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RESEARCH ARTICLE
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# Pseudomonas that Causes Otitis in Dogs: An Increasing Opposition

Baraa Qaism Mohammed[1], Asmaa Hamoody Abdullah[1] and Ahmed Raheem Rayshan*[,2]

[1]Department of Microbiology, College of Veterinary Medicine, University of Baghdad, Iraq
[2]Department of Physiology and Pharmacology, College of Veterinary Medicine, University of Al-Qadisiyah, Iraq
*Corresponding author: ahmed.raheem1206h@covm.uobaghdad.edu.iq

## ABSTRACT

This study investigated the prevalence and antibiotic susceptibility of *Pseudomonas aeruginosa* isolated from canine otitis externa in Baghdad, Iraq. Twelve ear swabs were collected from symptomatic dogs between October 2021 and February 2022. Identification of *P. aeruginosa* isolates was confirmed by a combination of phenotypic (morphology, Gram stain, biochemical tests) and genotypic (Vitek2 and PCR) methods. All isolates exhibited complete resistance to Erythromycin, Trimethoprim, and Chloramphenicol, and high resistance to Nalidixic acid (91.6%). Conversely, susceptibility was observed against Imipenem and Penicillin G (100%), Polymyxin B and Ceftazidime (91.6%), Streptomycin (58.3%), and Ceftriaxone (58.3%). Additionally, all isolates demonstrated the ability to produce hemolysin and phospholipase C, indicating potential virulence properties. The aforementioned results underscore the frequency of multidrug-resistant *P. aeruginosa* in cases of canine otitis externa and stress the significance of testing for antibiotic susceptibility in order to make well-informed treatment decisions.

**Keywords:** Pseudomonas aeruginosa, Dog, Otitis, PCR.

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## INTRODUCTION

The *Pseudomonadaceae* are a very large group of Gram-negative bacteria. Most members of this group are free-living saprophytes in soil, fresh water, marine environments, many other in natural materials, or in association with plants or animals serve as agents of diseases (Aysel et al., 2012; Borriello et al., 2020; Nadi et al., 2024). *Pseudomonas aeruginosa* is a common conditional pathogen on human skin and in the respiratory tract. It is a gram-negative bacillus that is widely distributed in nature. Gersard first isolated *P. aeruginosa* from wound pus in 1882 and discovered that it could live in both moist and dry conditions (Woodford and Livermore 2009; Salih and Eesa 2017). *P. aeruginosa* is a gram-negative, motile, non-fermenting member of the *Pseudomonadaceae* family. In the 1850s, Sédillot noticed that surgical wound dressings frequently had a blue-green discharge that was linked to infection (Lyczak et al., 2000). *P. aeruginosa* is a gram-negative, bacillus-shaped bacterium (size from 0.5 to 3.0µm), with an aerobic metabolism and a single flagellum that helps in movement. It is a non-fermentative bacterium that uses the glycolytic pathway under aerobic circumstances to break down glucose, with oxygen acting as the final electron acceptor. However, in anaerobic settings, nitrogen can be used as an electron acceptor (Ramos 2011). The auditory canal, which connects the middle ear to the pharynx and secures air entrance into it to achieve equal pressure on the eardrum membrane, makes the middle ear the region of the ear that is most susceptible to infection. In this way, numerous illnesses—particularly those that affect children, such as the common cold and throat and sinus infections—contribute to the disease (Mora et al., 2002). *P. aeruginosa* enters host tissue using a variety of virulence factors that are classified into two categories: extracellular (such as proteases, hemolysins, exotoxin A, exoenzyme S, and pyocyanin) and cellular (such as flagella, pilus, nonpilus adhesions, alginate, and lipopolysaccharide). These elements allow the bacteria to evade the host and produce toxins like endotoxin and exotoxin A, which cause the fever and shock associated with septic septicemia and result in the production of the pigments pyocyanin and bioferrin (Sudhakar et al., 2015). These studies have been reported by Kipnis et al. (2006) and Markey et al. (2014). In addition, having a biofilm and an alginate layer make Pyoverdin more resistant to antibiotics (Alhazmi 2015; Al-Taee et al., 2022; Paterson and Matyskiewicz, 2018). Because of this, the goal of the study was to identify and characterize *P. aeruginosa* isolates from canine ear samples with otitis from various parts of Baghdad metropolis.

