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Identification and detection of virulence genes among *Pseudomonas*spp isolated from Wayanad#

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Abstract

A study was conducted to identify virulence genes of Pseudomonas spp. from 20 cases of canine otitis externa in Wayanad district of Kerala. The samples were collected aseptically and bacterial isolates were identified using culture and biochemistry. The nine identified Pseudomonas isolates were selected and further confirmed by PCR, targeting Opr I gene at genus level. Presence of virulence genes viz. Las A, Las B, Lec A, Lec B and Apr were examined in the confirmed isolates. All the samples were positive for Las B and Apr. Five among the isolates were having Lec A gene. None of the isolates possessed Lec B and Las A gene. Presence of Las B and Apr gene indicate biofilm forming ability of the isolated Pseudomonas organisms.

Keywords: *Canine otitis externa, Pseudomonas, PCR, virulence genes*

Otitis externa can be defined as the acute or chronic inflammation of the external ear and dogs are more prone to this disease (August, 1988). It is noticed that bacteria and yeast are opportunistic pathogens and the disease occurs when the fast growth of organisms occurs in a short period of time due to favourable conditions caused by external factors like higher relative humidity, increased pH and accumulation of ceruminous discharge (Rosser, 2004).Though the infection is mainly caused by bacteria and yeast form of fungi, they cannot be considered as the initiating factors of otitis externa. These organisms are usually commensals of external ear and become infective only due to their over colonization during favourable conditions. The normal microflora of the external ear canal in dogs are*Staphylococcus* spp.*, Pseudomonas*spp,*Streptococcus*

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spp, and*Proteus* spp and in case of fungi, *Malassezia pachydermatis* and occasionally *Candida* spp(Scott *et al.,* 2001).

The objective of this study was to determine the bacterial pathogens involved in canine otitis externa. For this purpose, 20 animals presented in Teaching Veterinary Clinical Complex (TVCC), Pookode of Kerala Veterinary and Animal Sciences University, with canine otitis externa were selected for this study.

Materials and methods

The study was conducted in Department of Veterinary Epidemiology and Preventive Medicine, College of Veterinary and Animal Science, Pookode, Wayanad during the period of September 2019 to March 2020. A total of 20 dogs presented at Teaching Veterinary Clinical Complex, Pookode with clinical signs suggestive of otitis externa *viz.* ear irritation, severe pain, head shaking, foul odour, discharge from ear and ear scratching were selected as subjects for this study.Ear swabs were collected from the infected ear of dogs and primarily cultured in Brain Heart Infusion agar (BHI). The organisms were identified using culture and biochemistry (Quinn *et al*., 2013). Genus specific confirmation of the Pseudomonas isolates were done by amplification of *Opr I* gene (Al-Ahmadi and Roodsari, 2016).

Isolation of bacterial DNA

Bacterial DNA was isolated from bacterial culture grown in Nutrient broth for 24 hours. Bacterial DNA was isolated from whole isolated bacteria using HiPurA® Bacterial Genomic DNA Purification Kit (M/s Himedia, catalog no: MB505).

Identification of genes of virulence factors

The isolates confirmed as Pseudomonas by genus level identification, were further examined for presence of virulence genes *viz. Las A , Las B, Lec A, Lec B* and *Apr* using the primers given in Table 1. The cycling conditions for the same are given in Table 2. Amplified PCR products were subjected to electrophoresis and visualized under Gel Documentation System.

Sequencing of amplified DNA

Nucleotide sequencing of the PCR products for *Opr I* gene of Pseudomonas were performed by Sangers dideoxy method using automated DNA sequencing facility available at M/s Agrigenome Labs Pvt. Ltd., Cochin, Kerala. On Basic Local Alignment Search Tool (BLAST) analysis of the obtained sequences,the species was confirmed.

Results and discussion

On culture and biochemistryof twenty samples of otitis externa from dogs,nine organisms were found to be *Pseudomonas* spp.The Pseudomonas isolates were catalase and oxidase positive and only citrate positive on IMViC test (Fig. 1, 2, 3). Majority of the organisms belonged to *Pseudomonas* spp. Similar findings were reported by Bugden (2013) who on isolation of bacteria from 3,451

Table 1. Primers of virulence genes of *Pseudomonas* spp.

Lec A F- 5'CGGAGATCACATATGGCTTGGAAAGG 3' *LecA R*-5'CCGAGACAAGCTTTCAGGACTCATCC 3'	394 bp	Chemani*et al.* (2009)
*Lec B F- 5'*GCACCAATAACGCCGTCATC 3' *Lec B R-* 5'GCTGACCTGGACCTGTACCT 3'	74 bp	Bartels *et al.* (2011)
Las A F- 5'CATCGAAGCCGCGTTTCGC 3' *Las A R-* 5'CAACTGGTATTCCTCGAAACCGTA 3'	161 bp	Kadhim (2020)
Las B F- 5'GGAATGAACGAAGCGTTCTCCGAC 3' *Las B R-* 5'TTGGCGTCGACGAACACCTCG 3'	284 bp	Faraji*et al.*(2016)
Apr F- 5'TGTCCAGCAATTCTCTTGC 3' *Apr R -* 5'CGTTTTCCACGGTGACC 3'	1,017 bp	Fazeli and Momtaz (2014)

