Journal of Animal Research: v.12 n.3, p. 323-329. June 2022

DOI: 10.30954/2277-940X.03.2022.2

Occurrence of Multidrug Resistant Coagulase negative *Staphylococcus* spp in Canine pyoderma, and Their Comparative Phenotype and Molecular Characterization

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Received: 27 April, 2022 **Revised:** 24 May, 2022 **Accepted:** 27 May, 2022

ABSTRACT

A study was conducted on 137 clinical cases of canine pyoderma from July 2021 to November 2021. Out of 137 bacterial isolates, 129 (94.16%) isolates were Gram positive. Among them 125 were of *Staphylococcus* spp and accounted for 91.24% of total isolates. Among these 125 *Staphylococcus* isolates, 48 (38.4%) were identified as coagulase positive and 77 (61.6%) were identified as coagulase negative. Biochemically identified spp collections (*S. lugdunensis* - S40, *S. simulans* - S11, *S. cohnii* subsp. *xylosus* - S41 and *S. cohnii* subsp. *urealyticus* - S44) used for phylogenetic analysis of 16S rRNA gene nucleotide sequences. Sequence results identified S40 as *S. saprophyticus,* S11 as *S. haemolyticus*, S41, S44 as *S. pseudintermedius* and the sequences of the 16S rRNA was submitted to Genebank with accession numbers as OM302140, OM302142, OM302146, OM302147. The *in vitro* antibiotic sensitivity tests showed highest resistance to streptomycin (94, 75.2%) followed by cefoxitin (83, 66.4%). Most of the isolates were found to be susceptible to enrofloxacin (62.4%), amoxicillin + clavulanic acid (58.4%), and gentamicin (58.4%). Among the methicillin resistant strains, 37 were CPS isolates (*S. aureus* 9 and *S. pseudintermedius* 28) and 46 were CNS isolates. The distribution of methicillin resistant gene *mecA* was observed in 19 isolates (22.89%) of *Staphylococcus*. Out of these 19 isolates, four were methicillin resistant *S. aureus* (CPS), 14 were methicillin resistant *S. pseudintermedius* (CPS) and the remaining one was methicillin resistant CNS isolates. The results showed an increased pathogenesis and methicillin resistance of SIG group *S. pseudintermedius* in Canine pyoderma.

HIGHLIGHTS

- Biochemically identified spp collections used for phylogenetic analysis of 16S rRNA gene nucleotide sequences.
- The methicillin resistance of SIG group *S. pseudintermedius* (*mecA* gene and *pse* gene) isolated from Canine pyoderma.

Keywords: Canine pyoderma, antibiogram, Methicillin-resistance, *Staphylococcus pseudintermedius,* Biochemical characterization, 16S rRNA sequencing

Canine pyoderma remains the most common and frustrating problem in pet animals and has public health significance. Among different pyoderma conditions, recurrent pyoderma is an important clinical skin problem and frequently occurs because of uncorrected underlying causes or use of inappropriate antibiotics or improper duration of antibiotic therapy (Reddy *et al.,* 2014). Though multifactorial canine pyoderma is primarily caused by *Staphylococcus* spp. Both coagulase positive

How to cite this article: Antony, N., Kommalapati, L.K., Nagaram, V.K. and Angalakuditi, J.B. (2022). Occurrence of Multidrug Resistant Coagulase negative *Staphylococcus* spp in Canine pyoderma, and Their Comparative Phenotype and Molecular Characterization. *J. Anim. Res.,* **12**(03): 323-329.

Source of Support: None; **Conflict of Interest:** None

and coagulase negative *Staphylococcus* cause canine pyoderma. Among Coagulase positive isolates in addition to *Staphylococcus aureus* there is increased significance of *Staphylococcus pseudintermedius* (SP). Furthermore, the association of CNS in canine pyoderma has increased the development of virulence and resistance. In recent years, Methicillin-resistance has emerged as an important problem in both animals and human beings. Methicillin-resistant *S. psuedintermedius* (MRSP) and *Staphylococcus aureus* (MRSA) are common multidrug resistant bacteria in dogs. (Hanselman *et al.,* 2008). In this scenario it is essential to continuously monitor the bacteria associated with canine pyoderma.

MATERIALS AND METHODS

Isolation and Identification of bacterial pathogen

A total of 137 skin swabs were collected aseptically from Canine pyoderma and were transported to the laboratory under a cold chain for the isolation of the causative agent.

