PHENOTYPIC AND MOLECULAR CHARACTERIZATION OF MULTIDRUG RESISTANT STAPHYLOCOCCUS PSEUDINTERMEDIUS FROM CANINE CLINICAL SAMPLES

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The increased use of antimicrobial therapy has resulted in the emergence of antibiotic resistance *Staphylococcus pseudintermedius* in dogs making treatments more challenging. In this study,phenotypic and genotypic analysis documented the pattern of multi drug resistance (MDR) amongst the *S. pseduintermedius* isolates from various clinical samples from dogs. It was observed that amongst the 35-coagulase positive *S. pseudintermedius* isolates, there are two methicillin resistant *S. pseudintermedius* (MRSP), one each for ESBL + AmpC, ESBL, and AmpC producers. Isolates from milk sample showed resistance to more than one class of antibiotics but sensitive to piperacillin/tazobactam and ofloxacin; uropathogenic isolates showed sensitivity to gentamicin but are resistant to all other class of antibiotics screened; and isolates from ear swab showed sensitivity only to cefotaxime, ceftriaxone, doxycycline, linezolid, or piperacillin/tazobactam. Isolates from wound/abscess showed resistance to all the antibiotics used for screening.

Keywords: AmpC, Canine, ESBL, Intimicrobial resistance, Methicillin resistance *Staphylococcuspseudintermedius,* Multidrug resistant, 16S rRNA sequence.

S*taphylococcus* are commensal bacteria which colonize and cause major opportunistic infection in humans and animals worldwide (Vengust *et al.,* 2006). Among the coagulase positive staphylococci, *Staphylococcus aureus* and *Staphylococcus pseudintermedius* are the most common isolates (Paul *et al*., 2011) and the leading cause of infections in skin, ear, catheter site, urinary tract, pneumonia and post-operative wound sepsis in dogs (van Duijkeren *et al*., 2011).

Beta-lactam antibiotics (penicillins, cephalosporins, monobactams and carbapenems) are among the most prescribed drugs worldwide (Meletis, 2016). Repeated use of these antibiotics in recurrent infections has resulted in the emergence of methicillin resistant *Staphylococcus pseudintermedius* (MRSP) with some strains developing multidrug resistance to commonly used antibiotics in veterinary practice (Glajzner *et al*., 2023). This resulted in increased challenge and even more complicated treatment due to the inherent ability of the bacteria to produce biofilms (Wang *et al*., 2022). Generally, *Enterobacteriaceae* family are the leading beta-lactamase producers, however, staphylococci are also known to have the ability to produce the enzyme amongst the gram-positive bacteria (Andrade *et al*., 2022; MRSP in particular, poses high risk to dogs and may inadvertently cause transmission between the animals and owner/Veterinary professionals causing recurrence of infection and transmission of the bacterial infection in dogs and handlers, respectively (Starlander *et al*., 2014). Furthermore, enhanced understanding of the antimicrobial resistance of *S. pseudintermedius* is required to design effective treatment to control infections caused by MDR *S. pseudintermedius*. Therefore, this study aimed to document the antimicrobial resistant

pattern of the bacteria in the clinical samples of dogs received from Veterinary Clinical Complex, Khalsa College of Veterinary and Animal Sciences, Amritsar.

Materials and Methods

Sample collection:

A total of 53 clinical samples including urine, milk, and swabs from ear, wounds, paws, and perianal region from dogs received from Veterinary Clinical Complex, Khalsa College of Veterinary and Animal Sciences, Amritsar were processed for isolation of staphylococci. The isolates were stored at -20°C for further studies.

Isolation and Identification:

The identity of the bacterial strain was confirmed with conventional microbiological phenotypic tests. The samples were inoculated on Brain Heart Infusion (BHI) broth and then sub-cultured on selective media Mannitol Salt Agar (MSA) at 37°C for 24 hrs. Presumptive identification of the isolate was based on colony morphology, gram stain reaction, catalase and coagulase test. Gram positive cocci with typical grapelike clusters were presumed to be *Staphylococcus* spp. Phenotypic tests such as coagulase, clumping factor, pigment production, pyrrolidonylacrylamidase, acetoin production, maltose fermentation, and polymixin B resistance was performed to differentiate the isolates from *S. aureus* according to the method..The selected isolates were confirmed by 16S rRNA sequencing at Bio Kart Pvt. Ltd, Bangalore, using primer pair 16s Forward: GGATGAGCCCGCGGCCTA and 16S Reverse: CGGTGTGTACAAGGCCCGG. Phylogenetic tree was constructed with BLAST neighbour joining distance tree method with maximum sequence difference of 0.75.

