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Research Article

Isolation and identification of *P. aeruginosa* from clinical samples of dogs

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Abstract

In the present study, the infections caused by *Pseudomonas aeruginosa* in different clinical cases were represented historically, clinically and microbiologically. Isolation of *Pseudomonas aeruginosa* from the respective clinical infection and both phenotypical and genotypical studies were carried out. A total of sixty two (n=62) clinical cases of otitis (n=41) and pyoderma (skin infections) (n=21) from dogs of different age groups were collected for the retrospective study. Skin lesions in the affected dog were characterized by haemorrhagic bullae, erythematous papules and ulcers, and otitis cases consisted head shaking, redness of skin, and discharges were observed. Phenotypically, microbiological and biochemical confirmation tests were carried out for the isolation of *Pseudomonas aeruginosa* and molecular based detection on species specific genes was done. According to their confirmed microbiological and molecular assays, 45 samples (72.58%; 45/62) were positive for *Pseudomonas aeruginosa*.

Key words: Dogs, *Pseudomonas aeruginosa*, Pyoderma, Otitis

Highlights

- The present emerging problems in health care aspect seem to be the antimicrobial resistant infections caused by bacteria. One such important gram-negative bacterium is *Pseudomonas aeruginosa*, responsible for 20% of the dogs with ear infections and on progression, leads to extensive ulcers.
- Pyodermal infection in dogs initially may be superficial but later leads to allergic dermatitis and endocrine disorders.
- The outer membrane of the *Pseudomonas* sps. acts as a permeability barrier, paving the way for natural resistance towards antibiotics; as the bacterium is highly resistant, treating the infections is difficult. Moreover, resistance phenotypes occur more in dogs and are multi-drug resistant.

INTRODUCTION

Pseudomonas aeruginosa is a ubiquitous and opportunistic gram-negative bacillus found in soil, water, and decaying organic matter. It is responsible for distinct clinical infections in dogs, such as otitis externa, superficial skin infections, chronic deep pyoderma, perianal abscesses, and wound/urinary tract infections (Steen and Paterson, 2012; Pye, 2018; Degi *et al.*, 2021). The most common bacterial pathogens causing infectious otitis externa and pyoderma are *Pseudomonas aeruginosa*, *Staphylococcus intermedius*, *Escherichia coli and Klebsiella* spp., among which *Pseudomonas aeruginosa* being the most common gram-negative isolate (Miller *et al.*, 2013; Barnard and Foster, 2017). It is not a normal inhabitant of the canine ear and skin, but when it leads to infection, it could be challenging to manage due to the bacterium's resistance to many antibiotics. Otitis externa is a common complaint in veterinary medicine, and the infection ranges from 7.5% to 16.5% (Miller *et al.*, 2013). *Pseudomonas aeruginosa* singly or in combination with other microbes, mostly *Proteus mirabilis* or yeasts, is the dominant species in the infection (Barrasa *et al.*, 2000). *Pseudomonas aeruginosa* in dogs is more likely

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to cause otitis and pyodermal infections; if proper care and treatment are not provided at the right time, it happens to possible sequelae of extensive ulcers and allergic dermatitis. Early identification and confirmation of the *Pseudomonas* pathogen is of dire need as it is one of the common multi-drugs resistant pathogens. Appropriate use of antibiotics can reduce the antimicrobial resistance and thus aids in combating the *Pseudomonas* species infection at the earliest.

MATERIALS AND METHODS

Sixty two samples were collected from different clinical conditions, such as abscess material from otitis externa, otitis media, otitis interna and skin swabs from superficial pyoderma of dogs, from Veterinary Clinical Complex of NTR College of Veterinary Science, Gannavaram, Veterinary Dispensary (VD) / Veterinary Hospital (VH) / Veterinary Polyclinic (VPC), Veterinary Super Specialty Hospital, Vijayawada (VSSH), pet clinics in Krishna district of Andhra Pradesh during the period April 2021 to 1st week of May 2021 and from July 2021 to December 2021. The samples were collected under sterile conditions as described by Phuektes *et al.* (2001). Collected samples were transported on ice and immediately cultured or stored at 4°C for a minimum of 24 h until cultured/enriched.

Isolation and characterization of *Pseudomonas* species

Ear and skin swabs were inoculated directly in BHI broth and incubated at 37°C for 24 h. After observing the turbidity, the BHI broth culture was inoculated on Pseudomonas isolation agar. The inoculated plates were incubated at 37°C for 24 h to 48 h. The plates showing translucent colonies with green pigment were initially examined for their morphology and staining characters by employing Gram's method. Positive cultures were processed further for biochemical identification.

