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Molecular confirmation of *Pseudomonas aeruginosa* in dogs affected with otitis externa

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Abstract

The present study was carried out to confirm *Pseudomonas aeruginosa* isolates and detect extended-spectrum β -lactamase (ESBL) genes in dogs affected with otitis externa. A total of 52 cases were clinically diagnosed as otitis externa amongst 263 dogs of various ages and breeds examined at the Teaching Veterinary Clinical Complex, College of Veterinary Science and Animal Husbandry, Anjora, Durg, as well as at several Government Veterinary Hospitals and private pet clinics in and around Durg. Among these, *Pseudomonas* species were isolated from 19 cases, which were further subjected to molecular confirmation. Amplification of the species-specific 16S rRNA gene (956 bp) confirmed *Pseudomonas aeruginosa* in all 19 samples. Tentatively confirmed *P. aeruginosa* isolates were screened for ESBL-producing genes (*bla*_{TEM}, *bla*_{SHV} and *bla*_{CTX-M}) using PCR. The prevalence of ESBL genes was found to be 36.84% (*bla*_{TEM}, n = 7), 21.05% (*bla*_{SHV}, n = 4), and 5.26% (*bla*_{CTX-M}, n = 1). The study confirms the molecular presence of *P. aeruginosa* in canine otitis externa and highlights the occurrence of ESBL-producing strains, indicating the need for prudent antimicrobial use in veterinary practice.

Keywords: *Pseudomonas aeruginosa*, otitis externa, dog, ESBL genes, molecular detection

Introduction

Otitis externa (OE) is a commonly encountered and clinically important inflammatory disorder of the external ear canal in dogs which may extend from the auricle to the tympanic membrane. It significantly affects animal welfare and can predispose to otitis media or interna if inadequately treated. The overall prevalence of canine OE has been reported between 5-20 % of the canine population in various studies (Rougier *et al.*, 2005; Korbélik *et al.*, 2019) [1, 2]. It is a multifactorial disease influenced by predisposing, primary, secondary, and perpetuating factors, with microbial infection being a critical component in most chronic or recurrent cases (Hariharan *et al.*, 2006) [3].

Among the bacterial pathogens, *Pseudomonas aeruginosa* has emerged as a predominant cause of chronic or treatment-resistant OE (Barnard *et al.*, 2017) [4]. This opportunistic Gram-negative bacterium is capable of forming biofilms, producing tissue-damaging enzymes, and expressing multiple antibiotic resistance mechanisms, making infections difficult to eradicate (Garcias *et al.*, 2025) [5]. Studies have demonstrated that *P. aeruginosa* accounts for approximately 15-20 % of bacterial isolates from canine OE and is frequently associated with severe, purulent otic discharges, ulceration, and persistent inflammation (Morris *et al.*, 2021; Raus *et al.*, 2023) [6, 7].

The empirical use of antimicrobials without culture and sensitivity testing remains common in veterinary practice, which can contribute to the emergence of various multidrug-resistant (MDR) and extended-spectrum β -lactamase (ESBL)-producing strains (Pye, 2022; Kashyap *et al.*, 2023) [8, 9]. The identification of β -lactamase genes, including *bla*_{TEM}, *bla*_{SHV} and *bla*_{CTX-M} in *P. aeruginosa* isolates obtained from dogs underscores the growing issue of antimicrobial resistance and raises concerns regarding possible zoonotic transmission (Kadhim *et al.*, 2023) [10].

Considering the rising incidence of resistant otic infections, molecular confirmation and characterization of *P. aeruginosa* and associated resistance determinants are essential for accurate diagnosis, effective therapy, and antimicrobial stewardship.

Hence, the present study was undertaken to confirm *P. aeruginosa* isolates molecularly from dogs affected with otitis externa through 16S rRNA gene amplification and to detect ESBL-producing genes.