Antimicrobial Susceptibility Testing:

Antimicrobial susceptibility tests were performed on the isolates by disk diffusion method on Mueller-Hinton agar, as per Clinical and Laboratory Standards Institute (CLSI), and the zone sizes were interpreted per CLSI guidelines. The antibiotics used for the test included Amikacin (AK 10mcg), Amoxycillin/Clavulanic acid (AMC 30 mcg), Ampicillin (AMP 10 mcg), Cefotaxime (CTX 30 mcg), Cefoxitin (CX 30 mcg), Ceftazidime (CAZ 30 mcg), Cefpodoxime (CPD 30 mcg), Cotrimoxazole (COT 25 mcg), Clindamycin (CD 2 mcg), Doxycycline HCl (DX 30 mcg), Enrofloxacin (EX 5 mcg), Ofloxacin (OF 5 mcg), Oxacillin (OX 1 mcg), Linezolid (LZ 30 mcg), Penicillin G (10 IU), Ticarcillin/Clavulanic acid (TCC 75/10 mcg), Tetracyclin (TE 30 mcg). The zone of inhibition was read as per the standard of Clinical Laboratory Standard Institute. The isolate was interpreted as multidrug resistance if it was found to be resistant to more than 3 classes of antibiotics.

AmpC and ESBL screening was performed on all isolates of coagulase positive Staphylococci by using the CAZ and Ceftazidime-Clavulanic acid (CAC) and CX and Cefoxitin-Cloxacillin (CXX). A difference of $\geq$ 5 mm and $\geq$ 4 mm between the zone of inhibition of the single disk and the combination disks are interpreted as a confirmation of ESBL and AmpC positive isolates, respectively.If an ESBL is detected, all penicillins, cephalosporins, and Aztreonam are reported as resistant. Oxacillin was used as an alternate antibiotic to methicillin for screening methicillin resistance.

Results and Discussion

A total of 53 clinical samples were screened out of which 35 mono microbial colonies of coagulase positive *Staphylococcus* spp was isolated on Mannitol Salt Agar from urine (3), milk (2), swabs from ear (10), wounds (12), and paws (8). Growth of yellow colonies and change of color of the medium was taken as positive mannitol fermentation and designated as presumptive *Staphylococcus aureus*and isolates with pink colonies were presumed to be *S. pseudintermedius*. Various publications

reported misidentification of *S. pseudintermidius*with other CPS by conventional microbiological/phenotypic tests (Perez-Sancho *et al*., 2020). However, phenotypic differential screening in our study correctly identified the isolate as *S. pseudintermidius* which was confirmed by 16S rRNA sequencing using ABI 3130xl platform (Fig.-1) and phylogenetic analysis confirmed the sequence homology with strain of *S. pseudintermedius* (Fig.-2). A zone of growth inhibition of ≤17 mm against oxacillin 1 mcg disk was considered indicative of resistance as also mentioned by Gold *et al.,* (2013).

Resistance to antimicrobial drugs was observed in a majority of the isolates (Table-2). It was observed that amongst the 35 coagulase positive *S. pseudintermedius* isolates, there are two methicillin resistant *S. pseudintermedius* (MRSP), one each for ESBL + AmpC, ESBL, and AmpC producers (Table-3). Isolates from milk sample showed resistance to more than one class of antibiotics but sensitive to piperacillin/tazobactam and ofloxacin; uropathogenic isolates showed sensitivity to gentamicin but are resistant to all other class of antibiotics screened. Isolates from ear swab showed sensitivity to cefotaxime, ceftriaxone, doxycycline, linezolid, or piperacillin/tazobactam; whereas isolates from wound/abscess showed resistance to all the antibiotics used for screening. Among the 35 isolates screened, ESBL and AmpC genes (2.86%), ESBL alone (2.86%), and AmpCalone (2.86%) were observed (Table-3). Different studies have reported emergence of MDR*S. pseudintermedius* which correspond with our findings.

Table - 1 Phenotypic test for differentiation of coagulase positive Staphylococci

Test	*S. aureus*	*S. pseudintermedius*
Tube coagulase	+	+
Clumping factor	+	-
Pigment production	+	-
Pyrrolidonylacrylamidase	-	-
Acetoin production	+	-
Mannitol fermentation	+	-
Maltose fermentation	+	+
Polymixin B resistance	R	S

Table 2 Percentages of antimicrobial resistance amongst the *S. pseudintermedius*isolates from various clinical samples.

No. of isolatesfrom clinical samples	*S. pseudintermedius* isolates (n = 35)		
	Resistance (%)	Intermediate (%)	Sensitivity (%)
Urine (3)	3 (8.75)	-	-
Milk (2)	2 (5.71)	-	-
Ear swab (10)	8 (22.85)	-	2 (5.71)
Wound (12)	10 (28.57)	1 (2.86)	1 (2.86)
Paws (8)	4 (11.43)	1 (2.86)	3 (8.57)

Table 3 Antimicrobial resistance observed in ESBL and AmpCproducers

Clinical Sample	Antimicrobial resistance pattern	No. of isolates (n = 35)		
		ESBL + AmpC	ESBL	AmpC
Urine	AK, AMC, AMP, CTX, CTR, CX, CPD, CAZ, COT, EX, NX, PIT, TCC, TE	-	-	-
Milk	AK, AMC, CX, CPD, CAZ, COT, CD, GEN,	-	-	1

