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Pathogenicity Profiling of *Pseudomonas aeruginosa* from Canine Otitis Cases from two Veterinary Centers in Kerala, India

Anju J R [a++], Neeraja E [a++], Rathish R L [a#], Rajasekhar R [b#], Janus A [a#], Deepa P M [a†] and Bipin K C [a‡*]

[a] Department of Veterinary Epidemiology and Preventive Medicine, College of Veterinary and Animal Sciences, Kerala Veterinary and Animal Sciences University, Pookode, Wayanad, India.
[b] Department of Veterinary Microbiology, College of Veterinary and Animal Sciences, Pookode, Wayanad, Kerala Veterinary and Animal Sciences University, Kerala, India.

Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Background and Aim: Otitis externa, characterized by inflammation of external ear canal, is a common condition affecting dogs worldwide. The condition is further exacerbated by infections caused by various bacteria, among which *Pseudomonas aeruginosa* is of critical importance due to

[++]MVSc Scholar;
[#]Assistant Professor;
[†]Associate Professor and Head;
[‡]Associate Professor;
**Corresponding author: E-mail: bipinkc@kvasu.ac.in;*

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its ability to form biofilms and produce different virulence factors resulting in chronic infections. The aim of this study is to isolate *Pseudomonas spp*. from cases of canine otitis and analyze their pathogenicity profiles.

Study Design: Dogs exhibiting clinical signs of otitis were included in the study. Pathogens were identified using biochemical and molecular methods, and their pathogenicity profiles were assessed biochemically and clustered using K-means clustering.

Methodology: This Cross-sectional study involved 30 specimens from dogs presented at the clinics with signs such as pruritus of the ear, severe pain, head shaking, foul odour, ear discharges and ear scratching suggestive of otitis externa from September 2023-march, 2024 carried out at Wayanad district, Kerala, India. Samples were collected aseptically. Isolation and identification of *Pseudomonas* spp., were performed using biochemical tests and detection of the *OprI* gene. Pathogenicity profiles were evaluated based on proteolytic, lipolytic, and hemolytic activities, biofilm production, pyoverdine production, and prevalence of elastase (*LasB*) gene. Degrees of association between these traits were calculated, and clustering analyses were performed.

Results: Nine isolates (31%) of *P. aeruginosa* were obtained. Proteolytic and lipolytic activities were present in seven of the nine isolates. Production of elastase and biofilms were observed in only two isolates. Statistical analysis revealed no significant correlation between the traits, except for biofilm formation and elastase production ($\chi^2 = 9.009$, $p = 0.003$). Clustering analysis identified an optimal k-value of 4. Two isolates with the most pathogenic traits formed separate clusters, while the one isolate with the fewest traits formed its cluster. All other isolates with varying pathogenicity factors were grouped into one cluster.

Conclusion: The study demonstrated that *P. aeruginosa* in canine otitis exhibits various pathogenicity factors that contribute to its invasion and nutrient acquisition. These traits appear to function independently. A larger sample size with more traits under consideration may provide better clustering resolution based on pathogenicity profiles.

Keywords: Canine otitis; P. aeruginosa; pathogenicity profiling; clustering analysis; Kerala; India.

1. INTRODUCTION

Otitis externa is a widely prevalent condition in dogs, often resulting in significant discomfort and distress among canine patients. It is observed as one of the most prevalent reasons for dogs to be brought to veterinarians, and they may affect up to 20% of dogs [1]. One fourth of the bacteria isolated from ear swabs collected from dogs with otitis from Pookode, Wayanad, Kerala [2]. Among the various bacterial species implicated in otitis externa, *Pseudomonas aeruginosa (P. aeruginosa)* is frequently identified as an opportunistic pathogen due to its adaptability and virulence [3]. The bacteria can thrive in diverse environments and produce an array of virulence factors, such as extracellular enzymes and toxins, facilitating its colonization and persistence in the ear canal. This pathogen's ability to resist treatment makes it a significant challenge for veterinarians, as it not only complicates therapeutic interventions but also leads to chronic cases that are difficult to manage. The virulence factors of *P. aeruginosa* play critical roles in its pathogenicity. Lipases degrade host cell membranes by hydrolysing lipids, promoting tissue invasion, and aiding in nutrient acquisition [4,5]. Proteases cleave structural and immunological proteins, helping in tissue invasion and immune evasion [6]. Haemolysins degrade hemoglobin and release iron, a vital mineral for bacterial growth and multiplication, leading to damage to the tissues and produce inflammation [4]. Biofilm formation, a hallmark of chronic infections, protects bacteria from environmental stresses, antibiotics, and the host immune system [7,8]. Elastase, a zinc metalloprotease, degrades host tissues, including elastin, collagen, immunoglobulins, and complement proteins, promoting bacterial invasion and dissemination while impairing the host's immune defences [9]. Pyoverdine, a siderophore, plays a critical role in iron acquisition and regulates the expression of other virulence factors, contributing to cellular damage and facilitating bacterial invasion [10,11].

