (8%) (Norström *et al.*, 2009). The isolates were 100% sensitive to both amoxicillin-clavulanic acid and cephalexin and can be considered as drug of choice for treatment of *S. pseudintermedius* infections. Similar rates of susceptibility were reported in a study carried out in dogs with tumours (Namikawa *et al.*, 2012) and dogs with pyoderma in Japan (Kawakami *et al.*, 2010).

In this study, the isolates were 100% susceptible to enrofloxacin which is similar to that reported in dogs in Croatia at 98% (Šeol, 2005). However the isolates from dogs and cats in Croatia showed 5.7% resistance (Matanović *et al.*, 2012) and in dogs in Norway with resistance up to 8% (Norström *et al.*, 2009). Ciprofloxacin is a member of flourquinolones group having broad spectrum activity against Gram-positive, Gram-negative and mycoplasma bacteria (Hannan *et al.*, 1997; Watts *et al.*, 1997). In this study, the isolates were 100% sensitive to ciprofloxacin again similar to those in Croatia at 96% (Šeol *et al.*, 2005).

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Also, all the isolates tested were 100% susceptible to oxacillin, rifampicin and vancomycin. However, Won *et al.* (2010) reported oxacillin susceptibility rate of 74.3% in canine patients in Korea. Similarly, Fernandes *et al.* (2012) reported susceptibility rate of 96.4% against oxacillin and 93% against rifampicin in dogs with pyoderma in Brazil. However, very low resistant rate (up to 8%) was reported in a study conducted in dog populations in Norway (Norström *et al.*, 2009). Rifampicin or rifampin is used in both human and veterinary medicine because of its activity against methicillin resistant *Staphylococcus*. Rifampicin is a bactericidal antibiotic with excellent tissue penetration. It has a broad spectrum of activity against many Gram-negative and most Gram-positive micro-organisms and is the most active antibiotic known against staphylococci (Frank, 1990). Even though resistant among canine isolates has been reported (Kadlec *et al.*, 2011), it was found that rifampicin is active against most strains of MRSP (Perreten *et al.*, 2010). The differences in the prevalence of antibiotic resistance rates between this study and others may be due to the different animals studied, the geographical area and environment of sampling, investigation period, the method used in the study and the usage of the antibiotics.

The most important resistance in *Staphylococcus* is to methicillin. It was reported that there is a correlation between detection of *mecA* gene by PCR and oxacillin resistance by disk diffusion test for *S. aureus* (Loeffler *et al.*, 2007; El Zubeir *et al.*, 2007). However, according to Pak (2003) some staphylococcal isolates have been reported to be methicillin resistant even though they are *mecA* positive and oxacillin-susceptible or *mecA*-negative and oxacillin-resistant. In this present study, all the isolates were susceptible to oxacillin by disk diffusion test and mPCR did not detect *mecA*, indicating absence of MRSP. This is probably due to the infrequent usage of antibiotics or because MRSP is not prevalent in the environment. Similar results were reported by Haythem *et al.* (2013) in healthy dogs in Tunisia and Youn *et al.* (2014) in companion animals in Zambia. However, Kawakami *et al.* (2010) reported 66.5% MRSP in dogs with pyoderma in Japan while Elena *et al.* (2011) reported 4.6% MRSP in healthy dogs in Spain. Although the exact mechanism behind this observation may require further investigation, it has been proposed that in *S. aureus* hyperproduction of β-lactamase or PBPs with altered activity may be involved (Bartelt, 2000). Alternatively, variable expression of *mecA* might be possible (Griffeth *et al.*, 2008). Since PCR detection of *mecA* has been considered the gold standard for detection of methicillin resistant staphylococcal species, it is suggested that oxacillin resistance by disk diffusion should also be recommended for the determination of methicillin resistance. The *blaZ* gene is considered to be the gene responsible for resistance to β-lactam antibiotics. In this study, all isolates which were resistant to penicillin harbored the *blaZ* gene (100%). Similar result of about 98% was reported by Youn *et al.* (2011) on *Staphylococcus intermedius* group (SIG) isolates in Korea. In addition, Feng *et al.* (2012) reported 64% resistance in pets in South China. Youn *et al.* (2014) reported 52% resistance due to *blaZ* gene in companion animals and environment in Zambia and Heytham *et al.* (2013) reported 57% in healthy dogs in Tunisia.

In the absence of MRSP but with *S. pseudintermedius* resistant to penicillin, tetracycline and erythromycin, the treatment options using available antibiotics against *S. pseudintermedius* still remain adequate or acceptable. However, in order to maintain or improve this situation, a guideline on appropriate antibiotic use is needed. The study underlines the importance of prudent selection of antibiotics for small animal patients by the veterinarian, particularly when long-term treatment is required and susceptibility of staphylococci should always be monitored to choose the best antibiotic for treatment.

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AUTHOR'S CONTRIBUTION

The design and execution of this research study is a collective effort of all the authors. All authors were also involved in the analyses and the first four authors made critical review of the manuscript. There is no conflicting interest.

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