After overnight incubation in brain heart infusion broth, the inoculums were streaked onto Mannitol salt agar, Blood agar and Baird Parker agar plates and incubated at 37°C overnight. The isolated Mannitol fermenters and non fermenters on Manitol salt agar were subjected to Gram's staining and conventional biochemical methods. The biochemically confirmed *Staphylococcus* isolates were further subjected to coagulase test and molecular screening. A simplified biochemical test scheme devised by Sah *et al.* (2018) was employed to identify CNS isolates.

Molecular characterization

Molecular Screening of *Staphylococcus aureus*

A single colony from Mannitol Salt agar plates was inoculated into BHI broth and incubated at 37°C for overnight. Then the DNA was extracted by lysis method (boiling method). *S. aureus* isolates were confirmed using species-specific primer targeting the *23s rRNA* gene described in Table 1. The reaction conditions were initial denaturation at 94°C for 5 mins followed by 30 cycles of denaturation at 95°C for 45 secs, annealing at 60°C for 30 secs and elongation at 72°C for 45 secs and final elongation at 72°C for 10 mins as per the method of Shome *et al*., 2011. The PCR products were analyzed by 1.5% agarose gel electrophoresis, visualized under UV transilluminator for 894bp product, and were documented.

Molecular Screening of *Staphylococcus pseudintermedius*

S. pseudintermedius isolates were confirmed using species-specific primer targeting the *pse* gene (Table 1) using template DNA extracted by boiling method. The reaction conditions were initial denaturation 95°C for 5 mins, followed by 35 cycles of denaturation at 95°C for 30 secs, annealing at 52°C for 45 secs and elongation at 72°C for 1 min 30 secs and final elongation at 72°C for 10 mins (Jayalakshmi *et al*., 2020). The amplified product of 926 bp amplicon, was CNSidered positive for *S. pseudintermedius*.

Sequencing of *S. pseudintermedius*

Randomly selected one positive isolate was subjected to Sequencing. Sequencing was carried out at IRA Biotech, Hyderabad, Telangana, India. The chromatograms of the forward and reverse strands were analyzed for chimera detection and the CNSensus sequences were obtained after editing the sequence errors in Chromas. The sequences obtained were submitted in FASTA to the GenBank via Bank IT online.

Molecular Screening of 16S rRNA gene

The identification of individual species in the CNS group was validated by the amplification of 16S ribosomal RNA and sequencing. The reaction mixture and oligonucleotide primers for 16S rRNA PCR were mentioned in Table 1. The reaction conditions include initial denaturation at 95ºC for 5 min, 35 cycles of denaturation at 94ºC for 45 sec, annealing at 55ºC for 45 sec, elongation at 72ºC for 90 sec and final elongation at 72ºC for 7 min.

Sequencing of 16S rRNA gene

The ampliCNS of 1515 bp region of 16S rRNA gene of the four representative CNS isolates were sequenced in automatic sequencer using gene specific primers.

Table 1: Details of the Primers used in this study

Gene	Primer sequence 5'-3'	Amplicon size (bp)	Reference
pse	*pse*-F TRGGCAGTAGGATTCGTTAA	926	Jayalakshmi *et al.* (2020)
	pse-R CTTTTGTGCTYCMTTTTGG		
mecA	*mecA*-F GTAGAAATGACTGAACGTCCGATAA	310	Vishnu priya *et al*. (2014)
	mecA-R CCAATTCCACATTGTTTCGGTCTAA		
23S rRNA	23SrRNA-F AGCGAGTCTGAATAGGGCGTTT	894	Shome *et al*. (2011)
	23SrRNA-R CCCATCACAGCTCAGCCTTAAC		
16S rRNA	16SrRNA-F AGAGTTTGATCCTGGCTCAG	1515	Sah *et al*. (2018)
	16SrRNA-R ACGGCTACCTTGTTACGACTT		

Sequencing was carried out at IRA Biotech, Hyderabad, Telangana, India. Sanger sequencing method was used for nucleotide sequencing using Applied Biosystems, model number 3730, CA, USA. The chromatograms of the forward and reverse strands were analyzed for chimera detection and the CNSensus sequences were obtained after editing the sequence errors in Chromas. The sequences obtained were submitted in FASTA to the GenBank via Submission Portal online.

Antimicrobial susceptibility test

Antimicrobial susceptibility testing was performed by disc diffusion method on Mueller-Hinton agar and isolates were classified as sensitive, intermediate and resistant based on recommendations of CLSI 2021, EUCAST 2021.