## MATERIALS & METHODS

### Sample Collection

The study was carried out through October 2021 to February 2022. In this study, 120 samples were collected from dogs with otitis from different regions of Baghdad city.

### Isolation of *Pseudomonas aeruginosa*

Ear samples were transferred to the laboratory and identified based on the morphological and microscopically

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characteristics of the colonies culture media as well as biochemical tests following the methods described by MacFadden et al. (2000) and confirmed by VITEK2 system and molecular identification.

**Vitek® 2 Compact Identification System**

According to the guidelines published by the BioMérieux, bacteria were exposed to the VITEK 2 compact system for identification. Negative pressure was used to transfer the bacterial suspension to cassettes that contain 64-wells with 43 colorimetric substrates for the phenotypic identification of bacterial species. To calculate and adjust the turbidity to be equal to 0.5 Macfarlane standard, each isolate's three to five colonies were moved to a glass tube holding 3mL normal saline. Post 12 hours of the biochemical process, the results were recorded using specialized software to determine the species and strain of bacteria.

**Antibiotic Sensitivity Test**

Antibiotic susceptibility test was performed using disk diffusion method on the Mueller Hinton Agar according to Vandepitte et al. (2003), using commercially antimicrobial sensitivity disks (Bio analyze, Turkey) such as Streptomycin (S/25µg), Imipenem (IMI/10µg), Ceftazidime (CAZ/30µg), Nalidixic acid (NA/30µg), Trimethoprim (TMP/10µg), Ceftriaxone (CRO/10µg), cefotaxime (CTX/30µg), Chloramphenicol (C/10µg), Polymyxin-B (BP/300µg), Penicillin-G (P/10µg) and Erythromycin (E/10µg). The results were recorded as resistant or susceptible by measuring the inhibition zone and the results were compared with the standard diameter of inhibition zones for each antibiotic according to NCCLS.

**Determination of some Virulence Factors**

The *Pseudomonas aeruginosa* isolates were tested for their ability to produce hemolysin and lecithinase following the methods described by Collee et al. (1996) and Muerer (2003).

**Bacterial DNA Extraction and PCR Method**
**Extraction of Genomic DNA**

Genomic DNA was extracted according to the commercial purification system (Genomic DNA Mini Kit, Geneaid, Taiwan) with some minor modifications.

**Detection of Genomic DNA**

The purified DNA was detected by electrophoresis in 1.5% agarose gel with addition of red safe stain, ethidium bromide added to the DNA sample and visualized by U. V. light.

**Protocol of Agarose Gel Electrophoresis**

After genomic DNA extraction, agarose gel electrophoresis was adopted to confirm the presence and integrity of the extracted DNA (Sambrook and Russell 2001).

**Preparation of Agarose Gel 1.5%:** Following were the steps of the procedure:

a) Agarose (1.5gm) was added to the buffer.
b) The amount of 1X TBE (100mL) was taken in a beaker.
c) The solution was heated to a boiling point, using a microwave until all gel particles were dissolved.
d) The agarose was stirred to mix and avoid bubbles.
e) The solution was left to cool down at 50-60°C.
f) 2µL of ethidium bromide (10mg/mL) was added to the agarose solution.

**DNA extraction:** Using a commercial DNA extraction kit, (Genomic DNA Mini Kit, Korea) following manufacturer's instructions, the bacteria isolates were subjected to nucleic acid extraction.

**Nanodrop:** Extracted DNA was estimated using a nanodrop instrument at 350nm before storage at -20°C for PCR processing.