The aim of this study is to isolate and identify *P. aeruginosa* from cases of canine otitis externa in dogs and to characterize the pathogenic traits of these isolates. Understanding these traits provides insights into the bacterium's role in disease pathogenesis and its resistance mechanisms, potentially guiding more effective treatment strategies for managing otitis in dogs.

2. MATERIALS AND METHODS

2.1 Study Area and Sampling Criteria

The present study was conducted at the Teaching Veterinary Clinical Complex, Pookode, Wayanad, Kerala, India from September 2023 to March 2024. A total of 30 dogs presented at the clinics with signs such as pruritus of the ear, severe pain, head shaking, foul odour, ear discharge and ear scratching suggestive of otitis externa were selected were selected using convenience sampling, wherein cases were selected based on their availability and accessibility during the study period, rather than randomization or specific criteria.

2.2 Sample collection

The samples were taken aseptically from the ear canal using sterile swabs, placed in screw-capped vials and immediately transported to the laboratory. The collected samples were inoculated on Brain Heart Infusion Agar (BHIA) (Hi-media, Mumbai) and incubated at 37℃ for 24 hours.

2.3 Isolation and Biochemical Identification

Colonial morphology on culture, media and microscopic morphology of Gram-stained smears of the representative colonies were studied. Gram-negative bacteria were tested for oxidase and citrate utilization for presumptive identification of *Pseudomonas* spp.

2.4 Molecular Confirmation

Polymerase Chain Reaction (PCR) targeting *OprI* Gene confirmed the identity of *P. aeruginosa*. The total DNA was extracted from overnight broth culture using Hi-PurA ® Bacterial Genomic DNA Purification Kit (MB505-HIMEDIA, Mumbai). The PCR reaction mix consisted of five microliters of total DNA, one microliter of forward and reverse primers each, 12.5 µL Master mix (Takara) and the final volume adjusted to 25 µL. The primers were OprI F, 5'ATGAACAACGTTCTGAAATTCTCT3' and OprI R-5' CTTGCGGCTGGCTTTTTCCAG 3'. The reaction was carried out in Bio-Rad™ Thermocycler equipment. The reaction protocol was set up as initial denaturation at 95℃ for five minutes, followed by 30 cycles of denaturation at 95℃ for 30 seconds, annealing at 57℃ for 30 seconds and extension at 72℃ for 1 minute and a final extension for 10 minutes at 72℃. The presence of an amplified product with a size of 249bp visualized by agar gel electrophoresis using 1.2 percent agarose gel stained in ethidium bromide confirmed the identity of the isolate as *Pseudomonas aeruginosa* (*P. aeruginosa*). A known positive sample served as positive control, while sterile distilled water functioned as the template for negative control.

2.5 Pathogenicity Profiling

All the *P. aeruginosa* isolates were tested to detect their lipolytic, proteolytic, haemolytic pyoverdine production and biofilm production characteristics.

2.5.1 Lipolysis

The isolates were cultured on Tween 80 substrate plates, prepared by adding peptone (10 g/L), sodium chloride (5 g/L), Calcium Chloride (CaCl2·2H2O 0.1 g/L), agar (20 g/L) and Tween 80 (10ml/L) and incubated at 37°C for 2-4 days. The presence of white precipitation around the margins of the colony suggested lipolytic activity.

2.5.2 Proteolysis

Isolates were streaked on skimmed milk agar plates, prepared using nutrient agar supplemented with 10% skimmed milk powder and incubated at 37°C for 24 hours. Clear zones surrounding the colonies indicated proteolysis.

2.5.3 Haemolysis

Isolates were streaked on 5% Blood Agar plates and incubated at 37°C for 24 hours. Haemolysis was identified by observing clear zones surrounding the colonies.

2.5.4 Biofilm Formation

The isolates were inoculated in Congo Red Agar, containing 5 per cent sucrose. The appearance of slimy and shiny black colonies within 24 hours of incubation indicated biofilm formation.

2.5.5 Pyoverdine Production

Pyoverdine gives a bluish-green fluorescence on exposure to UV light.

Overnight cultures of the isolates in BHI Agar colonies exposed to UV light from a Wood's lamp

gave bluish-green fluorescence, indicating the presence of pyoverdine.