Molecular screening of Methicillin-resistant *S. pseudintermedius* and *S. aureus*

Methicillin resistance was observed by amplifying *mecA* gene with specific primers (Table 1). The amplification was done with an initial denaturation at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 45 sec, annealing at 60°C for 30 sec and elongation at 72°C for 30 sec and final elongation at 72°C for 10 min as per the method of Vishnu *et al*. (2014) and observed for 310 bp amplicon.

RESULTS AND DISCUSSION

Out of 137 bacterial isolates, 129 (94.16%) isolates were Gram positive, which were nearer to Chaudhary *et al.* (2019) who observed predominance of Gram positive bacteria from canine pyoderma cases.

Among the 129 Gram positive isolates 125 were *Staphylococcus* isolates (Fig. 1a) and accounted for 91.24%. The higher incidence of *Staphylococcus* isolates in canine pyoderma was also noticed as 71.15%, 67.2% and 92.3% by Shah *et al.* (2017), Ankita *et al.* (2018) Chaudhary *et al.* (2019), respectively.

Among the 125 isolates, 48 (38.4%) were identified as coagulase positive *Staphylococcus* (CPS) by observing growth on MSA (Fig. 1b,1c), Baird-Parker agar medium (Fig. 1d,1e) and by conducting tube coagulase test (Fig. 1f). The result was in accordance with Hariharan *et al.* (2014) who reported 29 CPS isolates (25 %) from canine pyoderma cases. Whereas Shah *et al.* (2017) reported a higher prevalence of CPS (67.87%).

Later the CPS isolates on genotype confirmation by 23S rRNA (Fig. 2a) and *pse* gene PCR showed 11 (22.91%) as *S. aureus* and 30 (62.51%) isolates as *S. pseudintermedius*, which does not correlate with the results of phenotype study that showed 11 (22.91%) as *S. aureus* and 32 (66.6%) isolates as *S. pseudintermedius*. This is primarily because *S. pseudintermedius* cannot be distinguished from *S. intermedius* by biochemical methods as reported by several workers (Sasaki *et al*., 2010 and Videla, 2013). Further, due to the lack of standardized and specific phenotypic tests, the routine presumptive identification of *S. pseudintermedius* is based on the fact that it is the only member of the *Staphylococcus* intermedius Group (SIG) that has been isolated from dogs. Thus, definitive identification of *S. pseudintermedius* relies on molecular methods (Bannoehr and Guardabassi, 2012).

The confirmation of *S. pseudintermedius* were further ascertained by sequencing the PCR amplified product of 926 bp (Fig. 2b). The sequence was analyzed and

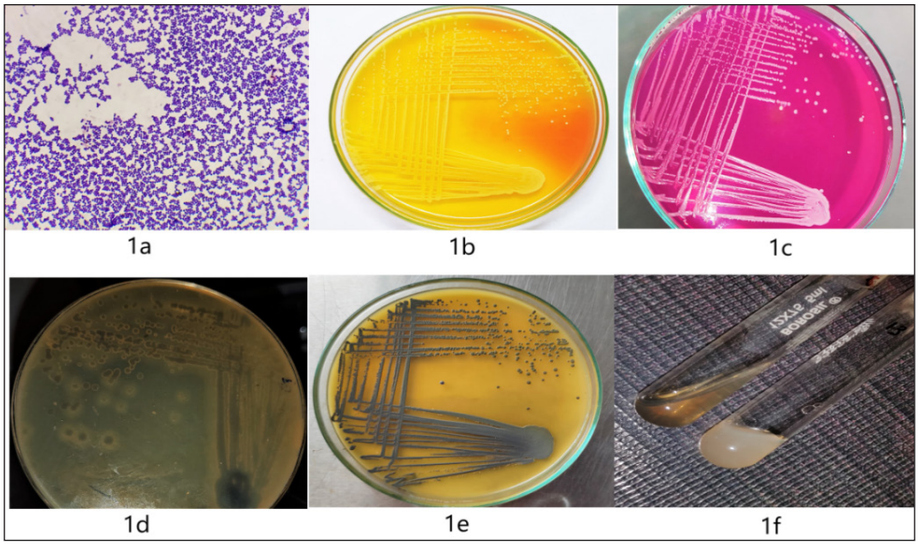

Fig. 1: Characterisation of *Staphylococcus* spp. **(a)** Gram positive cocci in clusters, bunch of grapes; **(b)** Golden yellow colonies of *Staphylococcus* spp. on MSA indicating mannitol fermenters; **(c)** Pink colonies of *Staphylococcus* spp. on MSA indicating mannitol non-fermenters; **(d)** A characteristic clear zone (opaque zone) of *S. aureus* colonies on Baird-Parker medium. [Opaque halos surrounding them are due to the action of Coagulase]; **(e)** A characteristic grey colonies on Baird-Parker agar; **(f)** Tube coagulase test for *Staphylococcus* isolates [Negative (Left) and Positive (Right) with fibrin clot formation]