**Primer:** Specific primers were used to detect the *Pseudomonas aeruginosa* (Table 1).

**PCR Master Mixture Preparation:** Master reaction mix was prepared in Eppendorf tube (1.5mL) as shown in Table 2.

**PCR Thermocycler Conditions**

The amplification was conducted in Thermocycler (PCR) with the following temperatures profile consisting of an initial denaturation at 95˚C for 5min, followed by a 30-cycle program with denaturation at 95˚C for 30s, annealing at 56˚C for 30s, extension at 72˚C for 1.5min and final extension at 72˚C for 7min (Hong et al., 2009). After amplification, a 10µL sample was electrophoresed for 1 hour with a molecular size marker, dyed with Red Safe, and illuminated by UV trans for detection.

## RESULTS

**Isolation and Identification of *Pseudomonas aeruginosa***

Dogs affected with *P. aeruginosa* was 10% between areas, including 4(8.88%) in Al_Kadhimiya, 4(8.88%) in Al-Amriya, 3(30%) in Al-Sayidia, and 1(10%) in Al-Aadhamiya Table 3. In addition, out of 120 ear samples of dogs, adult 70, and young 50, the isolates of *P. aeruginosa* were 8(11%) in adult and 4(8%) in young dogs. That means high infection rate of *P. aeruginosa* in adult-dogs.

According to cultural and microscopic features, the positive number of primary *P. aeruginosa* isolates as an initial isolate obtained from a total of 120 samples from dogs were 12 isolates that represent (10%) *Pseudomonas aeruginosa*. The percentage of isolated target bacteria were *P. aeruginosa* 12(10%) and the other samples gave negative result 108(90%).

Molecular detection was done for the (12) isolates of *P. aeruginosa* with Polymerase chain reaction technique. Vitek2 identification, the results of biochemical tests using the Vitek2 compact, *P. aeruginosa* were diagnosed as follow: 12 isolates from dogs.

The colony appeared circular mucoid smooth with sweet grape odor on nutrient agar (Fig. 2), gave beta hemolysis on blood agar (Fig. 4) but did not ferment lactose sugar on MacConkey (Fig. 1), and appeared as greenish yellow on cetrimide agar (Fig. 3), the suspected colony was re-culture to do more tests, the *P. aeruginosa* isolate was depending on differed biochemical test.

The *P. aeruginosa* isolated in this study showed high resistance to Erythromycin, Trimethoprim, Chloramphenicol and Nalidixic acid (100,100, 91.6 and 91.6%) and was sensitive to Impenem, Penicillin-G, Polymyxin-B and Ceftazidime (100,100, 91.6 and 91.6%) and moderate sensitive to Streptomycin and Ceftriaxone (58.33, 58.33%) (Table 3).

**Table 1:** Specific primer used to detect the *Pseudomonas aeruginosa*

| Primer | Size of Product (bp) | Sequence | Primers sequences | GC% | Tm (°C) |
|---|---|---|---|---|---|
| *16s RNA* | 1250 | R | 5'- GGTTACCTTGTTACGACTT- 3' | 42.1 | 49.4 |
| | | F | 5'- AGAGTTTGATCCTGGCTCAG- 3' | 50.0 | 54.3 |