2.6 Molecular Detection of Elastase Gene

The PCR assay for detecting the *Las B* gene confirmed the presence of elastase. The PCR reaction mix contained five microliters of total bacterial DNA, one microliter each of forward and reverse primers, 12.5 µL Master mix (Takara) and the final volume adjusted to 25 µL with nuclease-free water. The primer sequences used were 5'GGAATGAACGAAGCGTTCTCCGAC 3' as forward primer and 5'TTGGCGTCGACAC ACCTCG 3' as reverse primer. The reaction was done in Bio-Rad™ Thermocycler equipment. The reaction protocol was set up as initial denaturation at 94℃ for three minutes, followed by 30 cycles of denaturation at 94℃ for 30 seconds, annealing at 55℃ for one minute and extension at 72℃ for 90 seconds and final extension for five minutes at 72℃. The amplified product was visualised by agar gel electrophoresis using 1.2 per cent agarose gel stained in ethidium bromide. DNA from a known positive sample formed the positive control, and sterile distilled water served as a negative control. The presence of the elastase gene in the isolate was demonstrated by the presence of a 284 bp-sized amplicon.

2.7 Statistical Analysis

The data from the pathogenicity profiles were tabulated and subjected to statistical analysis using Python-based Jamovi and Orange software. Analysing the correlation matrix associated with each character provided the relationship between pathogenicity traits. Further, the degree of association between pairs of pathogenicity factors was assessed by the c2 test of association for independent samples. The association between isolates based on the presence or absence of pathogenicity characteristics was analysed by K-means clustering analysis and hierarchical clustering of distances.

3. RESULTS AND DISCUSSION

3.1 Isolation, Biochemical Characteristics and Molecular Confirmation

Bacterial growth was present in all the samples cultured in BHI Agar. Among them, 14 isolates were Gram-negative bacilli, and the rest 15 were Gram-positive cocci. Twelve of the 15 Gram-positive isolates were Staphylococci, and 3 were Streptococci. Among the 14 Gram-negative bacilli obtained, nine were oxidase-positive and turned Simmon's citrate agar slants blue, indicating a positive reaction, and thus identified as *Pseudomonas* spp. remaining isolates included two *Escherichia coli* and one *Proteus* sp. (Fig. 1).

Otitis is the inflammation of the ear caused by many bacterial species. *Pseudomonas aeruginosa*, is frequently implicated as the etiological agent in canine otitis due to several factors favouring its proliferation in the ear environment. The external ear canal of canines provides a warm, moist, and nutrient-rich milieu,

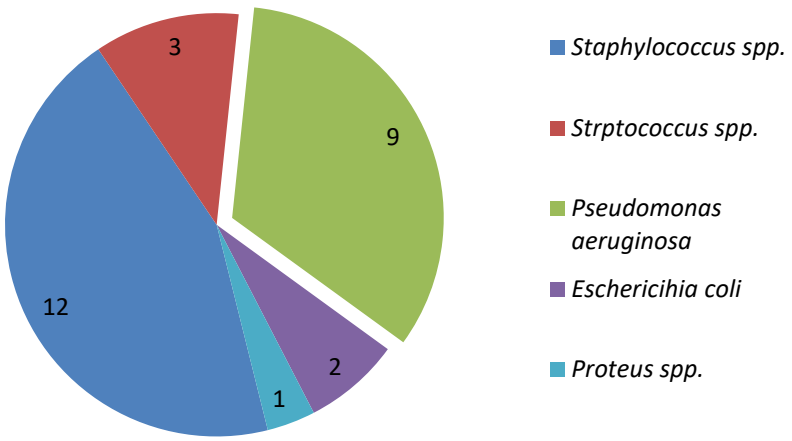

Fig. 1. Bacterial pathogens isolated from cases of canine otitis externa

ideal for the growth of *P. aeruginosa* [10]. This bacterium is highly adaptable and can thrive in diverse environments, including water and soil that provide plenty of oxygen, which are common sources of infection for dogs. Moreover, *P. aeruginosa* virulence factors enhance its ability to adhere to and persist on the epithelial surfaces of the ear canal and help it to survive in infected tissue for a prolonged time [12].

The extracellular enzymes and toxin production by bacteria, including proteases, lipases, and hemolysins, further aids tissue invasion and damage, exacerbating the infection [13]. Additionally, the inherent resistance of Pseudomonas to many common antibiotics complicates treatment, making it a persistent pathogen in canine otitis cases. This combination of environmental resilience, virulence mechanisms, and antibiotic resistance makes Pseudomonas a predominant pathogen in canine otitis [12], necessitating diligent veterinary care and targeted therapeutic strategies.

