MOLECULAR IDENTIFICATION AND DETECTION OF VIRULENCE GENES AMONG *PSEUDOMONAS AERUGINOSA* ISOLATED FROM CANINE OTITIS

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Pseudomonas aeruginosa possesses a variety of virulence factors that contribute to its pathogenicity. In order to find out any relationship between virulence factors and manifestation of *P. aeruginosa* infections, we detected virulence factors among these isolates by PCR. The Opr F, Exo A, Pcr V genes of *P. aeruginosa* were targeted for the study. A total of 70 ear swabs, from dogs suffering from chronic otitis with signs such as unilateral and bilateral ear damage, were collected from different regions of Mumbai. Out of 70 ear swabs processed, 33 number of (47.1%) *Pseudomonas* isolates were recovered and their confirmation was done by biochemical tests like MR, VP, Indole, etc. PCR assay was carried out for detection of virulence genes of *P. aeruginosa* isolates. Results indicate that, out of 33 isolates, 27(81.8%), 24 (72.7%) and 13 (39.3%) isolates showed presence of Exo A, Opr F and Pcr V genes respectively, by PCR assay. The findings of this study indicate that the PCR assay is useful for rapid and accurate detection of Exo A, Opr F and Pcr V virulence genes of *Pseudomonas aeruginosa* isolates from canine otitis.
Keywords: Canine otitis, Exo A, Opr F, *Pseudomonas aeruginosa*, Pcr V.

***P**seudomonas aeruginosa* is an opportunistic, ubiquitous bacterial pathogen causing otitis externa, dermatitis, conjunctivitis, lower urinary tract infections, septicemia and bacterial endocarditis. Chronic otitis externa in dogs is often associated with *P. aeruginosa* (Secker *et al.*, 2023). Chronic otitis is characterized by erythema, pruritus and a discharge of pus with different colors and varied viscosity and odour. *Pseudomonas* is among the most common etiological agents for chronic suppurative otitis externa and its incidence is reported between 11 to 13%. *P. aeruginosa* is widely recognized as having an arsenal of virulence factors that help to facilitate successful infection and colonization across a wide range of environments. The virulence factors include lipopolysaccharide, phospholipases, exoproteases, phenazines, outer membrane proteins like Opr F, type III secretion system (T3SS) effectors, flagella, pili, exotoxins like Exo A, biofilms, resistance to antibiotics, etc. Opr F is a structural protein of *P. aeruginosa* which functions as porin with relevant important roles in virulence. It is also found to be associated with the peptidoglycan layer and helps to maintain the cell wall integrity and cell shape. It is one of the few general porins of *P. aeruginosa* which allows nonspecific diffusion of ions and small polar nutrients, including polysaccharides. The protein is partially exposed on the external surface of the bacteria and participates in host-pathogen interactions. It is involved in adhesion to host cells and biofilm formation under anaerobic conditions Cassin *et al*., 2023). Pcr V is a soluble protein found in the bacterial cytoplasm and extracellular region and is highly responsible for the bacterial virulence. It functions by forming a complex with the highly unstable Pcr G protein. This Pcr V: Pcr G complex plays a significant role in regulation of the T3SS in *P. aeruginosa*. Pcr V has also been reported as a potent immunogen which provides protection against the pathogen in a study conducted on

burned mouse model. It is more frequently found in *P.aeruginosa* isolates from acute ill patients than in strains with chronic infection, Pcr V shows decreased virulence in chronic infection. Exotoxin A (Exo A) is produced by the majority of *P.aeruginosa* strains which can inhibit eukaryotic protein biosynthesis at the level of polypeptide chain elongation factor. More than 90% of *P. aeruginosa* produces Exo A. It is a 66 kDa protein that acts as a major virulence factor of *P. aeruginosa* (Jurado-Martin *et al.*, 2021).. Exo A belongs to a group of bacterial toxins known as ADP-ribosyltransferases (ADPRT), which transfers the adenosine diphosphateribosyl (ADPR) moiety from oxidized nicotinamide adenine dinucleotide (NAD+) into target proteins in eukaryotic cells. ADPR attaches to a modified histidine 3 residue (diphthamide) in elongation factor II (EF-II) and inhibits protein synthesis in toxin-sensitive cells.

The aim of this study was isolation of *P. aeruginosa* and detection of Opr F, Pcr V and Exo A virulence genes by Polymerase Chain Reaction.

Materials and Methods

Collection of ear swabs

For the present investigation, 70 ear swabs were collected from dogs suffering from chronic otitis in and around Mumbai region. Dogs exhibiting clinical signs like scratching of ear pinna, pain in the ear, greenish purulent discharge with pungent odour were selected for collection of samples. All swabs collected in sterile tubes were transported on ice and processed within 24 hrs after collection.