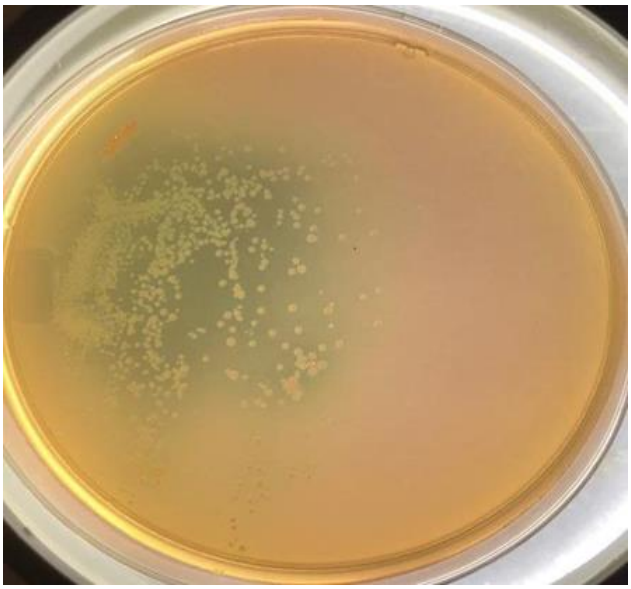

**Fig. 1:** *P. aeruginosa* on MacConkey's agar.

**Fig. 2:** *P. aeruginosa* on nutrient agar.

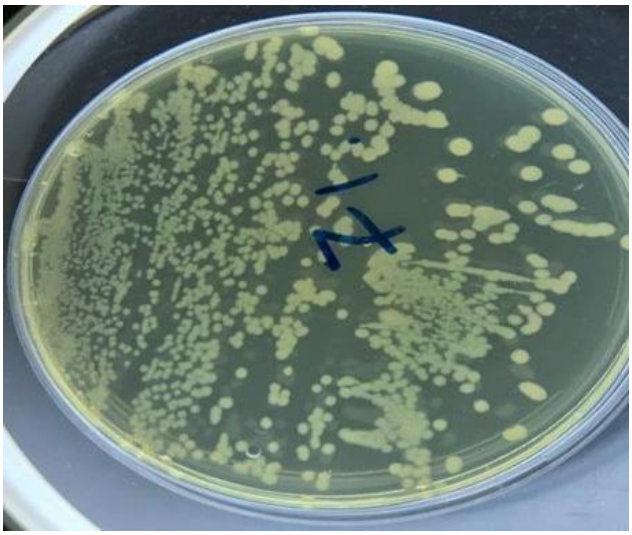

**Fig. 3:** *P. aeruginosa* on cetrimide agar.

**Fig. 4:** Beta-hemolysis produce on blood agar.

**Table 2:** Master reaction mix reaction component.

| Component | 25 µL (Final volume) |
|---|---|
| Reverse Primer | 10 picomols /µL (1µL) |
| Forward Primer | 10 picomols /µL (1µL) |
| DNA | 1.5µL |
| Taq PCR PreMix | 5µL |
| Distill water | 16.5µL |

The *P. aeruginosa* isolated in this study showed high resistance to Erythromycin, Trimethoprim, Chloramphenicol and Nalidixic acid (100,100, 91.6 and 91.6%) and was sensitive to Impenem, Penicillin-G, Polymyxin-B and Ceftazidime (100,100, 91.6 and 91.6%) and moderate sensitive to Streptomycin and Ceftriaxone (58.33, 58.33%) (Table 3).

**Determination of some Virulence Factors Produce by *P. aeruginosa***

Detection of some virulence for all isolated of *P. aeruginosa* gave Bela-hemolysin enzyme, formation of yellowish opaque zone around colonies for all isolate indicated production of phospholipase enzyme, production of pyoycainin, pyoverdin and pheomelanin pigment for isolates. Distribution of virulence factors in clinical isolates of *Pseudomonas aeruginosa* showed all isolates were (100%) β-hemolysis bacterial colonies appeared on blood agar, 100%. Positivity for Lecithinase On the egg yolk agar the bacterial colonies appeared as an opaque zone around the colonies indicating phospholipase producing as shown in Fig. 5.

The identify suspected isolates were confirmed by VITIK 2 compact system: The VITEK2 small system was used for this test (Biomerieux - France). This system technology has advanced the field of bacterial inspection by delivering more dependable technology, high speed, and high sensitivity for bacterial identification, with 99% accuracy findings. As shown in Fig. 6.

