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Emerging and Re-Emerging Animal Health Challenges in Low and Middle-Income Countries

Molecular Identification of Several *Pseudomonas aeruginosa* Virulence Genes

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Abstract | The investigation was carried out to identify virulence factors activity and to diagnose *Pseudomonas aeruginosa* from otitis infection of dog and its susceptibility to essential antibiotics. For this purpose, a total of twelve ear swabs were taken from affected canines. The isolates were initially identified based on their morphological characteristics, such as size, color, and shape, as well as by Gram's staining, biochemical tests, and Vitek2 system confirmation. *Pseudomonas aeruginosa* was then confirmed by PCR assay. Importantly, all isolates tested positive for the ability to produce the virulence factor hemolysin, a phospholipase enzyme. The 16S rRNA gene was first identified as a virulence gene by polymerase chain reaction study, followed by the identification of the exotoxin T, outer membrane protein I, and phenazine+ M genes. Three (27.27%) of the isolates were *P. aeruginosa* carrying exoT genes, four (36.36%) *P. aeruginosa* isolated carried oprI genes, and three (27.27%) isolates of *P. aeruginosa* carrying phz+M genes. By using traditional PCR, oprI had the highest prevalence of virulence genes with 504 bp fragments, followed by exoT with 153 bp fragment and phz+M with 128 bp fragment. High levels of significance ($P < 0.01$) were found in all of the study's components. Finding of the study highlight the prevalence of *P. aeruginosa* from dog otitis infections and disease associated molecular marker which can guide future design of improved treatments.

Keywords | Dogs, Ear samples, *P. aeruginosa*, PCR, 16Sr RNA**Received** | July 12, 2024; **Accepted** | September 15, 2024; **Published** | October 31, 2024***Correspondence** | Ahmed Raheem Rayshan, Department of Physiology, Pharmacology, and Biochemistry, University of Al-Qadisiyah, Al-Diwaniyah, Iraq; Email: ahmed.rayshan@qu.edu.iq**Citation** | Mohammed BQ, Abdullah AH, Rayshan AR (2024). Molecular identification of several *Pseudomonas aeruginosa* virulence genes. J. Anim. Health Prod. 12(s1): 67-74.**DOI** | <http://dx.doi.org/10.17582/journal.jahp/2024/12.s1.67.74>**ISSN (Online)** | 2308-2801**Copyright:** 2024 by the authors. Licensee ResearchersLinks Ltd, England, UK.This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

INTRODUCTION

Pseudomonas aeruginosa (*P. aeruginosa*) is a Gram-negative, rod-shaped, extracellular, obligatory aerobic bacterium which may grow anaerobically in the presence of arginine or nitrate, motile by monotrichous flagellum. Some species of types *P. aeruginosa* carry 1-3 flagella, are non-capsulated and are non-spore forming bacteria (Hameed et al., 2014). The pathogenesis of *P. aeruginosa* is due to the production of extracellular virulence factors (such as pili, hemolysins, pyocyanin, proteases, alginate,

lipopolysaccharide, and the Type III secretion system) and effector proteins (such as Exotoxin U, Exotoxin T, Exotoxin Y, and Exotoxin S) (Chirani et al., 2018; Harrison et al., 2010). Dogs presenting with *Pseudomonas* otitis externa may exhibit one or a combination of the following clinical signs: head shaking, aural pruritus, malodor from the ear, erythema, alopecia, signs of self-trauma to the pinnae and pre-auricular region, shyness around the head, discharge from the ear canal, aural hematoma, and ulceration of the external ear canal (Miller et al., 2013; Al-Nassry, 2011; Barnard and Foster, 2017). It causes inflammation of the

eye and the ear, and soft tissue infections, endocarditis, bacteremia and other infections as well as a number of injuries, especially in hosts who suffer from burns and discouraged immunologically such as cancer patients (Rajan et al., 2003). *P. aeruginosa* is characterized by special odor with production of many soluble pigments that are helpful for identification of this bacterium such as greenish blue pyocyanin pigment, which has bacteriocin activity that inhibits other bacteria, in addition to radioactive pyoverdine pigment, red pyorubin pigment, pyomelanin pigment and greenish-yellow fluorescence pigment (Lau et al., 2004).

Ear infections are among the ten most frequent reasons for dogs to be presented to veterinarians and may affect up to 20% of dogs (Angus, 2004; Cole, 2004). Bacteria are one of the main causes of middle ear infections, as well as some other pathogens such as viruses and fungi. One or more microorganisms may participate in the infection events, and other factors such as age, sex, genetic, ethnic, social, economic and climate factors influence (Daiy et al., 1999). The polymerase chain reaction (PCR) technique has been widely used to diagnose and identify many pathogens in molecular biology (Hery-Arnaud et al., 2017). *P. aeruginosa* is resistance to many antimicrobials and it frequently causes infection in animals undergoing antibiotic treatment or in immunocompromised hosts. Additionally, the bacteria are found infrequently as a part of microbial flora of healthy animals (Abedey et al., 2009). As a more powerful technique for identifying bacteria, ribosomal RNA sequencing has been developed. In a prior study, 16S rRNA gene sequencing was successfully used in the clinical laboratory to identify bacterial pathogens (Woo et al., 2008; Suardana, 2014).