2. Materials and Methods

2.1 Study location: The study titled “Molecular confirmation of *Pseudomonas aeruginosa* in dogs suffering from otitis externa” was carried out at the College of Veterinary Science and Animal Husbandry, Anjora, under Dau Shri Vasudev Chandrakar Kamdhenu Vishwavidyalaya, Durg, Chhattisgarh, India.

Source of animals: Dogs brought with ear disorders to the Teaching Veterinary Clinical Complex (TVCC), College of Veterinary Science and Animal Husbandry, Anjora, Durg, as well as to different government veterinary hospitals and private pet clinics in and around Durg, were enrolled in the investigation. Details such as age, breed, sex, date of sample collection, type of lesion, and clinical signs observed in dogs diagnosed with otitis externa were systematically documented.

2.2 Sample collection: Cotton ear swabs (Astra Bioscience Ltd., Kerala, India) were used for collecting samples from dogs with clinical otitis externa. Under aseptic conditions, each sterile swab was gently introduced into the external auditory canal and rotated for 10-15 seconds at the point where the vertical and horizontal canals meet to ensure proper sample collection. Samples were collected irrespective of age, sex or breed and transported to the laboratory for bacteriological examination following Wilkinson and Harvey (1994)^[11].

2.3 Media for isolation of bacterial isolates

Media used for bacteriology included nutrient broth, nutrient agar, mannitol salt agar, MacConkey agar, *Pseudomonas* isolation agar and eosin-methylene blue agar (HiMedia Laboratories Pvt Ltd., Mumbai) for initial culturing.

2.4 Morphological and biochemical identification of isolates

Gram's crystal violet, Gram's iodine, Gram's decolourizer and safranin (0.5 % w/v; HiMedia Laboratories, Mumbai) were used for Gram staining. Presumptive identification of *P. aeruginosa* was done according to the methods of Goto and Enomoto (1970)^[12].

2.5 Isolation of *Pseudomonas aeruginosa*

The collected ear swabs were initially inoculated into CFC (Cephalothin-sodium fusidate-cetrimide) broth base enriched with CetriNix supplement and incubated at 25 °C for 48 hours. After incubation, the cultures were streaked onto cetrimide agar or *Pseudomonas* agar base supplemented with CetriNix and further incubated at 37 °C for 24-48 hours. Colonies producing characteristic blue-green or brown pigmentation were provisionally considered as *P. aeruginosa* and subsequently subjected to biochemical tests for confirmation Goto and Enomoto (1970)^[12].

2.6 Phenotypic confirmation of ESBL

Isolates were screened phenotypically for ESBL production using ESBL agar (HiMedia) with ESBL-selective

supplement (FD278). Pink or purple colonies indicated ESBL-producing *Pseudomonas* spp.

2.7 Genotypic characterization of *Pseudomonas aeruginosa*

DNA extraction, PCR amplification, and gene detection were carried out as follows:

2.7.1 Genomic DNA extraction: Pure bacterial isolates were cultured in 5 ml of Luria-Bertani (LB) broth and incubated at 37 °C for 12-16 hours. From this culture, 1.5 ml was centrifuged at 8,000 rpm (approximately 6,800 ×g) for 6 minutes. The resulting pellet was washed twice with phosphate-buffered saline (PBS), resuspended in 300 µl of distilled water, and subjected to boiling at 100 °C for 10 minutes. The samples were then immediately chilled on ice for 20 minutes and centrifuged at 10,000 ×g for 2 minutes. The supernatant containing DNA was collected and preserved at -20 °C. The concentration and purity of the extracted DNA (A260/280 ratio between 1.8 and 2.0) were assessed using a NanoDrop spectrophotometer (Nicklas *et al.*, 2003)^[13].