Isolation and Identification

Ear swabs were inoculated directly in BHI broth and incubated at 37°C for 24 hrs. After observing the turbidity, the BHI broth culture was inoculated on Nutrient agar, Cetrimide agar and MacConkey'sagar. The inoculated plates were incubated at 37°C for 24 hrs. Translucent colonies with green pigment were initially examined for their morphology and staining characters by employing grams staining. Gram Positive isolates were further subjected to biochemical identification using Catalase test, Oxidase test, MR test, VP test, Indole test, Nitrate test, Citrate test etc

Molecular characterization of *Pseudomonas aeruginosa*

DNA extraction

DNA was extracted from cultures as per the protocol described. Overnight grown cultures of *P. aeruginosa* were suspended in 400 µL of TE buffer. 20 µl of lysozyme (20mg/ml) was added to the suspension; the tube was vortexed briefly and incubated at 37°C for 1 hr. in water bath. Further, 200 µl of 10 % SDS and 20µl of Proteinase K (20mg/ml) was added, vortexed briefly and incubated at 65°C for 2 hours. Following incubation, 200µl each of 5M NaCl and pre warmed 200µl CTAB- NaCl solution was added. The mixture was vortexed and incubated at 65°C for 20 mins. Subsequently, equal amount of Phenol: Chloroform: Isoamyl alcohol (25:24:1) was added, vortexed for 10 sec and centrifuged at 10,000 rpm for 10mins. The aqueous phase containing DNA was carefully transferred to a fresh micro centrifuge tube and DNA was precipitated by addition of 0.6 volume of isopropanol. The tubes were then kept at -20°C for 3 hours or overnight followed by centrifugation of 12,000 rpm for 15 mins. The supernatant was discarded and the pellet was washed with 1ml of 70% Ethanol and centrifuged at 11000 rpm for 10min. The DNA pellet thus obtained was air dried at room temperature for 30min. The DNA was dissolved in 50µl of nuclease free water and kept at 65°C in water bath for 1hr. The DNA so extracted was stored at -20°C until use.

Primers

The primers used in this study for diagnosis of virulence genes. The sequences of primers and expected amplicon sizes were as below:

Primer	Sequence (5'-3')		Amplicon	Reference
Exo A	F	GACAACGCCCTCAGCATCACCAGC	396 bp	AI-Daraghi *et al.* (2013)
	R	CGCTGGCCCATTCGCTCCAGCGCT		
Opr F	F	CAGATGCGACCGAAACATAG	511 bp	Lagares *et al*. (2015)
	R	CTGTCGCTGTTGATGTTGGT		
Pcr V	F	TGCGTGGCTTGTTGATCTGA	933 bp	Allmond *et al*. (2004).
	R	TGCTGGTCGGTGTCGGAA		

Detection of *Pseudomonas aeruginosa*

1. Exo A PCR

The PCR was standardized in a reaction volume of 25 µl, containing 10X PCR buffer, 25 mM$MgCl_2$, dNTPs mix (10 mM), Primers ExoA- F (0.1 mM /µl) and ExoA- R (0.1 mM / µl), Template DNA (1 µl) and 0.3 µlTaq DNA polymerase (5 U/µl). The reaction was standardized in a thermal cycler (Master Cycler, Eppendorf) with initial denaturation at 95°C for 5 min, followed by 35 cycles at 95°C for 5 min, 60°C for 45 s and 72°C for 1 min. Final extension was carried out at 72°C for 10 min.

2. Opr F PCR

Reaction mixture for Opr F PCR was similar to that of Exo A PCR**.** The reaction was standardized with initial denaturation at 95°C for 5 min, followed by 35 cycles at 95°C for 5 min, 48°C for 45 s and 72°C for 1 min. Final extension was carried out at 72°C for 10 min.

3. Pcr V PCR

Reaction mixture for Pcr V PCR was similar to that of Exo A PCR. The reaction was standardized with initial denaturation at 95ºC for 5 min, followed by 35 cycles at 95ºC for 5 min, 50ºC for 45 s and 72ºC for 1 min. Final extension was carried out at 72ºC for 10 min. The amplified products were electrophoresed on 2% agarose gel stained with ethidium bromide (EtBr) and the products were visualized and documented using Automatic Computerized Gel Documentation and Analysis System.