## DISCUSSION

Following growth on MacConkey, blood, and nutrition agars, all of the isolates were oxidase and catalase positive; 84% of the isolates showed pyocyanin pigment. According to Al-Shamaa et al. (2011), 80% of the organisms formed pyocyanin in 24 hours when *P. aeruginosa* was cultured in media containing cetrimide, while it took 4-5 days on modified MacConkey agar and broth. The majority of isolates had mucoid colonies and a grape-like odor. Gram staining resulted in rod-shaped cells with a pink tint being visible under a microscope.

**Fig. 5:** Phospholipase (lecithinase) produced on egg yolk agar.

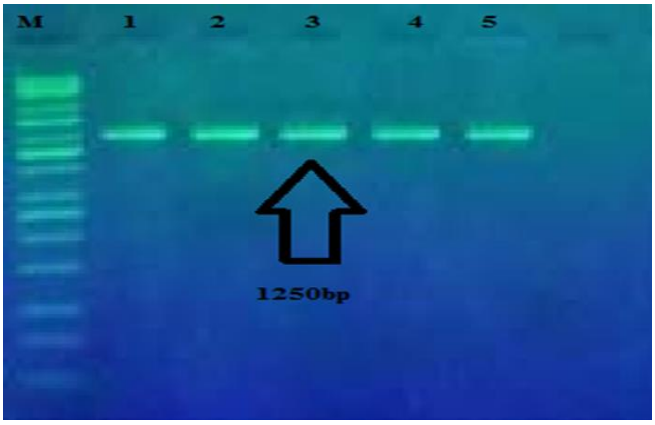


**Fig. 6:** PCR product the band size. The product was electrophoresis on 1.5% agarose at 5 volt/cm2. 1x TBE buffer for 1:30 hours. M: DNA ladder (100).

**Table 3:** The isolates of *P. aeruginosa* from dogs ear swabs according to area

| Area | Examined swab | Dog affected with *P. aeruginosa* | Percentage |
|---|---|---|---|
| Al_Aadhamiya | 10 | 1 | 10 |
| Al_Kadhimiya | 45 | 4 | 8.88 |
| Al_Sayidia | 10 | 3 | 30 |
| Al-Amriya | 45 | 4 | 8.88 |
| AlRahmaniya | 10 | 0 | 0 |
| Total No. | 120 | 12 | 10 |

**Table 4:** Results of antibiotic susceptibility testing of *P. aeruginosa* by disc diffusion method

| Antibiotic | Abbreviation | Disc Concentration (µg) | Intermediate I (n/%) | Susceptible S (n/%) | Resistant R (n/%) |
|---|---|---|---|---|---|
| Polymyxin-B | PB | 300 | 00.00 | 11 (91.66) | 1 |
| Trimethoprim | TMP | 10 | 00.00 | 00.00 | 12 |
| Nalidixic acid | NA | 30 | 00.00 | 1 | 11 |
| Streptomycin | S | 25 | 00.00 | 7 | 5 (41.66) |
| Chloramphenicol | C | 10 | 1 (8.33) | 00.00 | 11 |
| Penecillin-G | P | 10 | 00.00 | 12 (100.00) | 00.00 |
| Imipenem | IMI | 10 | 00.00 | 12 (100.00) | 00.00 |
| Polymyxin-B | PB | 300 | 00.00 | 11 (91.66) | 1 |
| Ceftriaxone | CRO | 10 | 3 (25.00) | 4 (33.33) | 5 |
| Cefotaxime | CTX | 30 | 7 (58.33) | 1 | 4 |
| Erythromycin | E | 10 | 00.00 | 00.00 | 12 |
| P-value | --- | --- | 0.0001 **_ | 0.0001 **_ | 0.0001 **_ |

* (P≤0.05). ** (P≤0.01).