2.7.2 16S rRNA PCR for species confirmation: Each PCR assay for detection of 16S rRNA gene (Table 1) was carried out in a total volume of 25 µl containing 12.5 µl of DreamTaq PCR Master Mix, 0.5 µl each of forward and reverse primers (0.5 mM), 1 µl of DNA template, and 10.5 µl of nuclease-free water. The amplification protocol involved an initial denaturation step at 94 °C for 2 minutes, followed by 25 cycles of denaturation at 94 °C for 20 seconds, annealing at 58 °C for 20 seconds, and extension at 72 °C for 40 seconds, with a final extension at 72 °C for 5 minutes. The amplified products were resolved on 1.5% agarose gel to detect the expected 956 bp fragment (Spilker *et al.*, 2004)^[14].

2.7.3 Detection of ESBL genes by multiplex PCR

Multiplex PCR for *bla*_{TEM}, *bla*_{SHV} and *bla*_{CTX-M} gene detection (Table 2) used reaction mixture of 25 µl: 3 µl 10× PCR buffer, 2.5 µl dNTP mix, 6 µl primers, 0.25 µl Taq polymerase, 3 µl genomic DNA and 10.25 µl nuclease-free water. Thermocycling: initial denaturation 95 °C for 5 min; 30 cycles of 94 °C for 2 min, 55 °C for 20 s, 72 °C for 40 s; final extension at 72 °C for 2 min (Apaka *et al.*, 2010; Paterson *et al.*, 2003; Boyd *et al.*, 2004)^[15, 16, 17]. Amplified products were resolved on 1.6 % agarose gel in 1× TBE buffer containing ethidium bromide (0.5 µg/ml), electrophoresed at 70 V for 45 min and visualised using Gel Doc™ XR system.

3. Results and Discussion

3.1 Molecular confirmation of *Pseudomonas aeruginosa*

All presumptively identified *Pseudomonas* spp. isolates (n = 19) on the basis of cultural and biochemical characteristics were subjected to molecular characterization by polymerase chain reaction using species-specific 16S rRNA primers. A clear amplification of a 956 bp fragment (Fig.1) specific to *P. aeruginosa* was obtained in all isolates (n = 19) recovered from canine ear swab samples, thereby confirming their molecular identity. The presence of a single distinct band of the expected amplicon size indicated the assay's specificity and reliability.

Universal primers designed to amplify conserved segments of the bacterial 16S rRNA gene are commonly employed for accurate species-level identification of *Pseudomonas* isolates (Tyler *et al.*, 1995) [18]. The present results are consistent with earlier reports on molecular confirmation of *P. aeruginosa* from canine otitis externa (Serrano *et al.*, 2016) [19], human infections (Elfadadny *et al.*, 2024) [20], bovine mastitis (Rawat *et al.*, 2021; Huang *et al.*, 2024) [21, 22], and environmental water samples (Kaszab *et al.*, 2019) [23].

The outcome of the present study substantiates that PCR targeting the 16S rRNA gene provides a sensitive as well as specific molecular method for the confirmation of *P. aeruginosa*. This approach overcomes the limitations of phenotypic identification, which often requires laborious and time-consuming biochemical assays that can be influenced by the organism's variable phenotypic traits. Similar observations have also been stated by Spilker *et al.* (2004) [14], who demonstrated the reliability of 16S rRNA gene-based PCR for the accurate identification and differentiation of *Pseudomonas* species.

The adoption of molecular diagnostic tools in Veterinary bacteriology enables early detection and precise identification of *P. aeruginosa* in otitis infections, facilitating rational antimicrobial selection and improved clinical management. Considering the high recurrence rate and antimicrobial resistance frequently associated with *P. aeruginosa*, the use of 16S rRNA-based PCR offers a valuable diagnostic advancement for veterinary clinical laboratories.