Ear swab	AMC, AMP, CX, CPD, COT, CD, EX, GEN, OX, TCC	-	1	-
Wound	AK, AMC, AMP, CTX, CTR, CX, CAZ, COT, CD, EX, OF, OX, LZ, P, TCC, TE	1	-	-
Paws	AMC, AMP, CX, CPD, COT, CD, EX, GEN, OX	-	-	-

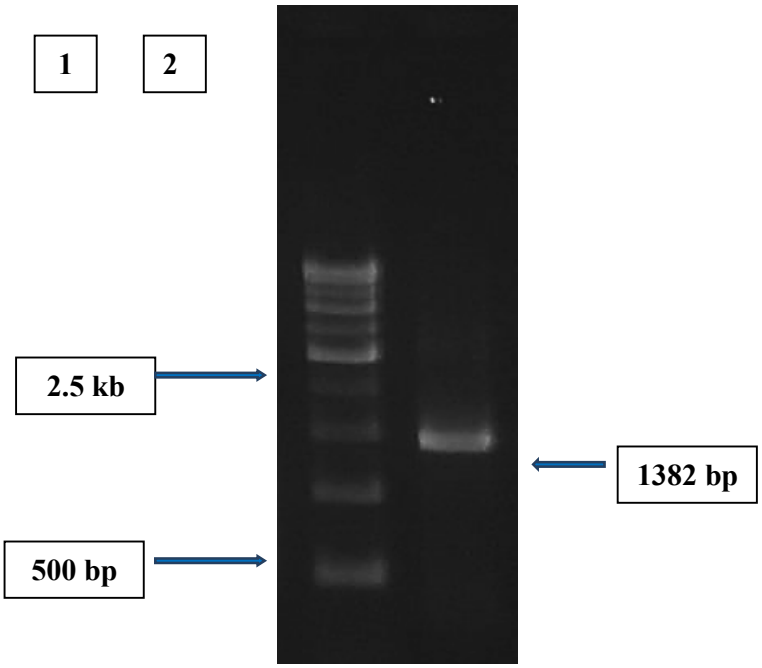

Fig 1. Lane description. Lane 1: DNA ladder (500 bp)
Lane 2: Bacterial isolate (1382 bp)

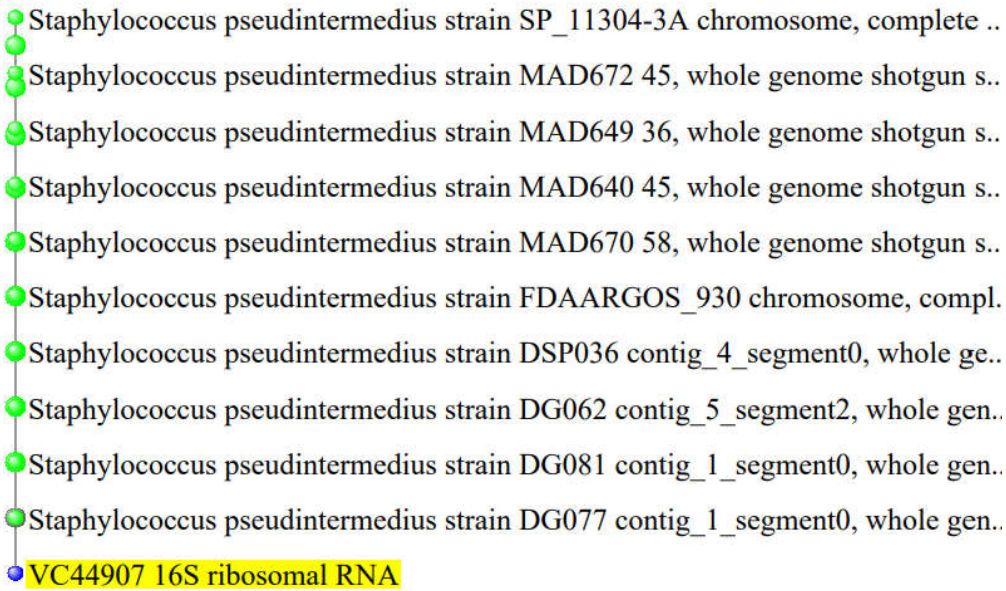

Fig 2 Phylogenetic tree of *S. pseudintermedius*isolate from canine clinical samples

Conclusions

The rapid emergence and dissemination of MDR *S. pseudintermedius* highlights the importance of monitoring the screening for MDR in clinical isolates from dogs. Emergence of ESBL, AmpC, or a combination of both ESBL and AmpC producing *S. pseudintermedius* presented a serious public health challenges which will result in critical shortages in the availability of treatment and its efficacy. Hence, prevention, strict surveillance and early identification of these MDR strains is critical and mandatory in Veterinary clinical set up. Judicious prescription of beta-lactam antibiotics should be followed with strict measures coupled with monitoring of the correct doses of each antibiotics used in treatment procedures.

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