MATERIALS AND METHODS

SAMPLING

One hundred twenty ear samples were collected from otitis infected dog of both sexes and were obtained from five cities of Baghdad province, Iraq (Hattin district, Al-Kadhimiya, Al-Mashatel Street, Al-Sidiya and Al-Rahmaniya). Samples were collected in a five-month period from October 2021 to February 2022. Samples were

collected from dogs suffering from clinical signs (the head tends frequently as a result of inflammation, itching, clear redness in the ear, earwax secretion and an unpleasant odor). Ear samples were transferred into sterile 10-ml universal tubes with transport medium (BHI broth) and labeled by a special number, then placed in a cool box and immediately, send to the Laboratory of Veterinary Medicine College in Baghdad University.

ISOLATION AND IDENTIFICATION OF *PSEUDOMONAS AERUGINOSA*

All samples were streaked on nutrient agar, MacConkey agar and sub-cultured on cetrimide agar and were incubated aerobically for 24 hrs at 37° C. The bacteria were identified depending on microscopical feature by using Gram stain to detect their response to stain, arrangement and shape (Markey et al., 2014). In addition, the morphological features on culture media such as beta-hemolysis on blood agar, non-lactose ferment on MacConkey agar also several biochemical tests were used to identify the *Pseudomonas* isolates, such as citrate utilization tests, methyl red / Voges - proskauer (MR/VP) tests, catalase test, gelatin liquefaction test, oxidase test, motility test and triple sugar iron test (Markey et al., 2014). Finally, samples were confirmed with Vitek2 system (Logan and Turnbull, 2013).

VITEK® 2 COMPACT IDENTIFICATION SYSTEM

The BCL identification cards (Bio Merieux) were used based on established biochemical methods and newly developed substrates. This device included 64 biochemical tests which were used in the diagnosis of bacteria to reach the 98 % degree of accuracy (Logan and Turnbull, 2013). Bacteria were subjected for identification by VITEK 2 compact system according to the instruction provided by the BioMérieux company were followed: 3-5 colonies from each isolate were transferred to a glass tube containing 3 ml normal saline to calculate and modify turbidity must be equal to 0.5 Macfarlane standard. Negative pressure was used to transfer bacterial suspension to cassettes which contains 64-wells with 43 colorimetric substrates for phenotypic identification of bacterial species. After complete biochemical reaction within 12 hours, the results were performed according to special software to identify bacterial species and strain.

Table 1: Primers set used for detection of *P. aeruginosa* (Macrogen/ Korea).

Primer	Primer sequence 5'- 3'	Product size bp	Annealing temp. (°C)
16S rRNA1	27F/ AGAGTTTGTATCCTGGCTCAG 1492R/TACGGTTACCTTGTACGACTT	1500	60
OprI	F/ ATGGAAATGCTGAAATTCGGC R/ CTTCTTCAGCTCGACGCGACG	55	504
exoT	F/ AATCGCCGTCCAACCTGCATGCG R/ TGTTCGCCGAGGTACTGCTC	56	153
Phz+M	F/ ACCTGGCCTTCCACGAGAT R/ CAACAGGCTGGAGAGGTTGT	59	128

MOLECULAR DETECTION OF *P. AERUGINOSA* ISOLATES

To study bacterial phylogeny and taxonomy, the determination of 16S rRNA gene sequences by polymerase chain reaction (PCR) was applied. With the gene presence in almost all bacteria, often existing as a multi-gene family, or operons, the function of the 16S rRNA gene over time has not changed, suggesting that random sequence changes are a more accurate measure of time and the 16S rRNA gene (1500 bp) is large enough for informatics purposes (Patel, 2001). Genomic DNA extraction of *P. aeruginosa* was performed by isolation of DNA from bacterial growth according to the protocol of G-spin DNA Extraction (Genomic DNA Mini Kit, Geneaid, Thailand). Amplification of a fragment of the *P. aeruginosa* 16SrRNA by PCR was conducted using primers: forward (5'- AGAGTTTGATCCTGGCTCAG - 3') and reverse (5'- TACGGTTACCTTGTTACGACTT - 3'). Primers were procured from IDT (Integrated DNA Technologies company, MacroGen (Korea)). The primers were diluted and used in the interaction according to Mackichan et al. (2015).