Table 2. Thermocycling conditions for identification of virulence genes by PCR

Gene	Initial denaturation	Denaturation	Annealing	Extension	Cycles	Final extension
Las A	95°C 5 min	95°C 45 sec	65°C 45 sec	72°C 55sec	35 cycles	72°C 7min
Las B	94°C 3 min	94°C 30 sec	55°C 1 min	72°C 1.5min	30 cycles	72°C 5min
Lec A	95°C 3 min	94°C 45 sec	55°C 1 min	72°C 1min	35 cycles	72°C 5 min
Lec B	95°C 5 min	95°C 40 sec	53°C 45sec	72°C 1 min	30 cycles	72°C 7 min
Apr	96°C 5 min	94°C 30 sec	62°C 30sec	72°C 1.5min	30 cycles	72°C 5 min

canine external ear infection samples found that the majority of organism involved in otitis externa infection was of *Pseudomonas* spp. (35.5%). Malayeri*et al.* (2010) also reported *Pseudomonas* spp. to be the second most common bacterial pathogen involved in otitis externa.

All the pseudomonas isolates were confirmed by PCR at genus leveltargeting the *Opr I* gene (Fig.4). The gene encodes outer membrane proteins which play an important role in interaction of bacteria with the environment and provide its inherent resistance to antibiotics. Similar observations were made by Noomi (2019), in a study conducted on virulence factors of *Pseudomonas aeruginosa* in different animals using bacteriological and molecular methods and by Douraghi*et al.* (2014) in Pseudomonas from cystic fibrosis patients, where this gene was used for identification of Pseudomonas at genus level.

The confirmed isolates were examined for presence of virulence genes. All the tested isolates were positive for *Las B* and *Apr* gene (Fig.5, 6). Five out of nine genes tested were positive for *Lec A*(Fig.7). *Las A* and*Lec B* genes were not identified in any of the tested samples. The three genes *Apr , Las A, Las B* encodes proteases, in which *Las A* and *Las B* encodes for elastase, and was reported in a study on genome diversity of *Pseudomonas aeruginosa* isolates from cystic fibrosis patients conducted

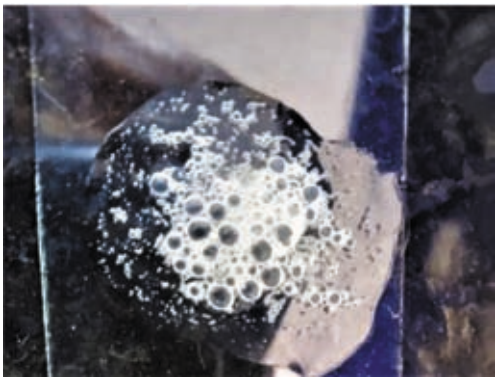

Fig.1 Catalase positive test

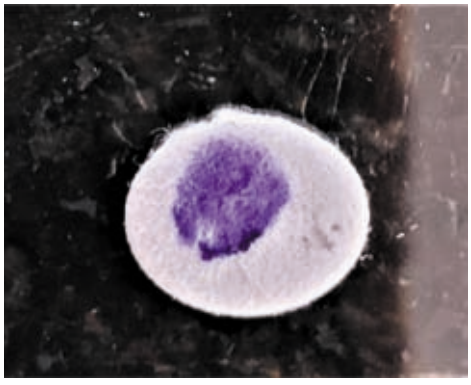

Fig.2 Oxidase positive test

Fig.3 Battery of biochemical tests for Pseudomonas

by Finnan *et al.(*2004). The authors also found that most of the clinical isolates were positive for *Las B* and *Apr*, and very few isolates were positive for *LasA* gene. Both *Las B* and *Apr* are quorum sensing genes that are crucial for biofilm formation according to Park *et al.* (2017). Martins *et al.* (2005)conducted a study on detection of *Apr* gene in raw milk, and states that the gene codes for heat-stable alkaline metaloproteases in proteolytic*Pseudmonas aeruginosa.*Chemani*et al.* (2009) had done a similar work on genes*Lec A* and*Lec B* in *Pseudomonas aeruginosa* and has stated that these genes, *Lec A* and *Lec B* encode two soluble lectins, and are two cytoplasmic proteins which specifically bind to galactose and fucose respectively and reported to be seen on the outer membrane of bacteria. Chemani*et al.* (2009) had stated that both *Lec A* and *Lec B*were found to be quite abundant in *P. aeruginosa* andthat it was suspected to play a role in adhesion of the bacteria to surfaces. In addition, *Lec A* causes epithelial injury which increases the absorption of exotoxins of bacteria. This was in partial agreement in case of *Lec A* gene, as there were only 55.56 per cent of the particular gene in the pseudomonas isolates and contradictory in case of *Lec B* gene as none were positive for this virulence gene.