3.2 Detection of Extended-Spectrum β -Lactamase (ESBL) producing *P. aeruginosa*

3.2.1 Phenotypic detection of ESBL producers

A total of 19 *Pseudomonas aeruginosa* isolates recovered from dogs affected with otitis externa were subjected to phenotypic screening for extended-spectrum β -lactamase (ESBL) production using ESBL agar (HiMedia Laboratories, Mumbai). All isolates (100%, 19/19) were found to be presumptive ESBL producers based on the appearance of characteristic pink to purple colonies on selective media (Fig. 2)

The high rate of ESBL positive samples observed in our study is indicative of the widespread antimicrobial resistance among *P. aeruginosa* strains infecting the canine ear canal. Similar findings were reported by Sharif *et al.* (2017) [24], who documented 91.3% (42/46) ESBL-producing *Pseudomonas* isolates from diarrhoeic dogs in Andhra Pradesh. The results obtained in the present investigation are thus consistent with earlier studies

suggesting that *P. aeruginosa* possesses intrinsic resistance mechanisms and a remarkable ability to acquire and disseminate β -lactamase-encoding genes (Raus *et al.*, 2023; Pye, 2022) [7, 8].

The 100% phenotypic ESBL positivity in the present study further suggests the influence of prior or empirical antibiotic exposure, as well as the chronicity of otitis externa cases, which favour the selection of multidrug-resistant bacteria (Kashyap *et al.*, 2023) [9]. Previous studies have also emphasized the role of irrational or prolonged antimicrobial therapy in the emergence of ESBL-producing *Pseudomonas* isolates in companion animals (Damborg *et al.*, 2016; Gomez-Santamarian *et al.*, 2021) [25, 26]. Such findings underline the need for continuous monitoring of antimicrobial susceptibility profiles to guide appropriate therapeutic decisions in clinical practice.

3.2.2 Molecular detection of ESBL genes

All 19 *P. aeruginosa* isolates were screened for the presence of ESBL-encoding genes (*bla*_{TEM}, *bla*_{SHV} and *bla*_{CTX-M}) by multiplex PCR which revealed that prevalence of ESBL genes was 36.84% (7/19) for *bla*_{TEM}, 21.05% (4/19) for *bla*_{SHV}, and 5.26% (1/19) for *bla*_{CTX-M} (Fig.3)

The detection of these genes provides molecular confirmation of the phenotypic resistance observed and demonstrates that *P. aeruginosa* from canine otitis externa can act as a significant reservoir for β -lactamase genes. Similar gene distributions were reported in *P. aeruginosa* isolates from canine infections and environmental sources (Raus *et al.*, 2023; Kadhim *et al.*, 2023 and Mohamed *et al.*, 2022) [7, 10, 27], reflecting the increasing prevalence of β -lactamase-mediated resistance in veterinary and environmental contexts.

The predominance of *bla*_{TEM} among the isolates in this study aligns with reports from other animal-origin *Pseudomonas* isolates, where *bla*_{TEM} was found to be the most frequently occurring β -lactamase gene (Apaka *et al.*, 2010; Boyd *et al.*, 2004) [15, 17]. The relatively lower detection of *bla*_{CTX-M} may be due to its sporadic occurrence in *Pseudomonas* spp., as it is more commonly associated with *Escherichia coli* and *Klebsiella pneumoniae* (Paterson *et al.*, 2003) [16].

The simultaneous presence of multiple ESBL genes in *Pseudomonas* species has been reported to increase resistance to a wide range of β -lactam antibiotics (Ranjbar *et al.*, 2020) [28]. Hence, the findings from this study emphasize the urgent need for prudent antimicrobial use, regular surveillance of resistance determinants, and molecular monitoring to prevent the dissemination of resistant clones in veterinary clinical settings.

