RESEARCH ARTICLE

Identification, Antimicrobial Sensitivity and Molecular Detection of *Staphylococcus aureus* and *Staphylococcus pseudintermedius* Isolated from Ear Infections of Canines

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Abstract

The present study was undertaken to identify by molecular detection and investigate the antimicrobial susceptibility of *S. aureus* and *S. pseudintermedius,* which are a major pathogens of public health concern in both animals and human beings. 132 ear swabs were collected from 92 dogs suffering with different types of ear infections. On isolation 85 (70.24%) isolates were identified as Staphylococcus. Through molecular profiling targeting *nuc* gene 23 isolates were identified as *S. aureus*, and 26 isolates were identified as *S. pseudintermedius*. On antimicrobial susceptibility testing *S. aureus* isolates exhibited greater sensitivity towards cephalothin (78.26%) followed by gentamicin and chloramphenicol (60.86% each), and ciprofloxacin (56.52%). *S. pseudintermedius* isolates exhibited greater sensitivity towards enrofloxacin (69.23%), followed by cephalothin and amoxiclav (61.53% each), and amikacin (57.69%). PCR studies targeting *mecA*, *tet*K, *tet*M genes revealed that 23 isolates of *S. aureus* and 26 isolates of *S. pseudintermedius* were positive for *mecA* gene. 14 isolates of *S. aureus* and 17 isolates of *S. pseudintermedius* were positive for *tet*K gene. 12 isolates of *S. aureus*, and 14 isolates of *S. pseudintermedius* were positive for *tet*M gene.

Key words: Antimicrobial susceptibility, Bacterial isolates, Dogs, Ear infections, PCR, Staphylococcus.

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Introduction

Dogs are the one among the companion animals living in close proximity to human beings. The frequent health problems faced by the dogs are the ear infections which accounts for 10-20% in dog populations. Different etiological factors are responsible for causing disease, of which bacterial etiology comes under the primary factor (Terziev and Urumova, 2018). The most frequent bacteria responsible for ear infection are *Staphylococcus*, *Pseudomonas*, *Streptococcus*, *E. coli*, *Proteus*, *Corynebacterium* and *Klebsiella* (Doshi *et al*., 2021). *Staphylococcus* responsible for ear infection in dogs, are Gram positive cocci arranged in irregular grape like clusters, tetrads with 0.5-1 µm in diameter. With the larger use of antibiotics for the treatment of infections, occurrence of mutations in the target genes, increase in number of antibiotic resistance genes had led to the cause of turning up of antimicrobial resistance (Hadi and Alabbas, 2023). Dogs are found to be the regular reservoirs of antimicrobial resistance determinants that can pass on to human beings either by direct or indirect contact leading to a serious public health concern (Bourély *et al*., 2019). Methicillin resistance *Staphylococcus aureus* and methicillin resistant *Staphylococcus pseudintermedius* are specific species of *Staphylococcus* that have developed resistance towards various beta-lactam classes of antibiotics which are frequently used for treatment of *Staphylococcal* infections in dogs and soon the bacteria started developing resistance to other classes of antibiotics like tetracyclines, aminoglycosides, fluoroquinolones, macrolides, fusidic acid etc (Morris *et al*., 2006). Tetracycline resistance is having a connecting relationship with the methicillin resistance. Studies reported that *tet*M gene was observed in methicillin resistant *Staphylococcus aureus*, while *tet*K gene was recorded more in methicillin susceptible *Staphylococcus aureus* (Schmitz *et al*., 2001). The rapid

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spread of multi-drug resistance leads to the persistence of infection and treatment failure ultimately causing increased morbidity and mortality rate. So, there is a need for the proper identification of different bacteria responsible for causing infection and their sensitivity to various antibiotics to help/prevent treatment failure. Hence, this study was targeted for identification, antimicrobial sensitivity and molecular detection of *Staphylococcus aureus* and *Staphylococcus pseudintermedius* isolated from ear infections of canines