PCR-based detection of *OprI* gene resulted in the amplification of the expected 249 bp amplicons from the nine isolates and confirmed as *P. aeruginosa*. (Fig. 2). Pseudomonas are Gram-negative obligate aerobes that are catalase-negative and oxidase-positive. These biochemical properties help in the easy differentiation of pseudomonas up to genus level from other bacterial pathogens. However, species-specific determination of *P. aeruginosa* is quite challenging by biochemical methods, as strains produce varying responses to different reagents [14]. Hence, PCR targeting the highly conserved *OprI* gene for P. aeruginosa is a suitable method for confirming the isolates. *OprI* gene codes for the Outer Membrane protein of *P. aeruginosa* and is conserved for the species, making it an ideal target site for PCR-based detection of *Pseudomonas aeruginosa* [15]. *OprI* amplification aided in detecting *P. aeruginosa* directly from tissues affected with cystic fibrosis and burn wounds [15, 16].

3.2 Pathogenicity Profiling

Seven out of nine isolates demonstrated lipolytic activity, as indicated by white precipitation around the boundaries of the colonies on the nutrient agar supplemented with Tween 80 and calcium chloride (Fig. 3A). Proteolytic activity occurred in seven out of the nine isolates. Clear zones around the colonies on the skimmed milk agar plates confirmed the presence of proteolytic enzymes (Fig. 3B). Five isolates exhibited haemolytic activity, as evidenced by clear zones around the colonies on the blood agar plates (Fig. 3C). Only two isolates demonstrated biofilm formation, indicated by the appearance of slimy and shiny black colonies on the Congo red agar (Fig. 3D). Five isolates tested positive for pyoverdine, indicated by bluish green fluorescence on exposure to wood's lamp (Fig. 3E). Two isolates possessed elastase (Fig. 4)

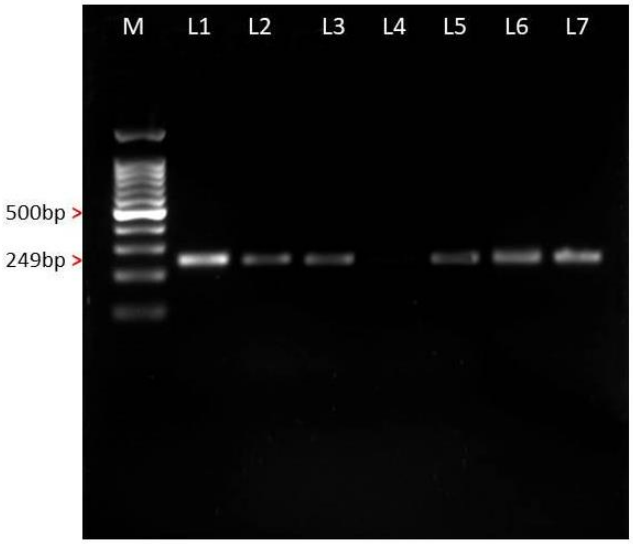

Fig. 2. Molecular confirmation of *P. aeruginosa.* Lane M- Molecular weight marker (100bp)
Lane L4: Negative Control
Lane L1: Positive Control
Lanes L2, L3, L5, L6, L7: Positive isolates