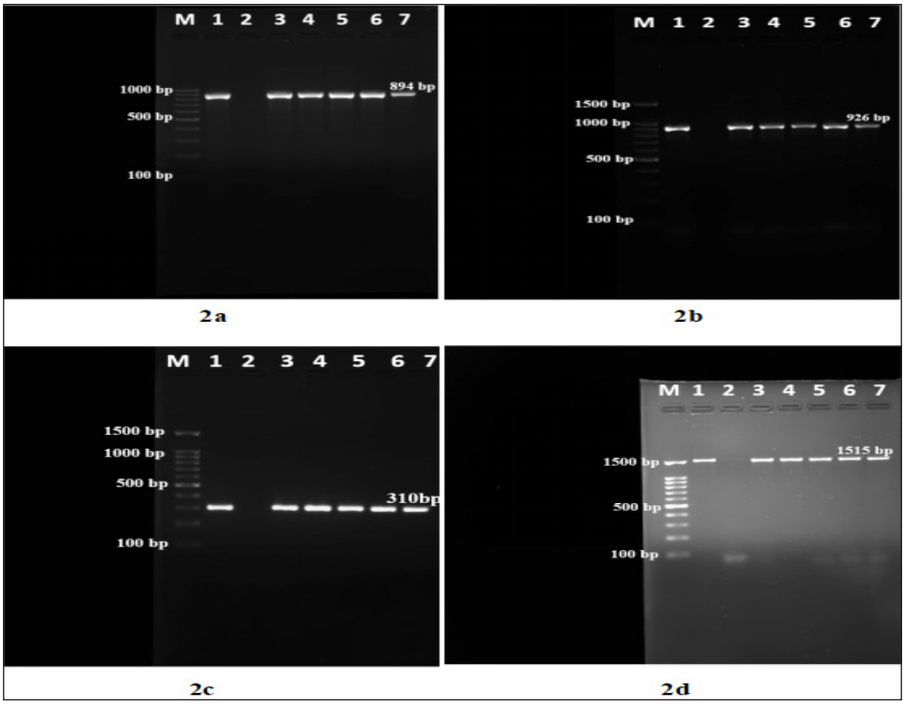

Fig. 2: Molecular characterization**. (a)** PCR for 23S rRNA gene of *S. aureus;* **(b)** PCR for *pse* gene of *S. pseudintermedius;* **(c)** PCR for *mecA* gene; **(d)** PCR for 16S rRNA gene

gave 100% identity with published sequenced data later sequence was submitted to genebank with accession number as OM320984.

The emergence of methicillin-resistant *Staphylococcus* spp. and its continuing spread worldwide, present significant clinical and public health challenges. Phenotypic methicillin resistance was observed in 83 (66.04%) of the present isolates. Among the methicillin resistant strains, 37 were CPS isolates (*S. aureus* 9 and *S. pseudintermedius* 28) and 46 were CNS isolates. Similarly, Shah *et al.* (2017) also recorded that methicillin-resistant *Staphylococci* accounted for 40.07% of the total isolates. They also reported that the emergence of methicillin resistance is high in *S. pseudintermedius* isolates. This was also in concurrence with the reports of Loeffler *et al.* (2007), Ruscher *et al.* (2008) and Joffe *et al*. (2015).

Out of the total isolates (n=125) 77 (61.6%) isolates were found to be CNS. According to the scheme employed to identify the CNS isolates, five tests are specific for identifying *S. epidermidis*. In the present study testing of the CNS isolates initially using five biochemical tests (trehalose, maltose, mannitol, mannose and novobiocin susceptibility) identified most of them (65/77) as phenogroup-1, two as phenogroup-2 and 10 as phenogroup-3 described in Table 2. None of the isolates were observed to have the biochemical test pattern characteristic of *S. epidermidis* and hence no isolate from our study is *S. epidermidis*. Similar schemes for the identification of CNS were reported by Bhavana (2019).