Materials and Methods

Following approval of the Institutional Animal Ethical Committee of Guru Angad Dev Veterinary and Animal Science University, Ludhiana (GADVASU /2023/IAEC/68/09), a total of 132 ear swab samples were collected from 92 dogs of either sex from different breeds of all age groups presented in small animal clinics unit of Multispecialty Veterinary Hospital, GADVASU, Ludhiana (India) which were having ear infections in either one or both the ears, and showing clinical signs like foul smelling pus discharge, erythema, swelling, formation of crusts in ear canal. The samples were brought to laboratory in cold chain and processed for bacterial isolation. The demographic data of 92 dogs such as age (0-4 ys, >4 to 8 yrs, >8 to 12 yrs), sex and breed were recorded.

Isolation and Identification of Bacteria

The samples were initially streaked on Brain Heart Infusion agar (BHI) and incubated for 18 to 24 h. Initial identification of isolates was done based on cultural characters, bacterial morphology on Gram stain and also by biochemical tests like catalase, oxidase, coagulase, indole, methyl red, voges-proskaeur, citrate, urease. The isolates which appeared as Gram-positive cocci arranged as bunch of grapes were identified as *Staphylococcus* and further sub-cultured on Mannitol salt agar and Baird Parker agar to obtain pure colonies of *Staphylococcus*. Colonies of both the isolates showed golden yellow colonies on MSA after 24 h of incubation. On Baird Parker agar the isolates of *S. aureus* and *S. pseudintermedius* produced black coloured colonies with opaque zone formation after 24-48 h of incubation. MALDI-TOF was also used to identify the organisms.

Antimicrobial Susceptibility Testing

Isolates were tested for sensitivity by Kirby-Bauer disc diffusion method on Muller-Hinton agar and results recorded according to the CLSI guidelines. Twenty four different antibiotic discs procured from Hi-Media were used in the study. Bacterial culture in broth was streaked on Muller-Hinton agar plates and the antibiotic discs were placed at an equidistance so as to avoid overlapping of the zones and incubated for 18-24 h. The diameter of zone of inhibition was measured in millimetre and results were compared with the interpretation chart and isolates were assigned as sensitive, intermediate, resistant.

Molecular Detection of *Staphylococcus aureus* and *Staphylococcus pseudintermedius*

For molecular detection the genomic DNA was extracted by Hot-cold lysis method of the culture colonies identified by Gram's staining. Genus identification as *Staphylococcus* was done by targeting *pta* gene, gel electrophoresis was performed with 1.5% agarose, PCR product yield which showed desired amplicon size at 320 bp was visualized under UV illuminator and also on gel doc was considered as positive for *Staphylococcus* (Fig. 1). Species identification of *S. aureus* and *S. pseudintermedius* was done by species specific primers targeting *nuc* gene, PCR product yield which showed desired amplicon at 359 bp and 926 bp were considered as positive for *S. aureus* and *S. pseudintermedius* (Fig. 2, 3). The isolates of *S. aureus* and *S. pseudintermedius* were further screened using species specific primers for *mecA*, *tet*K, *tet*M genes for methicillin and tetracycline resistance. The primer sequences used in the present study are shown in Table 1.

Results and Discussion

Incidence of Ear Infections

Age wise incidence of ear infections among 92 dogs studied was found to be more in age group of 0-4 yrs (52.17%), followed by >4-8 yrs (36.95%), and >8-12 yrs (10.86%). Similarly, Doshi *et al*. (2021) recorded the incidence of ear infections in 0-4 yrs age group dogs as 53.33%, in the age group of 5-10 yrs 40.00%, in the age group of 10-15 yrs as 6.66%.

