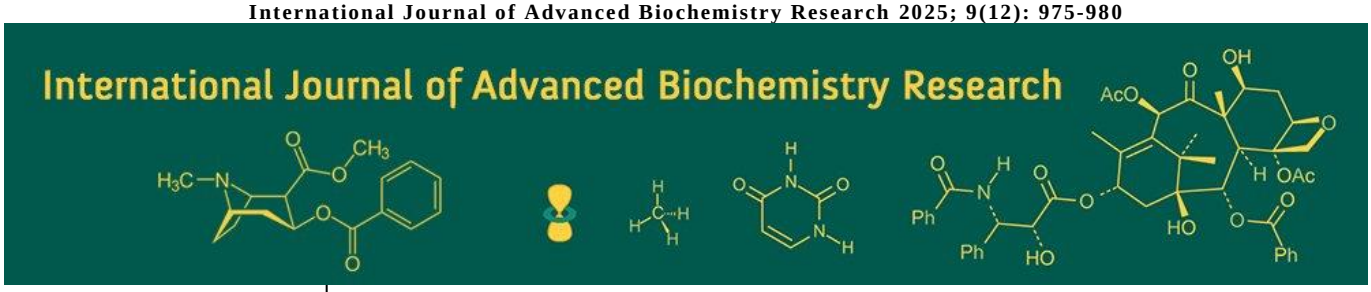

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Molecular detection of major coagulase-positive *Staphylococcus* isolates from the canine otitis externa

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Abstract
Canine otitis externa (COE) represents one of the most frequently diagnosed inflammatory diseases in dogs, with coagulase-positive staphylococci (CoPS) serving as major etiological agents. This study investigated the prevalence and molecular characterization of CoPS isolated from different breeds of dogs presenting with clinical otitis externa. A total of 87 ear swabs were collected, yielding 53 *Staphylococcus* isolates after primary staining. Of these, 47 (88.7%) tested positive for coagulase production using the tube coagulase test. All coagulase-positive isolates were confirmed as belonging to the genus *Staphylococcus* by 16S rRNA gene-targeted PCR. Species-level identification was performed using species-specific thermonuclease (nuc) gene PCR assays targeting *S. pseudintermedius* (*Pse-nuc*), *S. aureus* (*Au-nuc*) and *S. schleiferi* subsp. *coagulans* (*Sch-nuc*). *S. pseudintermedius* was the predominant species (67.92%, 36/53 isolates), followed by *S. aureus* (15.09%, 8/53), *S. schleiferi* subsp. *coagulans* (5.66%, 3/53) and coagulase-negative or unidentified *Staphylococcus* spp. (11.3%, 6/53). These results underscore the dominance of *S. pseudintermedius* among coagulase-positive staphylococci associated with COE.

Keywords: COE, CoPS, *S. pseudintermedius*, *S. aureus*, *S. schleiferi* subsp. *coagulans*, nuc gene, 16S rRNA