Catalase test: This test was performed with the help of slide method in which the loop was used to transfer a small amount of colony growth on the surface of a clean, dry glass slide. Then a drop of 3% hydrogen peroxide was poured on the glass slide. Immediate production of gas bubbles was considered a positive test, whereas, in the negative test, the absence of gas bubbles was observed.

Oxidase test: Standard oxidase discs were used to perform the test. The loopful of culture from a single colony was just touched on the disc. Immediate development of blue colour was considered a positive test, whereas in negative test shows no colour change.

Methyl red (MR) test: The test was performed using methyl red broth in which pure culture was inoculated. The tubes were incubated at 37ºC for 48 h. After incubation, 5 drops of methyl red indicator solution were added to the test tube. Positive results show bright red colour; however, yellow colour indicates negative results.

Voges-Proskauer (VP) test: The test was performed using Voges Proskauer (VP) broth in which pure culture was inoculated. The tubes were incubated for 48 h at 37ºC. After incubation, 3 mL of Barritt A reagent (alpha naphthol) and 1 mL of Barritt B (40% KOH) reagent were added. The tube was exposed to atmospheric oxygen for 10-15 minutes. Positive results show pink colour in 2-5 minutes, and negative result shows yellow or copper colour at the surface.

Indole test: The test was performed by using 5 mL indole broth in which pure culture was inoculated and incubated at 37ºC for 48h. After incubation, 0.5 mL of Kovac's reagent was added slowly by slightly tilting the test tube. A pink layer of xylene was considered a positive reaction, and no colour change with a yellow colour ring at the surface indicates the test as negative.

Citrate test: Simmon's citrate agar slants were inoculated from 18-24 h old culture

colony and incubated at 37ºC, and observed up to 7 days. A positive reaction was observed by colour change from green to blue in the slant and negative showing no colour change of the slant.

Molecular characterization of *P. aeruginosa* by polymerase chain reaction (PCR)

Template preparation by boiling and snap chilling method: About two mL of overnight grown *P. aeruginosa* culture was taken in a microcentrifuge tube and centrifuged at 10,000 rpm for five minutes. The pellet was suspended in 400 µL of nuclease free water and heated for 10 min in a boiling water bath. The microcentrifuge tube was transferred immediately onto the ice. After 20 min, the tube was centrifuged at 8000 rpm for five minutes at 4ºC, and the supernatant was used as a template for PCR reactions.

Measurement of DNA concentration and purity: The concentration of DNA was measured with Nanodrop 200C and adjusted to 50 ng for further molecular studies. Pure DNA samples with an optical density ratio of 1.8 to 2 at 260/280 nm were used.

Molecular confirmation of *P. aeruginosa*: In PCR test, the *P. aeruginosa* isolates were confirmed using species specific oligonucleotide primers under thermal cycler conditions as described in Table 1 & 2. All the reactions were put in a volume of 10 µL in 0.2 mL PCR tubes (Table 3).

The PCR amplicons were analyzed by electrophoresis on a 1.7% agarose gel stained with 0.5 µg of ethidium bromide/mL in Tris-Borate EDTA (TBE) buffer. Electrophoresis

Table 1. Nucleotide sequences and amplicon sizes of *P. aeruginosa* (Spilker *et al.*, 2004)

	Target gene	*Nucleotide sequence (52 -32)*	*Amplicon size (bp)*
P. aeruginosa	*16S rRNA*	Forward- GGGGGATCTTCGGACCTCA Reverse- TCCTTAGAGTGCCCACCCG	956

Table 2. Standardized thermal cycling conditions for *P. aeruginosa* (Spilker *et al.*, 2004)

Steps	Standardized conditions		No. of cycles
	Temperature	Duration	
Initial denaturation	95°C	5 min	1
Denaturation	95°C	45 sec	30
Annealing	55°C	45 sec	
Extension	72°C	60 sec	
Final extension	72°C	5 min	1

Table 3. Composition of PCR mix for *P. aeruginosa* species-specific PCR

S.No	Reagents	Quantity (µL)
1	2x Master Mix (Appendix)	5
2	Forward primer (20 pmol/µL)	0.5
3	Reverse primer (20 pmol/µL)	0.5
4	DNA template	1
5	Distilled water	3
	Total	10

was carried out at 90V for 60 min in a submarine gel electrophoresis unit, and the PCR products were visualized in Gel documentation system. The sizes of PCR products were verified by comparison with the quantitative DNA ladder. Negative control i.e., distilled water, was used in PCR tests.