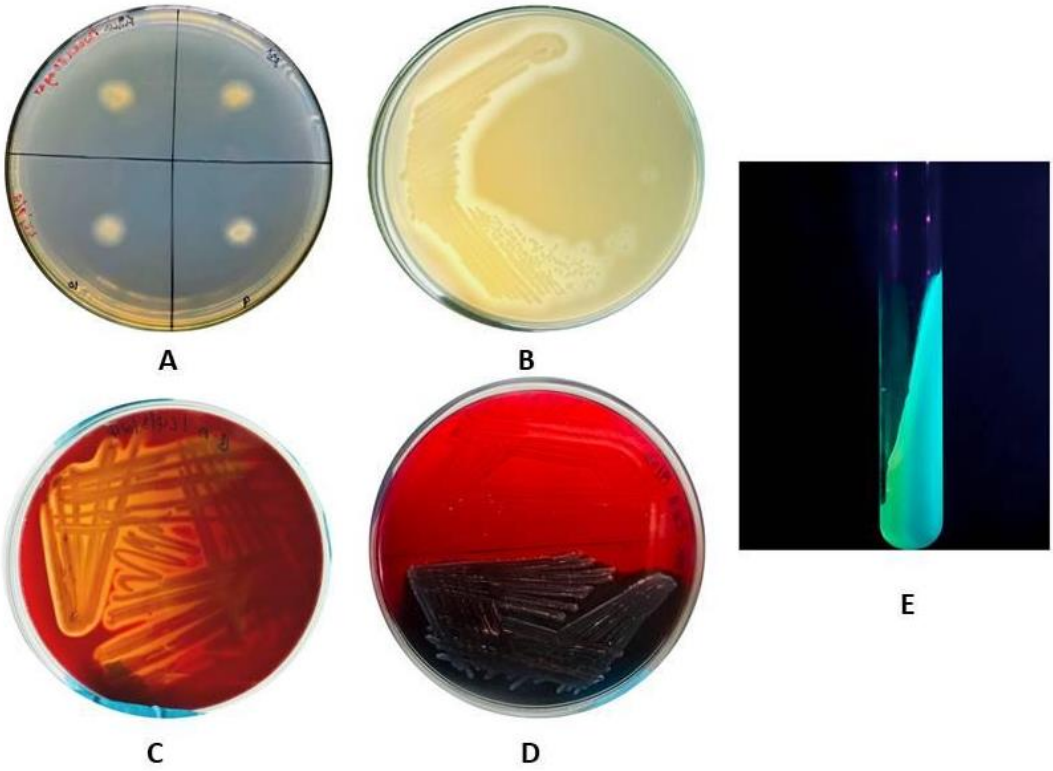

Fig. 3. Pathogenicity test for *P. aeruginosa*
A: Lipolysis in Tween 80 medium B: Proteolysis in skim milk agar
C: Haemolysis in Blood Agar D: Biofilm formation in Congo Red agar
E: Fluorescence on exposing to Wood's lamp indicating pyoverdine production

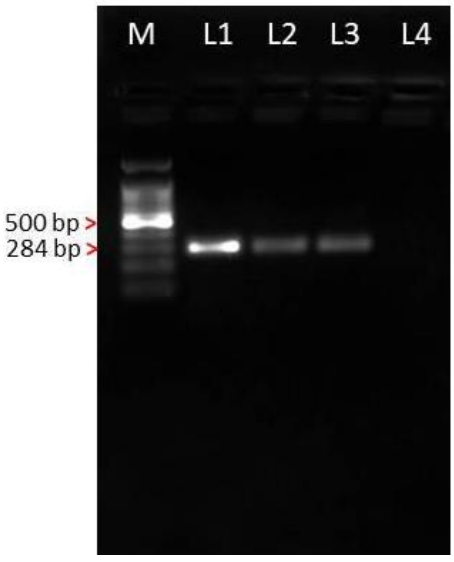

Fig. 4. Molecular detection of elastase gene (Las B) from P. aeruginosa isolates.
Lane M- Molecular weight marker (100bp)
Lane L1: Positive Control
Lanes L2, L3: Positive isolates
Lane L4: Negative Control

The isolate COPM5 possessed most virulence factors, including lipolytic, proteolytic, haemolytic activities, and biofilm formation. It also possessed elastase, however, did not produce Pyoverdine. COPM1 and COPM2 exhibited lipase, protease, and haemolytic activities and produced Pyoverdine but did not form biofilms or have elastase.

COPM3 and COPM4 displayed lipase activity and protease activity, but were negative for haemolytic activity, Pyoverdine production, biofilm formation, and *LasB* production. COPM8 possessed lipolytic and haemolytic activity, Pyoverdine and biofilm formation, and elastase. However, it did not exhibit protease activity. COPM9 exhibited haemolytic activity and Pyoverdine production but had no lipolytic or proteolytic activities, no biofilm formation or possessed elastase.

COPM6 and COPM 7 had the least number of pathogenicity traits. COPM 6 has protease activity and Pyoverdine production but no lipolytic, haemolytic activity, biofilm formation or elastase. COPM7 demonstrated lipase and protease activities but was negative for haemolytic activity, Pyoverdine production, biofilm formation, and LasB production.

Pathogenicity profiling of bacteria from specific infections helps to provide critical insights into the pathogenic process of infections [17]. Lipases degrade the host cell membranes by hydrolysing lipids and promoting tissue invasion. In addition to aiding in nutrient acquisition, lipases can contribute to biofilm formation, enhancing bacterial adherence and persistence on surfaces, which is critical for establishing infections [18]. Proteases cleave the structural and immunological proteins and help in tissue invasion and immune evasion. Hemolysin degrades haemoglobin and releases iron, a viral mineral required for bacterial growth and multiplication [19]. Haemolytic activity can lead to damage to the tissues and produce inflammation, contributing to the severity of infections. Biofilms are communities of bacteria enveloped by an organised extracellular matrix produced by the bacteria itself. This matrix protects bacteria from different stress factors such as, environmental stresses, including desiccation, antibiotics, and the host immune system. Biofilm formation is mostly associated with chronic infections, as biofilms can persist on medical devices, tissues, and surfaces, making infections difficult to eradicate [20]. Elastase, a zinc metalloprotease also known as elastase, degrades host tissues, including elastin, collagen, immunoglobulins, and complement proteins [7]. This degradation promotes bacterial invasion and dissemination and further impairs the host's immune defences. Pyoverdine is a critical pathogenicity factor for *P. aeruginosa* due to its role in acquisition of elements such as iron and regulation of other virulence factors. This siderophore provides essential iron for bacterial growth and controls the expression of exotoxin A and *PrpL*. The internalisation of pyoverdine in host cells leads to significant cellular damage, including mitochondrial disruption, facilitating bacterial invasion and pathogenicity [21].