Table 1: Details of the primer used for 16s rRNA gene in *Pseudomonas* isolates

Gene	Primer sequence	Amplicon size (bp)	Reference
16S rRNA	F-5'GGGGGATCTTCGGACCTCA 3' R-5'TCCTTAGAGTGGCCACCCG 3'	956	Spilker <i>et al.</i> (2004)

Table 2: Details of the primers used in multiplex PCR for ESBL genes

Gene	Primer Sequence	Length	Reference
<i>bla</i> _{SHV}	5'-ATG CGT TAT ATT CGC CTG TG-3' 5'-TGC TTT GTT ATT CGG GCC AA-3'	747-bp	(Paterson <i>et al.</i> , 2003)
<i>bla</i> _{TEM}	5'-TCG CCG CAT ACA CTA TTC TCA GAA TGA-3' 5'-ACG CTC ACC GGC TCC AGA TTT AT-3'	445-bp	(Apaka <i>et al.</i> , 2010)
<i>bla</i> _{CTX-M}	5'-ATG TGC AGY ACC AGT AAR GTK ATG GC-3' 5'-TGG GTR AAR TAR GTS ACC AGA AYC AGC GG-3'	593-bp	(Boyd <i>et al.</i> , 2004)

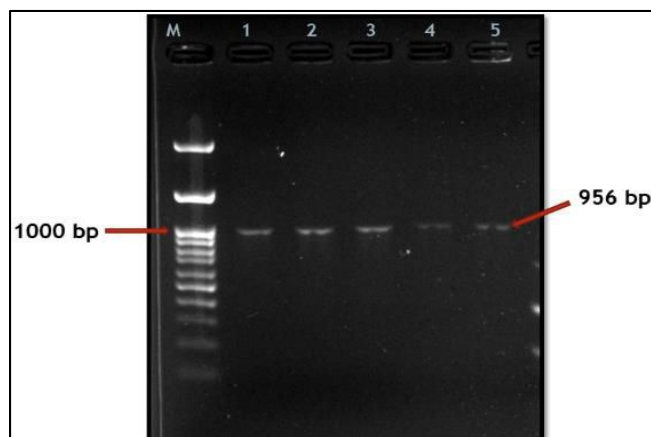


Fig 1: Molecular confirmation of *Pseudomonas aeruginosa* Agarose gel electrophoresis showing amplified PCR products of 16S rRNA gene of *Pseudomonas* isolates (Lane M-ladder, Lane 1 to 5-positive *Pseudomonas* isolates)



Fig 2. *Pseudomonas* isolates showing positive reaction on ESBL agar

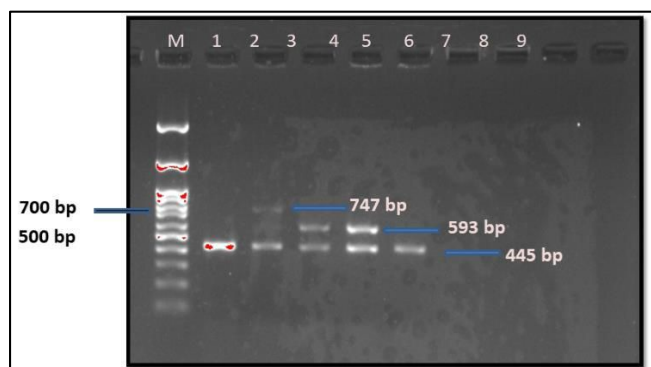


Fig 3: Agarose gel electrophoresis showing amplified PCR products of ESBL genes in *Pseudomonas* isolates (Lane M-ladder, Lane 1 to 5-positive *Pseudomonas* isolates)

4. Conclusion

The study confirmed *Pseudomonas aeruginosa* as a major pathogen in canine otitis externa through molecular characterization of the 16S rRNA gene. Phenotypic testing indicated that all 19 isolates produced extended-spectrum β -lactamase (ESBL) while PCR revealed the presence of *bla*_{TEM} (36.84%), *bla*_{SHV} (21.05%) and *bla*_{CTX-M} (5.26%) genes. The predominance of *bla*_{TEM} suggests its primary role

in β -lactam resistance. These findings highlight the emergence of multidrug-resistant *P. aeruginosa* in dogs and stress the importance of molecular surveillance and judicious antimicrobial use in veterinary practice.

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