RESULTS

The results on isolation, biochemical and molecular characterization of *P. aeruginosa* isolates from otitis and pyoderma cases of canines were detailed. Out of 62 clinical samples processed for isolation of the gram-negative bacillus, 45 samples (32 Otitis and 13 Pyoderma) were characteristic for *Pseudomonas*.

Cultural characterization of *P. aeruginosa*: In the present study, all the sixty two samples were inoculated in BHI medium preliminarily. Forty five samples exhibited greenish pigmentation which is characteristic for *P. aeruginosa* (Fig. 1). However, these 45 samples were further streaked onto Pseudomonas isolation agar (PIA). The test revealed that the 45 *Pseudomonas* isolates showed characteristic green pigmented smooth mucoid colonies and exhibited fluorescence under UV light. (Fig. 1) on PIA.

Biochemical profile revealed all the 45 isolates of *P. aeruginosa* to be negative for indole and MR-VP, and positive for catalase, citrate and oxidase (Table 4).

PCR test for detecting *P. aeruginosa*

All the 45 isolates that were culturally confirmed as *P. aeruginosa* were subjected to species specific (16S *rRNA*) and yielded a PCR product of 956 bp. The results are presented in Fig. 2.

DISCUSSION

Pseudomonas aeruginosa is the most common bacterial isolate found in the clinical samples, especially canine otitis and pyoderma (Arais *et al.*, 2016; Chatterjee *et al.*, 2016); very little nutrients were required for its survival, and it is highly unresponsive to antibiotics due to its multi-drug resistance. A total of 62 samples were collected from canines suggestive of otitis externa and pyoderma infections during this investigation. After performing cultural, biochemical and molecular tests, 45 clinical samples were confirmed as *Pseudomonas aeruginosa* isolates.

In the current study, 32 out 41 samples collected from canine otitis condition were positive for *Pseudomonas aeruginosa*. The isolation rate was found to be 78.04%. Several researchers had isolated *P. aeruginosa* from canine otitis conditions with varying rates of isolation. Schick *et al*. (2007) recorded the incidence of *P. aeruginosa* in 49% cases from ear swabs, Fernandez *et al*. (2006) reported an isolation rate of 22.22% in Venezuela, 17% by Turkyilmaz (2008) in Turkey. The isolation rate of *P. aeruginosa* in the present study was found to be higher than the isolation rate reported by Lakshmi and Tirumala Rao (2013), which was 29.16% from Hyderabad, 42.9% by Nwiyi *et al.* (2014) from Nigeria, 23.2% by Colombini *et al.* (2000) from Louisiana and Barrasa *et al.* (2000) reported an isolation rate of *P.*

Table 4. Biochemical sensitivity profile of *P. aeruginosa* isolates

Sl. No.	Name of the biochemical test	No. of isolates tested	No. of isolates (+)	No. of isolates (-)
1	Indole	52	0	52
2	MR-VP	52	0	52
3	Citrate	52	52	0
4	Catalase	52	52	0
5	Oxidase	52	52	0

Isolation of *P. aeruginosa* from dogs

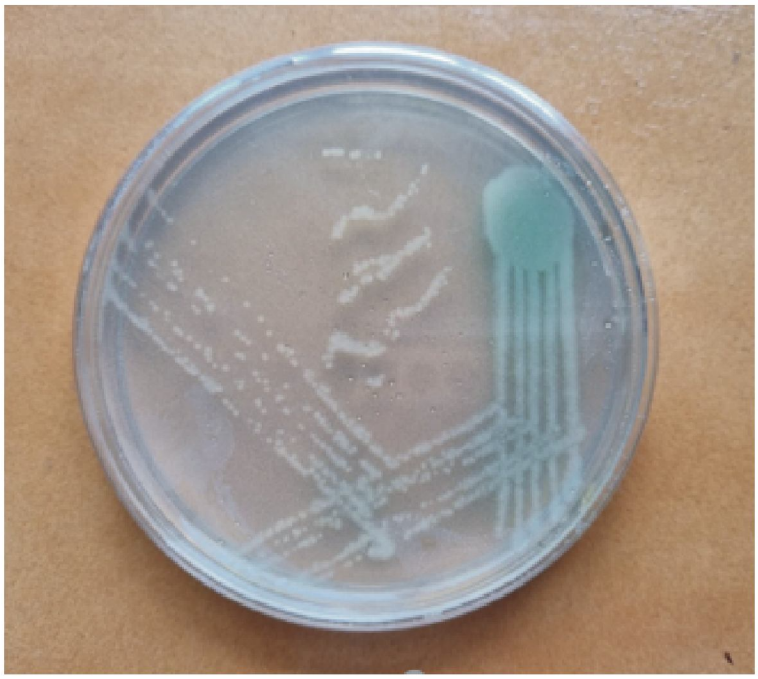

Fig. 1. Green pigmented smooth mucoid colonies of *Pseudomonas* on Pseudomonas Isolation Agar