Breed wise incidence was more in the Labrador breed of dogs which was reported to be 26 (28.26%), followed by 18 Golden retriever (19.56%), 18 Pug (19.56%), 10 Non-descriptive breeds (10.86%), 5 American bull dog (5.43%), 5 German shepherd (5.43%), 4 Beagle (4.34%), 2 Pomeranian (2.17%), 2 Dachshund (2.17%), 1 French bull dog (1.08%), and 1 Pit bull (1.08%). Similar results were recorded by Subapriya *et al.,* (2015), who studied the microbial profile of canine otitis and reported highest incidence of disease in Labrador breed of dogs which was about 25.35%.

Sex wise incidence was found greater in the male dogs (63.04%) compared to female dogs (36.95%), which was in accordance to studies carried out by Subapriya *et al.* (2015), who reported greater incidence in male dogs as compared to females dogs (61.03 vs 38.97%).

Isolation, Identification and AbST of Bacteria

Out of the 132 ear swabs, 121 isolates (91.6%) were obtained in the form of pure cultures, three isolates (2.27%) were obtained in the form of mixed growth, and no growth was found in eight samples (6.06%). In the present study, 85 isolates were identified as *Staphylococcus*, and of these 26 isolates were *Staphylococcus pseudintermedius* (21.48%), and 23 isolates were identified as *Staphylococcus aureus* (19.00%)

by biochemical tests and also genotypic study. These isolates were also confirmed through MALDI-TOF. Rests of the isolates of *Staphylococcus* were of other species. Incidence of *S. pseudintermedius* found in the present study was similar to that (19.0%) reported by Qekwana *et al.* (2017) in dogs. Dziva *et al.* (2015) reported the higher incidence of *Staphylococcus aureus* as 58.5% in dogs suffering with otitis against our observation of 19.0%. This variation in results may be due to geographical variation or because of sample size.

Staphylococcus pseudintermedius isolates exhibited maximum sensitivity towards enrofloxacin (69.23%), followed by cephalothin (61.53%), amoxyclav (61.53%), amikacin (57.69%), gentamicin (53.84%), cefotaxime (50%), co-trimoxazole (50%), cefadroxil (50%). Maximum resistance was exhibited towards methicillin (92.30%), oxacillin (92.30%), followed by kanamycin (80.76%), bacitracin (76.92%), tetracycline (73.07%), ampicillin (65.38%), trimethoprim (61.53%) (Table 2). Similar maximum sensitivity of *Staphylococcus pseudintermedius* isolates towards enrofloxacin, cephalothin, amoxyclav, amikacin and gentamicin was also reported by Rubin *et al.* (2011) and Matanovic *et al.* (2012).

Staphylococcus aureus isolates exhibited maximum sensitivity towards cephalothin (78.26%), followed by gentamicin (60.86%), chloramphenicol (60.86%), ciprofloxacin (56.52%), co-trimoxazole (52.17%) and trimethoprim (52.17%). Overall maximum resistance was exhibited towards methicillin (100%), oxacillin (100%), followed by ampicillin (73.19%), bacitracin (73.91%), enrofloxacin (69.56%), polymyxin-B (69.56%), penicillin-G (60.86%), rifampicin (60.86%) (Table 2). Chai *et al.* (2021) also recorded similar results of *S. aureus* exhibiting maximum sensitivity towards cephalothin, gentamicin, and chloramphenicol.

Molecular Detection of *S. aureus* and *S. pseudintermedius*

After confirmation of *Staphylococcus* isolates initially through Gram staining and biochemical tests, PCR was performed for genus identification targeting *pta* gene where all 85 isolates of *Staphylococcus* showed positive amplicon size at 320 bp (Fig. 1). Similarly, Bannoehr *et al.* (2009) successfully amplified the *pta* gene from all the strains of *Staphylococcus*.