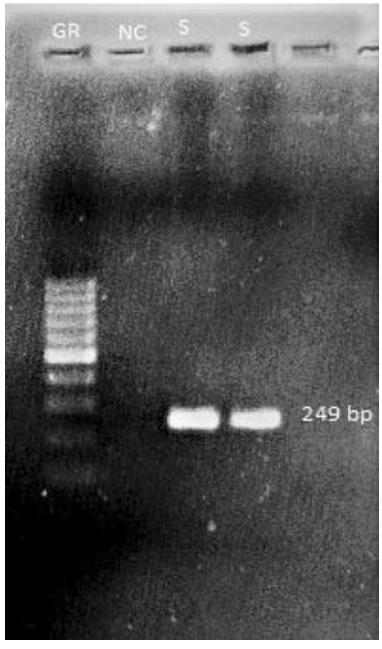

Fig.4 Genus specific identification of *Pseudomonas*

Lane GR – gene ruler of 100 bp
Lane NC – negative control
Lane S – Sample 1
Lane S – Sample 2

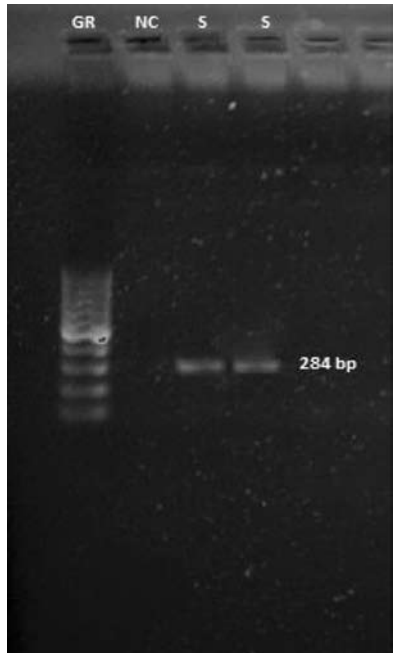

Fig.5 Amplicons of*Las B* gene

Lane GR – gene ruler of 100 bp
Lane NC – negative control
Lane S – Sample 1
Lane S – Sample 2

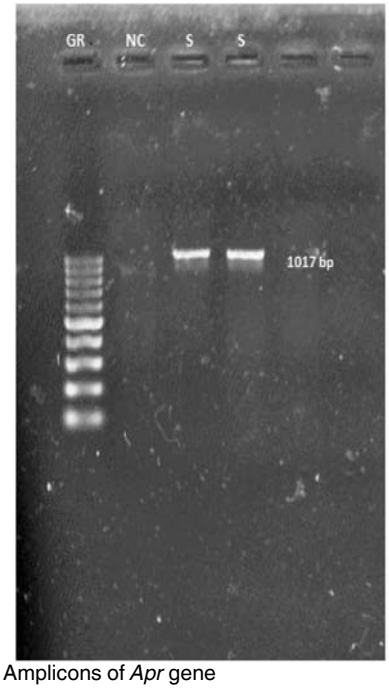

Fig.6 Amplicons of *Apr* gene
Lane GR – gene ruler of 100 bp
Lane NC – negative control
Lane S – Sample 1
Lane S – Sample 2

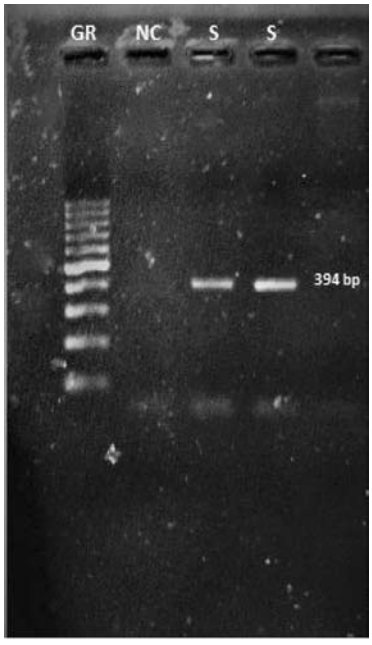

Fig.7 Amplicons of *Lec A* gene
Lane GR – gene ruler of 100 bp
Lane NC – negative control
Lane S – Sample 1
Lane S – Sample 2

As all the isolated Pseudomonas had*Las B* and *Apr* gene that are quorum sensing genes, they have a high chance of possessing the ability to produce biofilms which may cause chronic infections.

On Basic Local Alignment Search Tool (BLAST) analysis of nucleotide sequences of *Opr I* gene, it was confirmed as *Pseudomonas aeruginosa.*

Conclusion

Present study showed that Pseudomonas was the majororganisms involved in otitis exerna. Among the isolated Pseudomonas it was found that all of them possessedthe virulence genes *Las B* and *Apr. Lec A* gene was present in most of the isolates. None of the Pseudomonas isolates of this study possessed *Las A* and*Lec B* genes. Presence of *Las B* and *Apr* gene indicates biofilm forming ability of organism.

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Conflict of interest

The authors have no conflicts of interest to declare.

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