Results and Discussion

Isolation and identification of Pseudomonas aeruginosa

Thirty three *Pseudomonas* isolates were successfully recovered from the ear swabs (n=70). These isolates exhibited opaque, translucent with irregular borders and showing typical green pigment on nutrient agar and Cetrimide agar (Fig.1). The colonies on blood agar were non-haemolytic and non lactose fermenting on MacConkey agar (Fig.2). Gram's staining revealed pink colored, medium sized,rod shaped organisms. All isolates were found to produce Oxidase, Catalase, Citrate and Nitrate. However none of the isolates produced Urease, MR, VP and Indole tests. Thus, the biochemical characters were indicative of *P. aeruginosa*, as also reported by and Nwiyi *et al*., 2014. The isolation rate of *P.aeruginosa* from dogs was found to be 47.14%. Several workers have isolated *P.aeruginosa* from canine otitis with varying rates of isolation. Nwiyi *et al*., 2014, recorded 42.9% in Nigeria.

Fig 1-Growth of *Pseudomonas aeruginosa* on Cetrimide agar

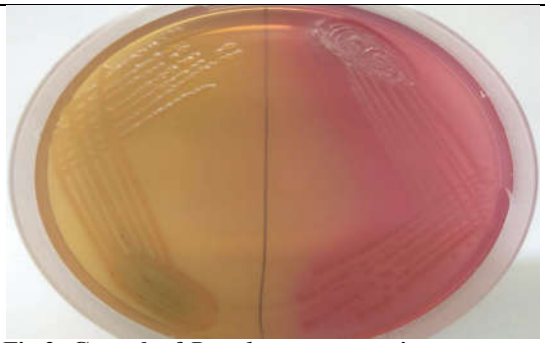

Fig 2- Growth of *Pseudomonas aeruginosa* on MacConkey agar (Left-Non-Lactose fermenting, Right- Lactose fermenting)

Molecular characterization of Pseudomonas aeruginosa

The PCR assays targeting Exo A, Opr F and Pcr V genes were carried out using published primers for detection of virulence genes of *P. aeruginosa*. The Exo A PCR assay showed amplification product of 396 bp in 27(81.8%) isolates (Fig.3). The results of our study are in agreement with Dekhil, 2017 and Ban *et al*., 2014. Opr F PCR revealed that 24 (72.7%) isolates showed amplification product of 511bp (Fig.4). The results were found to be similar with the reports of Lagares *et al*., 2015. The Pcr V PCR assay could amplify a product of 933bp inonly 13 (39.3%) isolates (Fig.5).

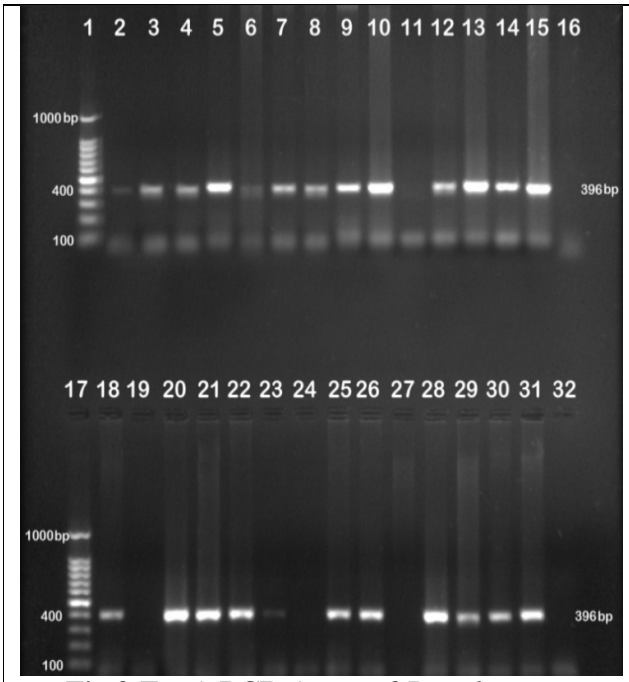

Fig 3-ExoA PCR Assay of *Pseudomonas aeruginosa*

Lane 1 and 17- 100bp ladder, Lane 2 to 15- Isolates 1 to 14, 16- Negative control, Lane 18 to 31- Isolates 15 to 28, 32- Negative control