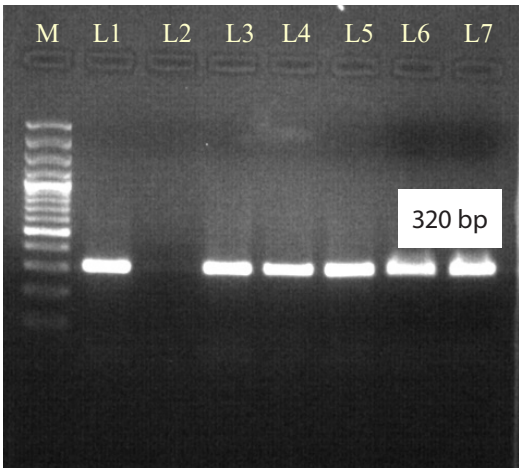

Fig. 1: Gel electrophoresis image of *pta* gene of *Staphylococcus.* M: Ladder (100 bp plus DNA ladder), L1: Positive control, L2: Negative control, L3 to L7: Samples

Table 1: Primer sequence of various genes used in the study

S. No.	Gene	Primer sequence	Amplicon size (bp)	Reference
1	*pta*	F- AAAGACAAACTTTCAGGTAA R- GCATAAACAAGCATTGTACCG	320	Bannoehr *et al.* (2009)
2	*nuc* (*S. aureus*)	F- TCGCTTGCTATGATTGTGG R- GCCAATGTTCTACCATAGC	359	Sasaki *et al.* (2010)
3	*nuc* (*S. pseudintermedius)*	F- TRGGCAGTAGGATTCGTTAA R- CTTTTGTGCTYCMTTTTGG	926	Sasaki *et al.* (2010)
4	*mecA* (*S. aureus*)	F- AGTTGTAGTTGTCGGGTTTGG R- GGCCAATTCCACATTGTTTC	454	Malik *et al.* (2006)
5	*mecA* (*S. pseudintermedius*)	F-TCCAGATTACAACTTCACCAGG R- CCACTTCATATCTTGTAACG	162	Tamilarasu *et al.* (2020)
6	*tet* K (*S. aureus*)	F- TCGATAGGAACAGCAGTA R- CAGCAGATCCTACTCCTT	169	Chai *et al.* (2021)
7	*tet* M (*S. aureus*)	F- GTGGACAAAGGTACAACGAG R- CGGTAAAGTTCGTCACACAC	406	Chai *et al.* (2021)
8	*tet* K (*S. pseudintermedius*)	F- TTAGGTGAAGGGTTAGGTCC R- GCAAACTCATTCCAGAAGCA	718	Ruzauskas *et al.* (2016)
9	*tet* M (*S. pseudintermedius*)	F- GTTAAATAGTGTTCTTGGAG R- CTAAGATATGGCTCTAACAA	646	Ruzauskas *et al.* (2016)

Table 2: Culture sensitivity test patterns of *Staphylococcus aureus* and *Staphylococcus pseudintermedius* isolates obtained from ear infections of dogs