Introduction
Canine otitis externa (COE) refers to inflammation of the external auditory canal, which can also involve the ear pinna. It can present as either acute or chronic, with the chronic form defined as persistent or recurring inflammation lasting three months or more. (Huang *et al.*, 2009) [1]. The canal is lined by stratified squamous epithelium similar to skin, containing hair follicles, sebaceous glands and ceruminous (modified apocrine) glands, and it harbours a resident microbiota; these features show considerable inter-breed variation, which represents a major predisposing factor (Miller *et al.*, 2012; Njaa *et al.*, 2012) [2, 3]. The prevalence in the general dog population is estimated to be between 8.7% and 20% (Saridomichelakis *et al.*, 2007; Topală *et al.*, 2007) [4, 5]. Clinically, it manifests as pruritus, erythema, oedema and otic discharge, frequently accompanied by pain and malodour. Affected dogs typically exhibit behavioural changes, including frequent head shaking, vigorous ear scratching and rubbing of the ears on surrounding objects or surfaces (Summers *et al.*, 2019) [6]. Early and precise diagnosis combined with targeted therapy is critical to achieve resolution, alleviate discomfort and minimize the risk of chronicity or recurrence. Otitis externa remains a common complaint in small animal practice and is often challenging to manage successfully because of its multifactorial and recurrent nature. Breeds with pendulous pinnae, such as Cocker Spaniels, Basset Hounds and Labrador Retrievers, are predisposed owing to poor ventilation, retention of moisture and debris within the ear canal and subsequent promotion of microbial proliferation (Rosser, 2004) [7].
Coagulase-positive staphylococci (CoPS) are gram-positive bacteria that commonly exist as commensals on the skin and mucous membranes of humans and animals while also acting as opportunistic pathogens (Fontana & Favaro, 2018) [8]. These organisms typically colonize areas such as the nasal cavity, throat and perianal region in a symbiotic relationship with their hosts (Nagase *et al.*, 2002; Wertheim *et al.*, 2005) [9, 10]. The CoPS group currently comprises ten recognized species: *Staphylococcus aureus*, *S. intermedius*, *S. pseudintermedius*, *S. coagulans* (formerly *S. schleiferi* subsp. *coagulans*), *S. hyicus* (variable

coagulase activity), *S. delphini*, *S. lutrae*, *S. agnetis* (variable coagulase activity), *S. cornubiensis* and *S. ursi* (González-Domínguez *et al.*, 2020; Perreten *et al.*, 2020) [11, 12].

S. pseudintermedius is the predominant CoPS species causing skin and soft-tissue infections in dogs. It commonly colonizes the skin, mucosa or GIT of over 50% of healthy dogs and fewer healthy cats. Normally commensal, it serves as an opportunistic pathogen and a primary cause of canine pyoderma and otitis externa (Paul *et al.*, 2012) [13].

Several methods exist for bacterial detection, but multiplex PCR (M-PCR) is the most rapid and convenient for identifying coagulase-positive staphylococci (CoPS). (Sasaki *et al.*, 2010) [14] analyzed nuc gene sequences in CoPS and related species to develop an M-PCR assay targeting the nuc locus for reliable species differentiation. Conventional identification involves cultural isolation, biochemical tests, and sugar fermentation patterns, which often require more than 72 hours for genus-and species-level confirmation. To overcome these delays, molecular approaches like M-PCR have become the preferred method for faster and more accurate results. This study is a molecular survey of CoPS isolates from COE, aimed at expanding our understanding of their phenotypic and genotypic identification.

Materials and Methods

Sample collection

Dogs exhibiting head shaking, downward ears, pinna swelling and purulent discharge were selected for sample collection attending the Veterinary Clinical Complex, Veterinary College, Junagadh, Gujarat, India. Sterile cotton-tipped swabs moistened with sterile 0.9% saline solution were used to collect ear exudate samples from 87 COE patients. These swabs were transported to the Department of Veterinary Microbiology, Kamdhenu University, Junagadh, on the same day while maintaining a cold chain or stored at 4 °C until further processing.

Isolation and biochemical characterization

The samples collected from the clinical cases of COE were streaked onto brain heart infusion (BHI) agar plates and incubated at 37 °C for 24 hours to obtain pure colonies. The bacterial isolates were identified at the genus level by observing colony characteristics, performing Gram's staining, examining microscopic morphology and assessing growth on Mannitol Salt Agar (HiMedia Laboratory, Mumbai). Additional biochemical tests (oxidase, tube coagulase and catalase tests) were performed according to the proper procedures (Quinn *et al.*, 1994) [15].

Genomic DNA extraction from bacteria

Genomic DNA was extracted from pure *Staphylococcus* cultures using the conventional Proteinase K-SDS method (Sambrook and Russell, 2001) [16]. The quality and concentration of the isolated DNA were determined using a μDrop™ Plate in a μDrop plate reader (Thermo Scientific).