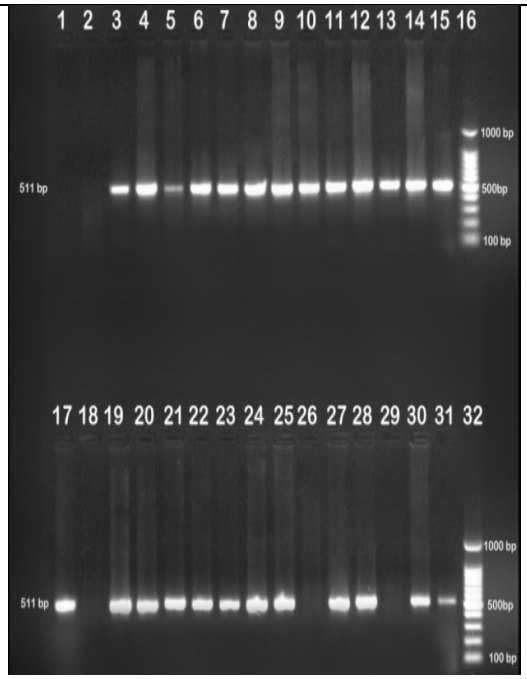

Fig 4- OprF PCR Assay of *Pseudomonas aeruginosa*

Lane 1- Negative control, Lane 2 to 15- Isolates 1 to 14, Lane 16 and 32- 100bp ladder, Lane 17 to 31- Isolates 15 to 29

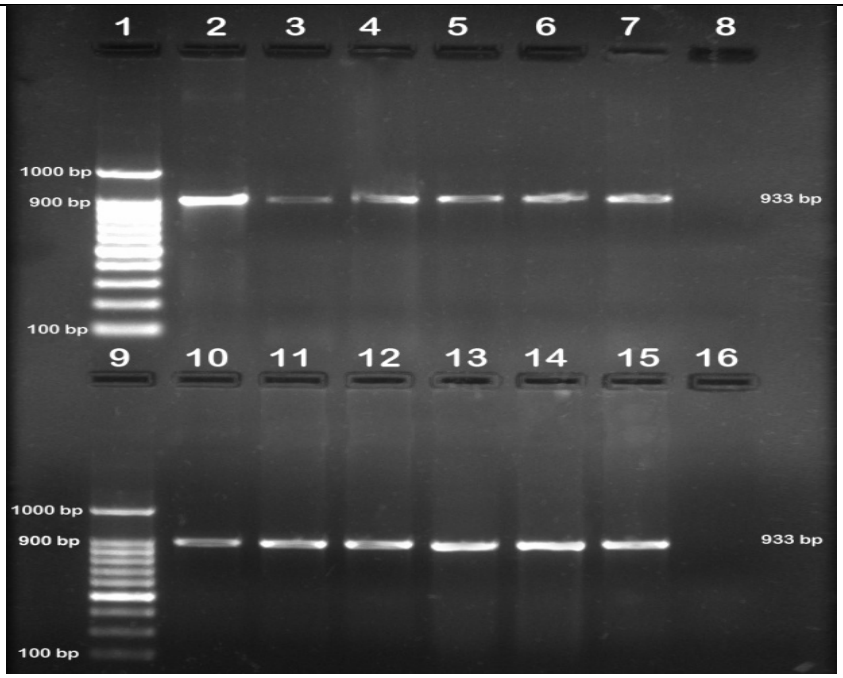

Fig 5- Pcr V PCR Assay of *Pseudomonas aeruginosa*
Lane 1 and 9- 100bp ladder, Lane 2 to 7 - Isolates 1 to 6, 8- Negative control
Lane 10 to 15- Isolates 7 to 12, 16- Negative control

The higher percentage of Exo A gene was noticed followed by Opr F and Pcr V genes. This shows that these two genes Opr F and Exo A are responsible for virulence in *P. aeruginosa.* In the present study 13 isolates found Pcr V gene which helps in injection of protein effectors into targeted host cell. More studies are needed to understand the mechanism of type III secretion system. It was observed that out of 33 isolates, 05 isolates presented all the 3 genes, 22 isolates possessed Opr F and Exo A genes, 10 isolates showed the presence of Exo A and Pcr V genes and 10 isolates revealed Pcr V and Opr F genes. The results reveal that Exo A gene, responsible for inhibition of host protein synthesis was present in maximum of the isolates under study followed by Opr F and Pcr V genes. These virulence factors play an important role in the pathogenesis *P.aeruginosa* infections. This may have different consequences on the outcome of infections.

Conclusion

The isolation rate of *Pseudomonas aeruginosa* was 47.1% from clinical cases of canine otitis. In this study, we concluded that the virulence genes, especially Exo A and Opr F genes, in *P. aeruginosa* are important to canine otitis infection. Detection of these virulence genes of *P. areuginosa* isolates suggests that they are associated with different levels of intrinsic virulence and pathogenicity. More detailed studies are required to find out the role of these genes in pathogenesis of otitis in dogs. Also PCR technique is very specific and reliable method in detection of virulence genes in *P. aeruginosa* isolates recovered from canine otitis samples.

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