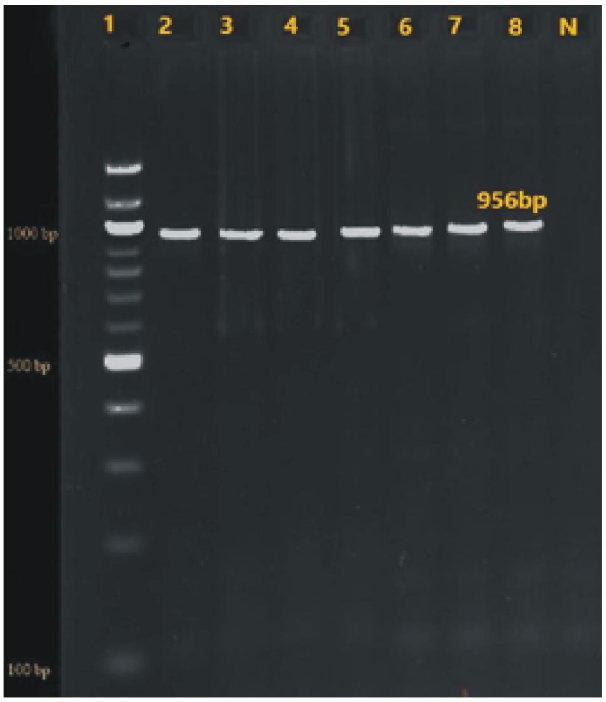

Fig. 2. Polymerase chain reaction of *Pseudomonas aeruginosa*

aeruginosa 52.8% from Spain. The isolation rate varies depending on the stage of disease, previous use of antibiotics, type of media used and climatic conditions of the region (Kowalski, 1988; Fernandez *et al.*, 2006).

P. aeruginosa isolates were further confirmed by employing specific biochemical tests like oxidase, urease, citrate, indole, catalase and MR-VP. All the isolates were positive for oxidase, catalase and citrate. However, none of the isolates produced urease, MR-VP and indole tests. Similar findings were observed by (Kovacs, 1956; Barrasa *et al*., 2000; Colombini *et al*., 2000; Fernandez *et al*., 2006; Turkyilmaz, 2008; Quinn *et al.*, 2011; Lakshmi and Tirumala Rao, 2013; Nwiyi *et al*., 2014).

Molecular studies were carried out based on the pseudomonas species specific oligonucleotide sequence. All the 45 samples that were isolated on specific agars and biochemically confirmed were further amplified by PCR, and all the samples produced a product size of 956 bp. Arais *et al*., 2016 employed PCR and RFLP- PCR for detecting the *P. aeruginosa* strains in 104 samples obtained from otitis and pyoderma infections. Degi *et al.* (2021) conducted a multicentric study on canine infections in Romania. Out of 142 samples, they were able to confirm 58 isolates of *Pseudomonas* species by PCR, and an amplified fragment of 956 bp was observed.

It can be concluded that the major bacterial pathogen invading canine otitis and pyoderma infections in dogs is *P. aeruginosa.* Timely detection and confirmation of the pathogen is of vital importance in decreasing the antimicrobial resistance of the microbe. As *P. aeruginosa* can act as an opportunistic one for human beings also, so proper care should be taken to control the infection at an early stage. Hence, suitable control strategies, both microbiological and molecular tests for superficial canine infections and otitis cases, must be performed early in preventing the disease. Dogs infected and treated with otitis externa of *Pseudomonas aeruginosa* infections should undergo regular checkups for the successful management of ear disease.

Conflict of interest: Authors have no conflict of interest in this study.

Author's contribution: PR: Data and sample collection, carried out the work, analysis and interpretation of the results; GDK and PAK: Implemented, supervised and regularly monitored the work; GDK, CBK and PAK: Corrected the manuscript and interpreted the data analysis.

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