PCR-based detection of the *Staphylococcus* genus and species

Primers specific for detecting the genus and species of CoPS were used according to protocols from various researchers. Details of the primers, including their names, oligonucleotide sequences, targeted genes and product sizes are provided in Table 1. The composition of the PCR reaction mixture followed the guidelines established by Mason *et al.* (2001) [17] and (González-Domínguez *et al.*, 2020) [11]. The PCR reaction mixture was prepared as per Table 2. The PCR conditions for detecting the *Staphylococcus* genus-specific 16S rRNA gene included an initial denaturation step at 95 °C for 5 minutes, followed by 35 cycles of 45 seconds at 95 °C for denaturation, 40 seconds at 55 °C for annealing and a 45-second extension at 72 °C. The final extension was done at 72 °C for 7 minutes. For *Staphylococcus* species-specific gene amplification (*Pse-nuc*, *Au-nuc* and *Sch-nuc*), the cycling conditions included an initial denaturation at 95 °C for 5 minutes, followed by 35 cycles of 10 seconds denaturation at 95 °C, 30 seconds annealing at 60 °C and 30 seconds extension at 72 °C, with a final extension at 72 °C for 5 minutes (Table 3). PCR amplifications were performed using a programmable thermal cycler (Verity, Applied Biosystems by Life Technologies, Singapore). The resulting PCR products (4 µL) were analyzed by agarose gel electrophoresis (1.5% w/v), and the gel visualization was done by using a gel documentation system (Bio-PrintST4® Vilber Lourmat).

Table 1: Oligonucleotide sequences (primers) used for the characterization of major CoPS by targeting genus-specific and species-specific target genes

Target gene	Organism	Primer sequence (5' to 3')	Product size	Reference
16S rRNA	Genus-*Staphylococcus*	F: CCTATAAGACTGGGATAACTTCGGG R: CTTTGAGTTTCAACCTTGCGGTCG	791bp	(Mason *et al.*, 2001) [17]
Pse-nuc	*Staphylococcus pseudintermedius*	F: TGATGCAGCTTTTCCGTATG R: AAAGATGGGCAAGATGAACG	99bp	(González-Domínguez *et al.*, 2020) [11]
Au-nuc	*S. aureus*	F: CAGAAACGGTGAAACCGAAT R: CCATAGCGGTCTTGCTTTTC	127bp	
Sch-nuc	*S. schleiferi* subsp. *coagulans*	F: TTAAAACGACGGAAGGCAGT R: CCAATCATACGCACACGTTC	115bp	

(F, forward; R, reverse)

Table 2: Amounts and concentrations of the components utilized in the PCR reaction

Sr. No.	Components	PCR Reaction
1.	PCR Master Mix (2X)	12.5 µl
2.	Forward Primer (10 pmol/µl)	1 µl
3.	Reverse Primer (10 pmol/µl)	1 µl
4.	Template DNA	3 µl
5.	NFW (Nuclease Free Water)	7.5 µl
	Total Reaction volume	25 µl

Table 3: Thermal cycling conditions for different primer pairs used in PCR for the identification of the *Staphylococcus* genus and major CoPS

Target gene	Cycling conditions				
	Initial denaturation	Denaturation	Annealing	Extension	Final extension
16SrRNA	95 °C for 8 min.	95 °C for 45 sec.	55 °C for 40 sec.	72 °C for 45 sec.	72 °C for 7 min.
		Repeated for 35 cycles			
Pse-nuc	95 °C for 5 min.	95 °C for 10 sec.	60 °C for 30 sec.	72 °C for 30 sec.	72 °C for 5 min.
		Repeated for 35 cycles			
Au-nuc	95 °C for 5 min.	95 °C for 10 sec.	60 °C for 30 sec.	72 °C for 30 sec.	72 °C for 5 min.
		Repeated for 35 cycles			
Sch-nuc	95 °C for 5 min.	95 °C for 10 sec.	60 °C for 30 sec.	72 °C for 30 sec.	72 °C for 5 min.
		Repeated for 35 cycles			