S. No.	Antibiotics	*Staphylococcus aureus* (%)			*Staphylococcus pseudintermedius* (%)		
		Sensitive	Intermediate	Resistant	Sensitive	Intermediate	Resistant
1	Amikacin (AK)	39.13	39.13	21.73	57.69	26.92	15.38
2	Amoxyclav (AMC)	47.82	21.73	30.43	61.53	19.23	19.23
3	Ampicillin (AMP)	13.04	13.04	73.91	19.23	15.38	65.38
4	Bacitracin (B)	17.39	8.69	73.91	11.53	11.53	76.92
5	Cefadroxil (CFR)	21.73	26.08	52.17	50.00	11.53	34.46
6	Cefotaxime (CTX)	52.17	17.39	30.43	50.00	11.53	34.46
7	Cephalothin (CEP)	78.26	3.84	17.39	61.53	7.69	30.76
8	Chloramphenicol (C)	60.86	0	39.13	34.46	11.53	50.00
9	Ciprofloxacin (CIP)	56.52	8.69	34.78	42.30	7.69	50.00
10	Cloxacillin (COX)	34.78	13.04	52.18	50.00	0	50.00
11	Co-Trimoxazole (COT)	52.17	3.84	43.47	42.30	3.85	53.84
12	Doxycycline (DO)	47.82	8.69	43.47	30.76	19.23	50.00
13	Enrofloxacin (EX)	52.17	26.08	21.73	69.23	7.69	23.07
14	Erythromycin (E)	30.43	0	69.56	42.30	0	57.69
15	Gentamicin (GEN)	60.86	3.84	34.78	53.84	15.38	30.76
16	Kanamycin (K)	21.73	21.73	56.52	15.38	3.85	80.76
17	Methicillin (MET)	0	0	100	7.69	0	92.30
18	Oxacillin (OX)	0	0	100	7.69	0	92.30
19	Penicillin -G (P)	21.73	17.39	60.86	26.92	15.38	57.69
20	Polymyxin -B (PB)	8.69	21.73	69.56	11.53	30.76	57.69
21	Rifampicin (RIF)	34.78	3.84	60.86	46.15	0	53.84
22	Streptomycin (S)	21.73	34.78	43.47	34.61	15.38	50.00
23	Tetracycline (TE)	43.47	8.69	47.82	26.92	0	73.07
24	Trimethoprim (TR)	52.17	3.84	43.47	34.61	3.85	61.53

Through species specific primers targeting *nuc* genes, 23 isolates were identified as *S. aureus* (Fig. 2) and 26 isolates were identified as *S. pseudintermedius* (Fig. 3). Using species specific primers targeting for detection of methicillin and tetracycline resistance, 23 isolates of *S. aureus* were positive genotypically by PCR and carried *mecA* gene (Fig. 4) exhibiting methicillin resistance on antibiotic susceptibility testing. A total of 26 isolates of *S. pseudintermedius* were positive genotypically and exhibited *mecA* gene (Fig. 5), of these 24 isolates exhibited methicillin resistance on antibiotic susceptibility testing. A total of 14 and 12 isolates of *S. aureus* were positive genotypically by PCR for *tet*K and *tet*M genes, respectively (Fig. 6, 7), and on CST 11 isolates exhibited tetracycline resistance. Likewise 17 and 14 isolates of *S. pseudintermedius* were positive genotypically by PCR for *tet*K and *tet*M genes, respectively (Fig. 8, 9) and all exhibited phenotypically tetracycline resistance on CST.

Similar studies were carried out by Hadi and Alabbas (2023) and Malik *et al.* (2006) for detection of *mecA* gene in *S. aureus* of canine isolates where the isolates positive for *mecA* gene also exhibited methicillin resistance by CST as in the present study. Yoon *et al.* (2010), Chitra *et al.* (2015) and Tamilarasu *et al.* (2020) carried out studies on *S. pseudintermedius* for the detection of *mecA* gene where all the isolates were positive for *mecA* gene genotypically by PCR and also exhibited methicillin resistance by antibiotic susceptible testing which are in contrast to present findings, where out of 26 isolates the correlation in CST and PCR was recorded in 24 isolates. Studies carried out by Ruzauskas *et al.* (2016) on 51 *S. pseudintermedius* isolates from diseased dogs for detection of methicillin and tetracycline resistance revealed 29.4% isolates *mecA* positive, 64.7% *tet*K, *tet*M genes positive by PCR, and exhibited methicillin and tetracycline resistance on CST. Chai *et al.* (2021) carried out studies on 30 samples of dogs with ear infections, which were identified as *S. aureus* through PCR and only 3 isolates exhibited methicillin and tetracycline resistance and were *mecA*, *tet*K and *tet*M gene positive by PCR. Present study recorded little variation in correlation between CST and PCR results in methicillin and tetracycline resistance, which may be due to

the concentration of antibiotic disc used, any temperature variation or amount of bacteria inoculated or dormancy of that gene.