From the study, lipolysis, proteolysis and haemolysis were predominant traits in the pseudomonas isolates. The presence of Pyoverdine could help in giving the isolates a competitive edge over the bacteria, indicating why *P. aeruginosa* was the most prevalent species isolated in this study. Elastase and biofilm formation were absent in the majority of isolates. The findings suggest the pathogenicity traits that the isolates used are predominantly for attachment and acquiring nutrition. The absence of biofilm formation in most isolates indicates a low risk of chronic infection in these cases. However, pseudomonas can produce adhesins that help them in tissue adhesion and persistence. The distribution of these pathogenic traits among the isolates shows a varied but significant presence of lipolytic, proteolytic, haemolytic activities and the pyoverdine production, with less common occurrence of biofilm formation, elastase, and presence of pyoverdine. This suggests that while some virulence factors are widespread, others are more specific to certain isolates, potentially reflecting their adaptation to different environmental niches or infection sites.

The combination of lipase, protease, and haemolytic activities, as seen in isolates COPM1 and COPM2, indicates a high potential for tissue invasion and nutrient acquisition. The ability to produce biofilms, coupled with *LasB* activity in COPM8, suggests a high resilience to environmental stresses and immune responses, making infections caused by such isolates particularly challenging to treat. The presence of multiple virulence factors in COPM5, including lipase, protease, haemolytic activity, and biofilm formation, underscores its high pathogenic potential, despite the absence of Pyoverdine production.

3.3 Statistical Analysis

3.3.1 Correlation matrix

The correlation analysis of six pathogenic traits (Lipase, Protease, Haemolysis, Pyoverdine, Biofilm, and elastase) among nine Pseudomonas strains revealed varying degrees of associations. The correlation between Lipase and Protease was weakly positive (r = .36, *P* = .35), while Lipase showed very weak correlations with Haemolysis (r = 0.06, *P* = 0.88) and Biofilm (r = .29, *P* = .46), and a moderate negative correlation with Pyoverdine (r = - .48, *P* = .19). Protease exhibited a moderate negative correlation with Haemolysis (r = - .48, *P* = .19) and Pyoverdine (r = - .48, *P* = .19) and a weak negative correlation with Biofilm (r = - .36, *P* = .35) and elastase (r = - .36, *P* = .35). Haemolysis was moderately positively correlated with Pyoverdine (r = .55, *P* = .13) and Biofilm (r = .48, *P* = .19) and had a moderate positive correlation with *LasB* (r = .48, *P* = .19). Pyoverdine showed a weak negative correlation with Biofilm (r = - .06, *P* = .88) and *LasB* (r = - .06, *P* = .88). Notably, Biofilm and *LasB* were perfectly correlated (r = 1.0, *P* < .001), indicating these traits always co-occurred. These results suggest that apart from the strong relationship between Biofilm and *LasB*, the pathogenic traits in Pseudomonas strains generally exhibit weak to moderate correlations, with none except the Biofilm-elastase pair reaching statistical significance (Table 1).

The correlation analysis of the six pathogenic traits (Lipolysis, Proteolysis, Haemolysis, Pyoverdine, Biofilm, and elastase) in nine Pseudomonas strains revealed varying degrees of associations. The results showed that most correlations among the traits were weak to moderate, with none except the Biofilm-elastase pair reaching statistical significance. Protease exhibited moderate negative correlations with Haemolysis and Pyoverdine, suggesting that proteolytic activity might inhibit the expression or functionality of these other traits, probably due to resource allocation within the bacterial cells or specific regulatory pathways that prioritise one activity over another [22]. The weak negative correlation of Proteolytic activity with Biofilm also indicates potential trade-offs in pathogenic trait expression [23]. Interestingly, haemolysis showed moderate positive correlations with Pyoverdine, Biofilm, and elastase, suggesting haemolytic activity might be more commonly associated with these other virulence factors, possibly contributing to more aggressive pathogenic profiles in certain strains. The perfect correlation between Biofilm and elastase could be due to the small sample size, as only two isolates were positive for both traits. Therefore, while the observed relationship is compelling, further testing with a larger sample size is needed to confirm and validate the finding.

Findings from the correlation matrix pointed out that the pathogenic traits studied generally functioned independently rather than synergistically or antagonistically. The independent functioning of pathogenic traits reiterates the complexity of Pseudomonas pathogenesis [24]. Each trait contributed uniquely to the bacterial ability to infect and persist. This finding suggests that pathogenicity profiling of pathogens may be essential in developing models of pathogenesis that may vary regionally [25]. To further substantiate the associations between these traits, or a lack thereof, an independent Chi-Square test was done to provide more robust evidence of the relationships, considering the limitations posed by the small sample size in the initial correlation analysis.