Table 2: Phenotypic profile for CNS isolates

No. of isolates	Pheno group	Man-nitol	Malt-ose	Man-nose	Treha-lose	Novobiocin Susceptibility
65	1	+	+	+	+	S/R
2	2	+	+	+	_	S
10	3	_	+	+	+	R

Later the CNS isolates were phenotypically characterized further by using a panel of four biochemical tests *viz.* ornithine decarboxylase (OD), urease (U), mannose(M) and novobiocin 5 µg susceptibility (N), based on which they were identified 48.05% as *S. cohnii* subsp. *urealyticus* (OD -, U -, M + and N-Resistant), 38.98% as *S. simulans* (OD -, U +, M + and N-Sensitive) as predominant CNS species followed by, 9.09% as *S. cohnii* subsp. *xylosus* (OD -, U +, M + and N-Resistant) and 2.59% as *S. lugdunensis* (OD +, U +, M + and N-Sensitive), 1.29% as *S. capitis* subsp. *capitis* (OD -, U -, M + and N-Sensitive) from canine pyoderma cases in the present study region. Shah *et al.* (2017) observed *S. epidermidis* (3.03% n=5) and *S. saprophyticus* (0.60% n=1) as the CNS from the cases of canine pyoderma. Similarly different CNS were also observed by Joffe *et al*. (2015).

Four representative isolates (*S. simulans* - S11, *S. lugdunensis* - S40, *S. cohnii* subsp. *xylosus* - S41 and *S. cohnii* subsp. *urealyticus* - S44 16S rRNA (Fig. 2d, Fig. 3) sequence phylogeny identified S11 as *S. haemolyticus,* S40 as *S. saprophyticus,* S41, S44 as *S. pseudintermedius*.

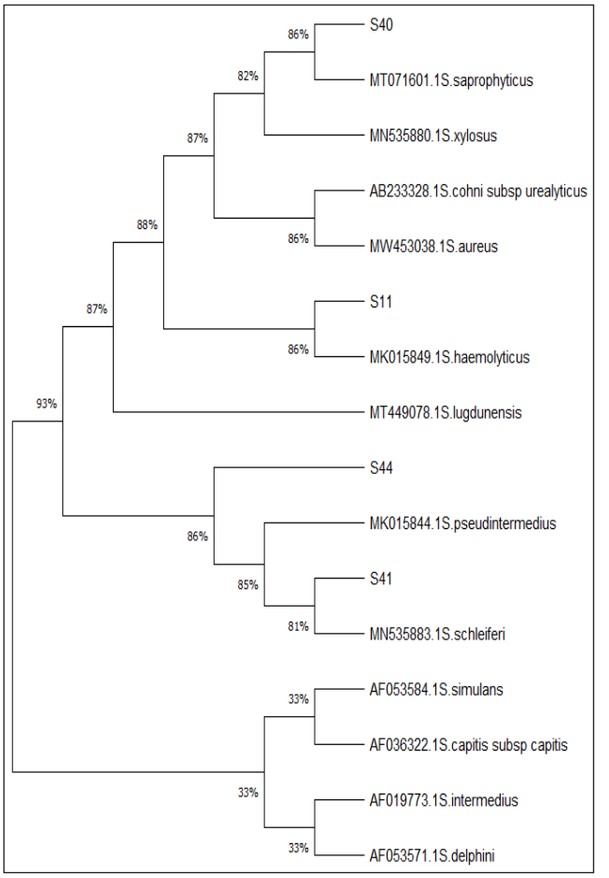

Fig. 3: Phylogenetic analysis of 16S rRNA sequences of four CNS isolates with reference sequences available in NCBI data base. The phylogenetic tree was Constructed using MEGA 11 software by Maximum Likelihood method with 1000 bootstrap replicates using Kimura 2-parameter model

The biochemical test results are in disagreement with the results of 16S rRNA sequence based identification of the four representative isolates. The 16S rRNA sequencing being the accurate method for species identification of bacteria, the present study indicated that the biochemical tests are non-specific and inadequate for the identification of different CNS species. More isolates from each species collection identified based on biochemical test scheme have to be sequenced in order to evaluate the accuracy of the biochemical test scheme for species identification (Bhavana, 2019). Similar misidentification of the isolates was reported by Sah *et al.* (2018). Further, detailed phylogenetic analysis of complete sequences of 16S rRNA sequences might help to show an accurate correlation between biochemical and gene sequencing for speciation of CNS isolates. Kim *et al.* (2018) used *16s rRNA* sequencing for the identification of CNS species, but it is not effective in discriminating the closely related species. They developed a rapid and accurate identification method based on *sodA* gene sequencing and *sodA*-specific multiplex PCR.