Results and Discussion

Isolation, identification and biochemical characterization

A total of 110 bacterial isolates were obtained from clinical cases of COE. Primary bacterial isolation was carried out on BHI agar. Out of which, 53(48.18%) isolates of *Staphylococcus* spp. were identified based on their morphologies, culture characteristics, Gram's staining and biochemical tests from clinical cases (E.g., oxidase test, catalase test and tube coagulase test) of COE (Fig.1, 2 and 3). In the tube coagulase test, 47/53 *Staphylococcus* spp. were found coagulase-positive, while 6/53 were coagulase-negative (other *Staphylococcus* species). A similar study on the COE by Parmar *et al.* (2020) [18] and Vanisree *et al.* (2025) [19] identified *Staphylococcus* spp. as the most common pathogen in 56.57% and 70.24% of cases, which was slightly higher than observed in the current study, 48.18%. Whereas Hassan *et al.* (2023) [20] found a lower incidence of *Staphylococcus* spp. (36%), which was slightly lower than this study.

S. pseudintermedius was the most prevalent species, accounting for 67.92% (36/53). *S. aureus* was identified in 15.09% (8/53), while *S. schleiferi* subsp. *coagulans* accounted for 5.66% (3/53). Additionally, 11.32% (6/53) of the isolates were categorized as other *Staphylococcus* species (Chart 1). Similar results were obtained by Hassan *et al.* (2023) [20], who reported 41.6% *S. pseudintermedius* and 22.2% *S. aureus*; Prošić *et al.* (2024) [21] reported 65.8% as *S. pseudintermedius*, 22.4% as *S. aureus*, 7.9% as *S. coagulans* and 3.9% as *S. intermedius*; Rana *et al.* (2022) [22] reported 78.16% *S. pseudintermedius*, 19.71% *S. aureus* and 2.11% other *Staphylococcus* spp.; Makwana *et al.* (2023) [23] also reported *S. pseudintermedius (40.63%), S. schleiferi* subsp. *coagulans* (18.75%) *and S. aureus* (15.63%). Whereas a higher percentage of incidence was reported by Tamakan & Gocmen (2022) [24], 87.5% *S. pseudintermedius*, 9.37% *S. aureus* and 3.12% *S. schleiferi*. Kadhim & Abdullah (2022) [25] and Núñez *et al.* (2025) [26] also found the highest prevalence for *S. pseudintermedius*, 28.5% and 37.5% respectively, among all *Staphylococcus* species, which was less in percentage for *S. pseudintermedius* than this study.

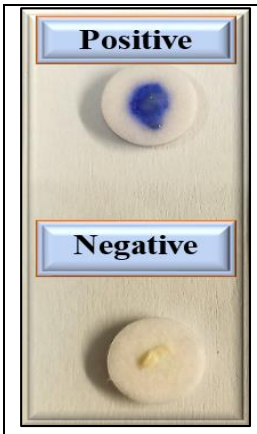


Fig 1: Oxidase test

Fig 2: Catalase test

Fig 3: Tube coagulase test

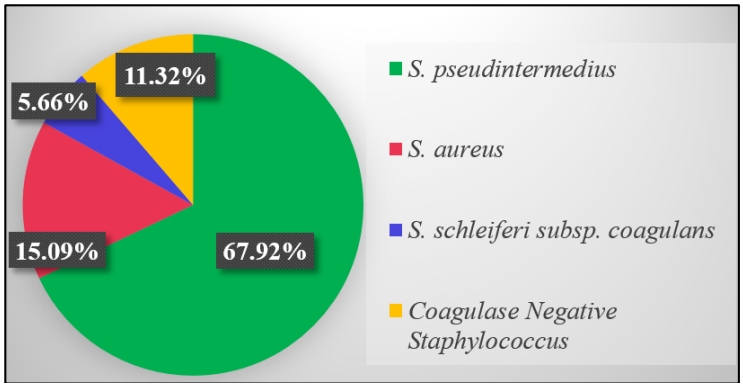
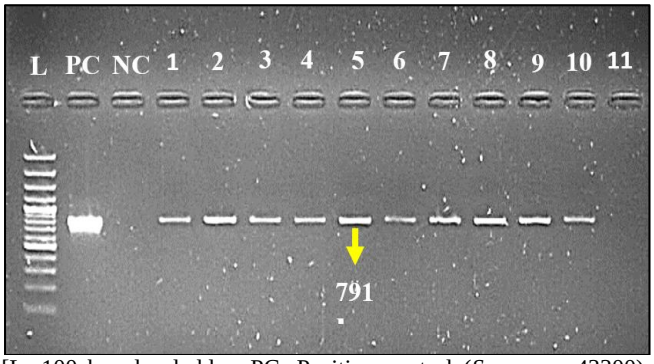