3.3.2 Degree of association between traits

The chi-square test determined the degree of association between various pathogenic traits among the Pseudomonas strains. The analysis revealed that most pairs of traits exhibited no significant association. However, a statistically significant association occurred between Biofilm and elastase (χ^2 = 9.009, *P* = .003) in the Pseudomonas strains analysed (Table 2). This association underscores the importance of these two traits in the pathogenic profile of the bacteria. Biofilm formation is a crucial factor in chronic infections and antibiotic resistance, and elastase is a critical virulence degrading host tissues and immune components. Their co-occurrence could indicate a particularly virulent strain. The significant correlation and association between Biofilm and elastase warrant further investigation, as it could have important implications for understanding the pathogenic mechanisms and developing targeted therapies. Future studies with larger sample sizes and more diverse Pseudomonas strains will be crucial to validate these observations and explore the potential interdependencies among virulence factors.

Table 1. Correlation Matrix between pathogenicity traits

		Lipase	Protease	Hemolysis	Pyoverdine	Biofilm
Protease	Pearson's r	.36	—			
	df	7	—			
	P-value	.35	—			
Hemolysis	Pearson's r	.06	- .48	—		
	df	7	7	—		
	P-value	.88	.19	—		
Pyoverdine	Pearson's r	-.48	-.48	.55	—	
	df	7	7	7	—	
	P-value	.19	.19	.13	—	
Biofilm	Pearson's r	.29	-.36	.48	-.06	—
	df	7	7	7	7	—
	P-value	.46	.35	.19	.88	—
LasB	Pearson's r	.29	-.36	.48	-.06	1
	df	7	7	7	7	7
	P-value	.46	.35	.19	.88	< .001

Table 2. $|^2$ test of association between pathogenicity traits

Row	Column	x2	*P*- value	inference
Lipase	Protease	1.15	.28	No association
Lipase	Pyoverdine	2.06	.15	No association
Lipase	Haemolysis	.032	.86	No association
Lipase	Biofilm	.74	.39	No association
Lipase	Elastase	.74	.39	No association
Protease	Pyoverdine	2.07	.15	No association
Protease	Haemolysis	2.07	.15	No association
Protease	Biofilm	1.16	.28	No association
Protease	Elastase	1.16	.28	No association
Pyoverdine	Haemolysis	2.73	.099	No association
Pyoverdine	Biofilm	.032	.86	No association
Pyoverdine	Elastase	.032	.86	No association
Haemolysis	Biofilm	2.07	.15	No association
Haemolysis	Elastase	2.07	.15	No association
biofilm	Elastase	9.009	.003	Association

3.3.3 Clustering analysis of pseudomonas pathogenicity profiles

This aim of this study was to classify nine Pseudomonas strains based on six pathogenic traits: lipolysis, proteolysis, haemolysis, pyoverdine, biofilm formation, and elastase by K-means and hierarchical clustering methods.

The K-Means clustering algorithm determined the optimal number of clusters, with the cluster number (k) ranging from 2 to 8. The highest average silhouette score (0.596) was obtained for k=4, indicating that a four-cluster solution best represented the underlying structure of the data (Fig. 1). Hierarchical clustering provides a visual representation of the hierarchical relationships among the Pseudomonas strains. The dendrogram supported the four-cluster solution suggested by the K-Means analysis, confirming the presence of four distinct clusters.

The Pseudomonas strains formed four clusters based on their pathogenic profiles. Cluster 1 (C1) included strain COPM5, which possessed Lipolytic, Proteolytic and haemolytic activities, possessed elastase and produced biofilm but no pyoverdine. Cluster 2 (C2) comprised strain COPM8, characterised by the presence of Lipase, Haemolysis, Pyoverdine, Biofilm, and *LasB*, and the absence of Protease. Cluster 3 (C3) included strain COPM9, which exhibited Haemolysis and Pyoverdine but lacked Lipase, Protease, Biofilm, and *LasB*. The presence of only Haemolysis and Pyoverdine set COPM9 apart from other strains. Cluster 4 (C4) contained

strains COPM1, COPM2, COPM3, COPM4, COPM6, and COPM7. COPM1 and COPM2 were positive for Lipase, Protease, Haemolysis, and Pyoverdine but negative for Biofilm and *LasB*. COPM3 and COPM4 had lipolysis and proteolysis but lacked Haemolysis, Pyoverdine, Biofilm, and *LasB*. COPM6 was positive for proteolysis and pyoverdine and negative for Lipase, Haemolysis, Biofilm, and *LasB*. COPM7 showed Lipolytic and Proteolytic activity but no haemolysis, Pyoverdine, Biofilm, and *LasB* (Fig. 5).