**Table 4:** The production of some virulence factor by *P. aeuroginosa*

| Results | Virulence factors | |
|---|---|---|
| | Hemolysin | Lecithinase |
| Positive | 12(100%) | 12(100%) |
| Negative | 0 | 0 |

These results were consistent with an Egyptian study, which found 20% *P. aeruginosa* isolates from canine ear infections. This examination discovered 10% of *P. aeruginosa* isolates from canine otitis. These findings corroborated a report from Egypt that found 20% *P. aeruginosa* isolates from canine ear infections. Additionally, *P. aeruginosa* resistance to b-lactam antibiotics complicates the treatment of *P. aeruginosa* infection (Al-Charrakh et al., 2016; Cavallo et al., 2000; Barnard and Foster 2018; KuKanich et al., 2022; Okonkwo and Achilike, 2022). According to Morales-Espinosa et al. (2012). *P. aeruginosa* produces a number of virulence factors, whose expression is controlled by a variety of methods (Empel et al., 2007).

The current study findings were in agreement with the findings of Mahmood (2015) as an opaque zone around colonies with percentage 100%, so it was suggested as an economical confirmative method for lecithinase production. For produce of lecithinase enzyme from *P. aeruginosa* isolates gave positive production with 100% accordance to the results of Atlas et al. (1995), who noted the formation of opaque zones around growing colonies of egg yolk agar.

The simplest method to confirm the hemolysin enzyme-producing virulence strains of *P. aeruginosa*, according to Selim et al. (2015) who also verified the formation of -hemolysis bacterial colonies, is to use blood agar.

The highlighted results suggested different degrees of virulence via pigments production by *P. aeruginosa* due to the environmental condition and investigation areas, in contrast to the findings of Nedeljkovi et al. (2011). In this study *P. aeruginosa* was isolated from dogs, otitis infection with the percentage of isolation 10% from collected swabs in Baghdad city, this percentage was according to the results of Abdullah et al. (2017) study when isolated *P. aeruginosa* from otitis in dogs with percentage 10% in Kalar city, while another study detection of *P. aeruginosa* in otitis at 18% in Wasit city (Alaa et al., 2018).

All antibiotics resistant 100% for six antibiotics and chose as multidrug resistance agreement to the findings of Alaa et al. (2018) who isolated *P. aeruginosa* are showed resistance for Cefotaxime 100% and was similar to the

findings of Mahmood (2015) who showed *P. aeruginosa* was resistance for Nalidixic acid, Cefotaxime, Ceftriaxone, Erythromycin and trimethoprim (100, 73, 89, 84 and 68.4%) respectively while sensitivity to impenem and polymixin-B (26%,36%).

While another investigation disagrees with the findings of Al-Ajeeli (2013) who isolated P. aeruginosa a resistance of 8% to Imipenem and Ceftazidime 29.3%.

Another result disagrees with the findings of Yassin et al. (2014) who isolated this bacterium infections and found that the percentage of resistance was 12.7% for Imipenem. Jaafar et al. (2014), found that imipenem resistance was 24.4%. Al-Shara (2013) in Najaf reported less resistance against imipenem to be 7.4%. A recent study by Al-khudhairy and Al-Shammari (2020) in Iraq stated that imipenem resistance rate was 12.4%.

**Conclusion**

The results presented in this study clearly demonstrated that dogs infected with otitis in Baghdad-Iraq likely constitute an ecological reservoir of antibiotic-resistant strains of *P. aeruginosa* causing otitis media that can pose a threat to dogs. The relatively low incidence of *P. aeruginosa* in ear samples may indicate that domestic dogs in Baghdad-Iraq may not play an important role in the epidemiology *of P. aeruginosa*. However, dogs can play an important role in multidrug-resistant *P. aeruginosa*.

**Authors' Contribution**

Abdullah HA conceived, designed the study, and carried out proofreading. Mohammed BQ collected samples, performed the experiments. Rayshan AR analyzed the data and wrote the paper. All authors reviewed and approved the final manuscript.

**Conflict of Interests:** None.

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