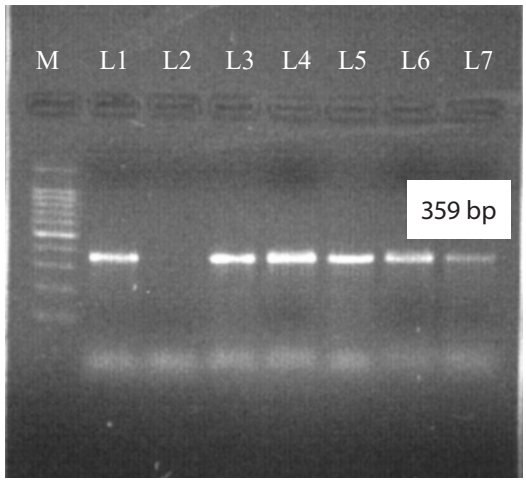

Fig. 2: Gel electrophoresis image of *nuc* gene of *S. aureus.* M: Ladder (100 bp plus DNA ladder), L1: Positive control, L2: Negative control, L3 to L7: Samples

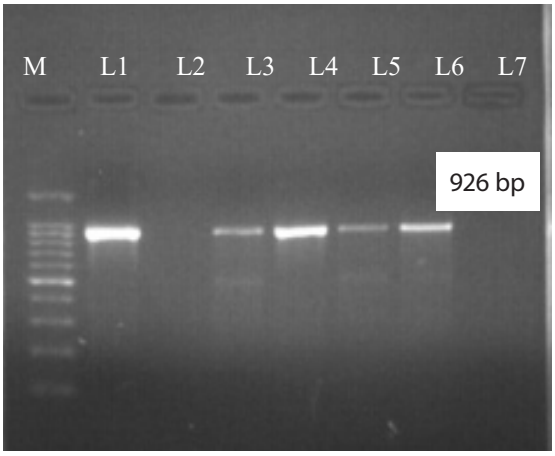

Fig. 3: Gel electrophoresis image of *nuc* gene of *S. pseudintermedius.* M: Ladder (100 bp plus DNA ladder), L1: Positive control, L2: Negative control, L3 to L7: Samples

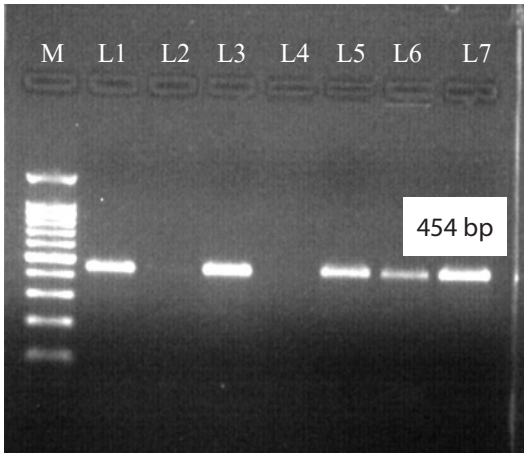

Fig. 4: Gel electrophoresis image of *mecA* gene of *S. aureus.* M: Ladder (100 bp plus DNA ladder), L1: Positive control, L2: Negative control, L3 to L7: Samples

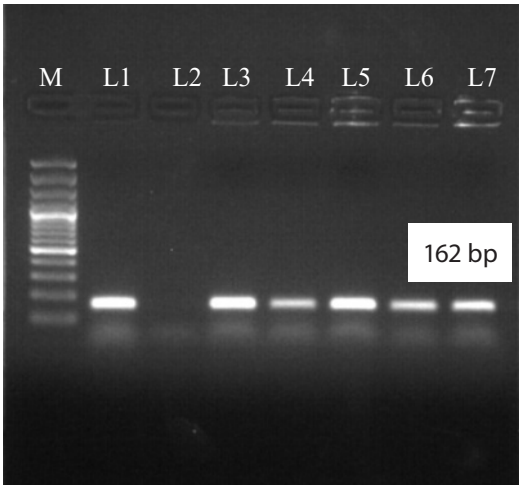

Fig. 5: Gel electrophoresis image of *mecA* gene of *S. pseudintermedius.* M: Ladder (100 bp plus DNA ladder), L1: Positive control, L2: Negative control, L3 to L7: Samples