K-Means clustering is a machine learning-based partitioning algorithm that divides a dataset into K distinct, non-overlapping clusters based on similarity between features [26]. By maximising the intra-cluster similarity, the algorithm iteratively assigns each data point to the nearest cluster centroid, recalculates the centroids as the mean of all points in a cluster, and repeats this process until convergence. The silhouette score quantifies an object's similarity to its own cluster in relation to other clusters [27]. A higher average silhouette score indicates better-defined and more cohesive clusters, guiding the selection of the most appropriate cluster count. On order to create compact and well-separated clusters, the goal is to minimize the sum of squared distances between data points and the centroids of each cluster. The clustering analysis of the pathogenicity profiles of nine Pseudomonas strains revealed several key insights into the diversity and virulence of these bacteria. The findings demonstrate the heterogeneity of various pathogenic traits within Pseudomonas strains and provide a foundation for further studies on adhesion potentials, virulence, and pathogenic mechanisms.

Cluster C1 represents a highly virulent profile with multiple pathogenic traits, suggesting strong adhesion and tissue degradation capabilities. Cluster C2 had a combination of various virulence factors that promote biofilm formation and haemolysis, critical for chronic infections and immune evasion. Cluster C3 appeared to have a different virulence strategy focused on toxin production rather than adhesion. Cluster C4 had varied pathogenic profiles, highlighting a high degree of diversity and complexity in their pathogenesis.

Strains with biofilm and elastase (C1 and C2) are likely to have enhanced adhesion capabilities, particularly in chronic conditions such as otitis in canines. The presence of distinct clusters indicates that Pseudomonas strains employ various virulence strategies, ranging from toxin production to biofilm formation and enzymatic degradation of host tissues. This heterogeneity is a significant challenge in clinical settings, as different strains may require different treatment approaches [28]. Strains like COPM5 and COPM8, which possess multiple virulence factors, are likely more aggressive and unresponsive to therapy. In contrast, strains with fewer virulence traits may employ different survival strategies that still contribute to their pathogenicity.

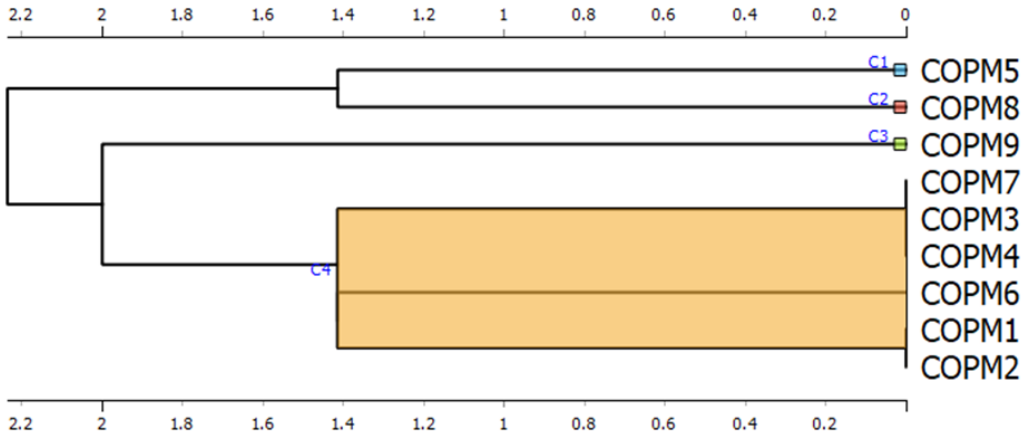

Fig. 5. Dendrogram showing hierarchical clustering of *P. aeruginosa* isolates based on pathogenicity traits

4. CONCLUSION

The study successfully isolated and identified *P. aeruginosa* from canine otitis externa cases, confirming its role as a predominant pathogen. Pathogenicity profiling revealed a diverse range of virulence traits, including lipolytic, proteolytic, and haemolytic activities, as well as biofilm formation and elastase production. The correlation and clustering analyses highlighted the complexity and heterogeneity of *P. aeruginosa* pathogenic mechanisms, indicating that these traits often function independently rather than synergistically. These findings emphasise the need for targeted therapeutic approaches tailored to the specific virulence profiles of *P. aeruginosa* isolates. Future research should focus on investigating the interaction among these pathogenic traits and their impact on disease progression, with the goal of developing more effective strategies for preventing and treating canine otitis externa.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declares that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of this manuscript.

ETHICAL APROVAL

IRB approval for the research work: KVASU/ DAR/A3/2457/2023(1) Dated 23/12/2023, of the Director of Academics and Research, Kerala Veterinary and Animal Sciences University, Pookode, Wayanad, Kerala.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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