Chart 1: Detection of various *Staphylococcus* spp.

Genus-confirmation and species-level identification of CoPS by PCR

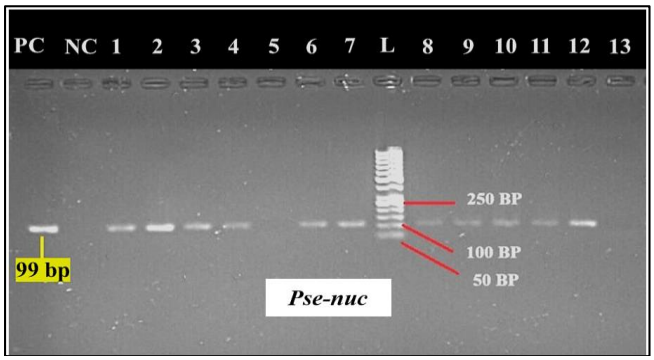
For confirmation of the genus *Staphylococcus*, 16S rRNA gene-targeted primers originally developed by Mason *et al.* (2001) [17] were utilized in this study. All 53 isolates that had been presumptively identified by phenotypic methods successfully amplified the expected 16S rRNA gene fragment, thereby confirming their identity as members of the genus *Staphylococcus* through PCR. Species-level differentiation among the major CoPS was achieved using species-specific thermonuclease (*nuc*) gene primers for *S. aureus*, *S. pseudintermedius* and *S. schleiferi* subsp. *coagulans*, as previously reported by González-Domínguez *et al.* (2020) [11]. Reference strains *S. aureus* ATCC 43300, *S. pseudintermedius* ATCC 49444 and *S. schleiferi* subsp. *coagulans* ATCC 49545 served as positive controls, whereas *Escherichia coli* MTCC-722 was included as a negative control to validate the specificity of the nuc gene-based PCR assays.

Among the 53 phenotypically presumptive staphylococcal isolates, 47 were successfully verified as members of the genus *Staphylococcus* through amplification of the genus-specific 16S rRNA gene in PCR (Fig. 4).



[L: 100 bp plus ladder; PC: Positive control (*S. aureus* 43300); Positive samples: 1 to 10; Negative samples: 11; NC: Negative control (*E. coli* MTCC 722)]

Fig 4: Genus-specific PCR of *Staphylococcus* spp. for 16S rRNA gene



[L: 50 bp plus ladder; PC: Positive control (*S. pseudintermedius*); Positive samples: 1 to 4, 6 to 12; Negative samples: 5, 13; NC: Negative control]

Fig 5: Species-specific PCR of *S. pseudintermedius* for *Pse-nuc* gene

Subsequent species-specific PCR targeting the *nuc* gene revealed the following distribution: 36 isolates (67.92%) produced the characteristic 99-bp amplicon diagnostic for *S. pseudintermedius*, 8 isolates (15.09%) showed the 127-bp product indicative of *S. aureus* and 3 isolates (5.66%) generated the 115-bp fragment specific to *S. schleiferi* subsp. *coagulans*. The remaining 6 isolates (11.32%) of the molecularly confirmed *Staphylococcus* population) did not amplify any of the three species-specific nuc gene targets and therefore could not be assigned to these major coagulase-positive species.