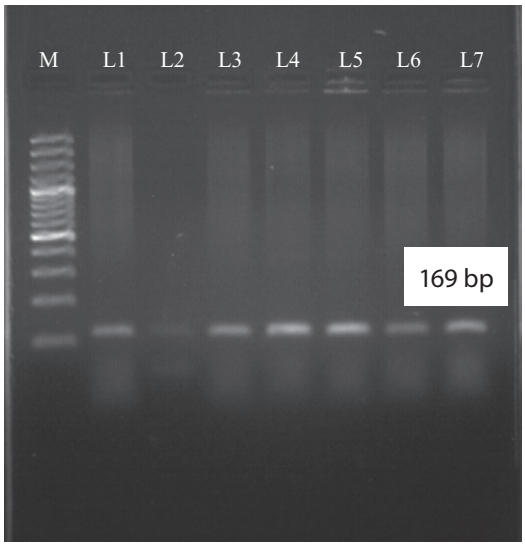

Fig. 6: Gel electrophoresis image of *tet*K gene of *S. aureus.* M: Ladder (100 bp plus DNA ladder), L1: Positive control, L2: Negative control, L3 to L7: Samples

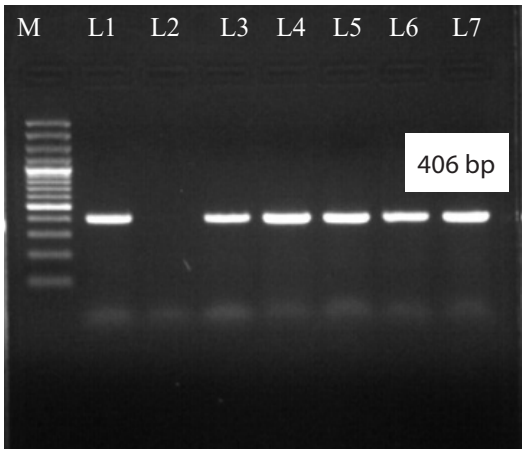

Fig. 7: Gel electrophoresis image of *tet*M gene of *S. aureus.* M: Ladder (100 bp plus DNA ladder), L1: Positive control, L2: Negative control, L3 to L7: Samples

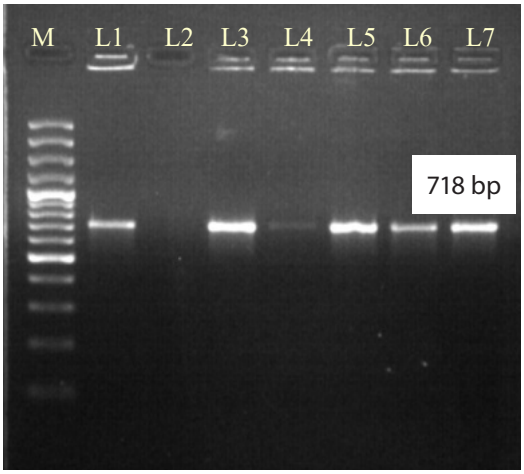

Fig. 8: Gel electrophoresis image of *tet*K gene of *S. pseudintermedius.* M: Ladder (100 bp plus DNA ladder), L1: Positive control, L2: Negative control, L3 to L7: Samples