Antimicrobial susceptibility and resistance trends of *Staphylococcus* spp showed majority of the CPS and CNS isolates of the present study were resistant to streptomycin (75.2%) followed by cefoxitin (66.4%), oxacillin (64%), erythromycin (64%), azithromycin (63.2%), cefpodoxime (61.6%) and ceftriaxone + tazobactam (60%). Most of the isolates were found to be susceptible to enrofloxacin (62.4%), amoxicillin + clavulanic acid (58.4%), and gentamicin (58.4%) (Fig. 4). These findings are in near agreement to Huerta *et al.* (2011).

The phenotypically identified cefoxitin resistant isolates (83) were subjected for the detection of *mecA* gene. In PCR only 19 isolates (22.89%) of *Staphylococci* were found to possess *mecA* gene with ampliCNS of 310 bp (Fig. 2c). Out of these 19 isolates, four were methicillin resistant *S. aureus,* 14 were methicillin resistant *S. pseudintermedius* and the remaining one were methicillin resistant CNS isolates. In concurrence Jayalakshmi *et al.* (2019) who reported the increased frequency of *mecA* in *S. pseudintermedius* in diseased dogs. Similar reports were also made by Anandachitra *et al.* (2018).

In the present study it was observed that not all the cefoxitin resistant isolates harboured *mecA* or *mecC* genes. Currently, 14 SCC *mec* types have been identified. The acquisition and expression of *mecA* gene alone do not make the cell uniformly resistant to β-lactam antibiotics. The expression of *mecA* confers on the bacterial strain a moderate level of resistance to β-lactam antibiotics. The absence of *mecA* or *mecC* genes doesn't indicate the absence of resistance genes in other cefoxitin resistant pathogens as their resistance may be encoded by other genes that confer methicillin resistance.

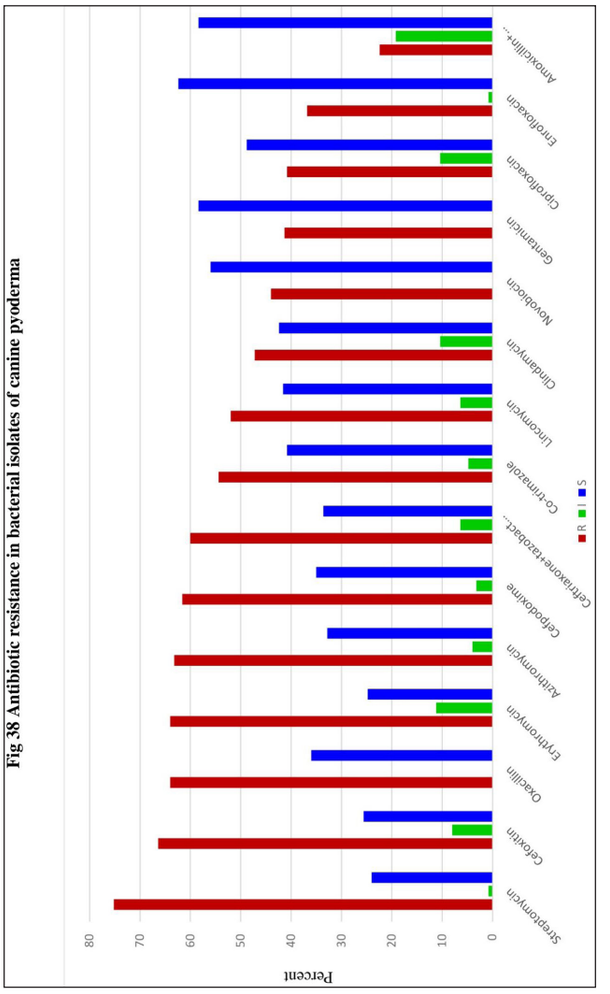

Fig. 4: Antibiotic resistance in bacterial isolates of canine pyoderma

CONCLUSION

This study gave a record on the prevalent pathogen and antibiotic of choice for treating canine pyoderma with a

record on an alarming increase in methicillin resistance. The results showed an increased pathogenesis and methicillin resistance of SIG group *S. pseudintermedius* in Canine pyoderma.

ACKNOWLEDGEMENTS

The authors are highly thankful to Sri Venkateswara Veterinary University, Tirupati for providing necessary facilities and funding to carry out this work.

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