In the present study, all staphylococcal isolates were molecularly confirmed as belonging to the genus *Staphylococcus* through amplification of the 16S rRNA gene. This complete concordance between phenotypic and genotypic identification is consistent with findings from several previous reports. Notably, Mathapati *et al.* (2016) [27], Chaudhary *et al.* (2021) [28] and Kadhim & Abdullah (2022) [25] similarly achieved 100% positivity for the 16S rRNA gene among their staphylococcal isolates from canine clinical samples. Likewise, Zedan *et al.* (2023) [29] successfully confirmed all 29 phenotypically presumptive *Staphylococcus* isolates via genotypic detection of the same marker.

In the present study, 76.60% (36/47) of CoPS isolates were confirmed as *S. pseudintermedius* through detection of the species-specific *nuc* gene. This prevalence is notably higher than that reported in several prior investigations using similar PCR-based methodologies. For instance, Weese *et al.* (2010) [30] and Dziva *et al.* (2015) [31] identified *S. pseudintermedius* in 39.2% and 38.5% of isolates, respectively, while Vincze *et al.* (2014) [32] and Bannoehr *et al.* (2015) [33] documented rates around 45%. In contrast, Schmidt *et al.* (2017) [34] reported a substantially higher positivity rate of 89.21%, whereas Kadhim & Abdullah (2022) [25] observed a lower prevalence of 28.5% (10/35) among staphylococcal isolates. More recently, Chehida *et al.* (2024) [35] detected S. pseudintermedius in 64.4% of 99 CoPS isolates based on thermonuclease gene profiling.

The high prevalence of *S. pseudintermedius* in our study (76.60%) confirms its status as the dominant pathogen in COE and the leading CoPS in canine skin and ear infections. Observed differences among studies likely stem from variations in geographic location, sample size, case type (acute vs. chronic), or specimen collection method, underscoring the value of ongoing molecular surveillance for regional epidemiological insights.

In the current study, 17.02% (8/47) of CoPS isolates were identified as *S. aureus* based on the detection of the *Au-nuc* gene. This proportion is somewhat lower than, yet comparable to, rates reported in previous investigations. For example, Weese *et al.* (2010) [30] documented *S. aureus* in 32.4% of canine staphylococcal isolates, while Davis & Thompson (2021) [36] reported a 29.2% incidence in cases of COE. Similarly, Nakamura *et al.* (2020) [37] and Ruzauskas *et al.* (2016) [38] observed incidences of 25.9% and 25%, respectively, while Chehida *et al.* (2024) [35] identified *S. aureus* in 20.2% of CoPS isolates. Although slightly reduced in our cohort, this prevalence falls within the typical range described in the literature and supports the recognition of *S. aureus* as an important, albeit secondary, pathogen relative to *S. pseudintermedius* in canine skin and ear infections.

In the present study, *S. schleiferi* subsp. *coagulans* accounted for 6.38% (3/47) of staphylococcal isolates. This proportion is moderately lower than the rates documented in prior reports, including 10.2% by (Ruzauskas *et al.*, 2016) [38], 15% by (Garcia *et al.*, 2022) [39] and 10.1% by (Chehida *et al.*, 2024) [35].

Conclusions

This study establishes Staphylococcus spp. as the foremost etiological agent in canine otitis externa (48.18% of isolates), with coagulase-positive staphylococci dominating (88.68%). Integrative phenotypic and genotypic analyses confirmed *S. pseudintermedius* as the predominant pathogen (67.92% overall; 76.60% of CoPS), followed by *S. aureus*, *S. schleiferi* subsp. *coagulans*. PCR-based species-specific nuc gene amplification proved superior in precision and sensitivity to biochemical methods, enabling accurate identification of non-specific isolates. This marked ascendancy of *S. pseudintermedius* reinforces its primacy in canine otic and cutaneous infections, often exceeding global prevalences. These findings mandate precise species-level diagnostics to guide targeted therapy and antimicrobial stewardship, while geographic variability underscores the need for sustained molecular surveillance to combat evolving resistance in staphylococcal populations.

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Conflict of Interest

All authors declare no conflict of interest.

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