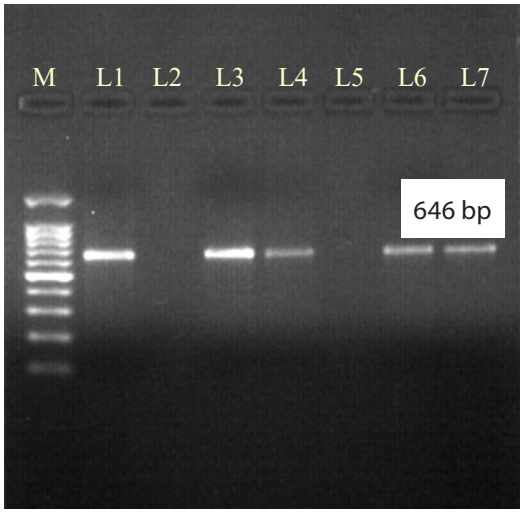

Fig. 9: Gel electrophoresis image of *tet*M gene of *S. pseudintermedius.* M - Ladder (100 bp plus DNA ladder), L1: Positive control, L2: Negative control, L3 to L7: Samples

Conclusion

The present study revealed a high incidence of *Staphylococcus* related ear infections in dogs. Isolates of *S. aureus* and *S. pseudintermedius* were identified by microbiological examination and exhibited variation in the sensitivity pattern on CST which suggest that the veterinary clinician should perform proper bacteriological examination for the identification of proper etiological cause which will largely help in choosing appropriate antibiotics for correct treatment. This will further prevent the development and spread of antibiotic resistance. The study focused on the molecular characterization of *S. aureus* and *S. pseudintermedius* associated with companion animal infections and also major public health concern because of the development of multi-drug resistance by these bacteria to several antibiotics. From public health point of view staphylococcal infections should be controlled as there are chances of the similar strains to cause infection in the people living in near contact with dogs and spread the disease which can be difficult to treat.

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Announcement - IV, Associate Fellow Award: SVSBT-2025

Applications in the prescribed formats are invited from the **Life Members** of the Society for the **Associate Fellow Awards of SVSBT-2025**. The awards will be honoured to the deserving candidates during the inaugural ceremony of ***XII Annual Convention and International Conference of SVSBT on "Bridging Science and Society: Biotechnology for Sustainable One Health"*** to be held **during December 3-5, 2025** at College of Veterinary Science, LUVAS, **Hisar-125 004**, Haryana, India.

The application format can be had on request from the President, SVSBT by e-mail at ajdhami59@gmail.com or whatsapp No. 9898262498. The application must be submitted as **soft copy in single pdf at** ajdhami59@gmail.com **along with one hard copy** on or before **1st October, 2025** in person or by speed post/courier **to the President, SVSBT, Dr. A. J. Dhami**, 48, Mangal Nagar, Vidya Dairy Road, Near Borsal Crossing, **Anand-388 001**, Gujarat. It is mandatory that the **applicant must be a life member of the Society, or else he/she should become life member by due date of application** through paying prescribed fees online in the UCO Bank A/C as detailed below. Incomplete applications and those received after the due date will not be entertained.

Eligibility Criteria & Rules for SVSBT Associate Fellow Award ((Scorecard on website: svsbt.com)

- The applicant must be having MVSc/PhD degree in Veterinary Science or Animal Biotech, and life membership of the SVSBT.
- Applicant should be between 40 and 50 years of age on the last date of application.
- He/She should be Indian national, who is working in the field of Veterinary Science & Animal Biotechnology in India.
- Application in the prescribed format must include Full name, Present Designation, Address with E-mail Id and Contact No., Date of birth, Qualifications, and other details as per the format that can be had from the President SVSBT on request.
- Processing fee of Rs. 1000/- is to be paid by the applicant on or before last date of application.
- Applicant must provide evidence of each claim he/she is making in this application.
- Selected candidate shall pay Rs. 5000/- online within 15 days of intimation of results.
- SVSBT reserves the rights for the final selection of the awardees.

Bank Details for Processing Fee: Online Payment i**n favour of** Society for Veterinary Science and Biotechnology, **payable at UCO Bank, Anand Branch, Account No. 18400110008843, IFSC code: UCBA0000082**.