RESULTS AND DISCUSSION

In the present study, a total of 120 swab of otitis infections in dogs were collected from different areas in Baghdad city and from different age groups between October 2021 and February 2022. Of these 120 samples, *Pseudomonas aeruginosa* only was detected in 12 (10%) samples while other samples were tested positive for *Staphylococcus* spp., 30 (25%), *Salmonella* spp., 10 (8.33%), *Streptococcus* spp., 4 (3.33%), *Klebsiella* spp., 8 (6.67%), *Proteus* spp., 20 (16.67%), *E. coli* 10 (8.33%), respectively. All other samples were negative for any bacterial growth (Table 2). This agrees with Al-Charrakh et al. (2016) who have recorded 20% *P. aeruginosa* isolates from ear infections in Egypt. The incidence of *P. aeruginosa* isolates according to the status of the examined dogs and ages either young or adults, as it showed higher incidence with *P. aeruginosa*, as reported by Gonzalez et al. (2004).

Table 2: The bacteria isolated from samples collected from infected dogs.

Percentages of isolates	Number of isolates	Identification of bacterium
10%	12	<i>Pseudomonas aeruginosa</i>
12.50%	15	<i>Salmonella</i> spp.
16.67%	20	<i>Proteus</i> spp.
6.67%	8	<i>Klebsiella</i> spp.
8.33%	10	<i>E. coli</i>
25.00%	30	<i>Staphylococcus</i> spp.
3.33%	4	<i>Streptococcus</i> spp.
17.50%	21	Negative samples
100%	120	Total No.
8.719 **	---	Chi-Square (χ^2)

** (P≤0.01).

The twelve samples were positive for *P. aeruginosa* (showing a percentage positivity of 10%). The infection rate of *Pseudomonas aeruginosa* was high 4 (33.33%) in Al_kadhimiya aerra, 4 (33.33%) in Al-amriya aera, 3 (25%) in Al-Sayidia area, and low infection rate 1 (8.33%) in Al-dhamiya aera, while the other samples observed negative for any bacteria as show in the Figure 1 and Table 3.

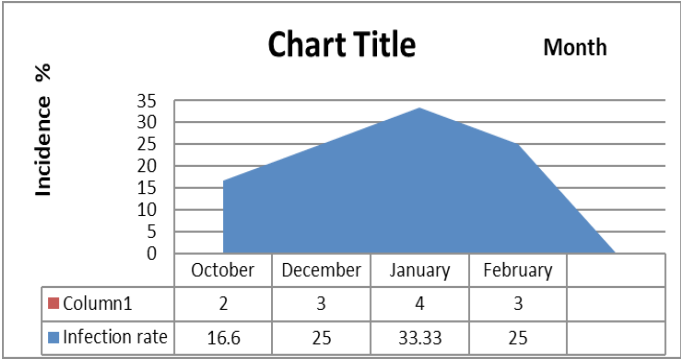


Figure 1: The incidence of *P. aeruginosa* isolated from dog's ear samples according to the area.

Table 3: The incidence of *P. aeruginosa* isolated from dog's ear samples according to area.

Percent-ages %	<i>Pseudomonas aeruginosa</i> isolates	Per-centage %	Exam-ined samples	Area
33.33%	4	37.50%	45	Al-Amriya
33.33%	4	37.50%	45	Al_Kadhimiya
8.33%	1	8.33%	10	Al_ Dhamiya
25.00%	3	8.33%	10	Al_Sayidia
	0	8.33%	10	Al-Rahmaniya
100%	12	100%	120	Total No.
4.0278*	---		---	Chi-Square (χ^2)

* (P≤0.05).

Table 4: The incidence of *P. aeruginosa* isolated from dog's ear samples according to date.

Percent-ages %	<i>Pseudomonas aeruginosa</i> isolates	Percent-ages %	No. of samples isolates	Months
16.67%	2	8.33%	10	October
0%	0	25.00%	30	November
25.00%	3	33.33%	40	December
33.33%	4	20.83%	25	January
25.00%	3	12.50%	15	February
100%	12	100%	120	Total
4.517 *	---	23.750 **		Chi-Square (χ^2)

* (P≤0.05), ** (P≤0.01).

The positivity rates in samples collected between October 2021 to February 2022 were higher in January (4, 33.33%) followed by February (3, 25%) and low rate of positivity

was observed in December (3, 25%) and even lower in October (2, 16.67%). These results indicate a low incidence of *P. aeruginosa* infection in October and high incidence of *P. aeruginosa* infection in January (Table 4).

Table 5: The incidence of *P. aeruginosa* isolated from young and adult dogs.

Percent-ages	<i>Pseudomonas aeruginosa</i> isolates	Percent-ages	Examined samples	Age of dogs
66.67%	8	58.33%	70	Adult
33.33	4	41.67%	50	Young
100%	12	100%	120	Total
0.248 NS	---	3.371 *	---	Chi-square (χ^2)

*($P \leq 0.05$).

Out of 120 ear samples of dogs, adult (70, 58.3%) showed higher positivity compared to young dogs (50, 41.67%). The isolation of *P. aeruginosa* was higher (8, 66.6%) in adult and low (4, 33.3%) in young dogs. This clearly articulate that a high infection rate of *P. aeruginosa* in adult dog with low infection rate in young (Table 5).

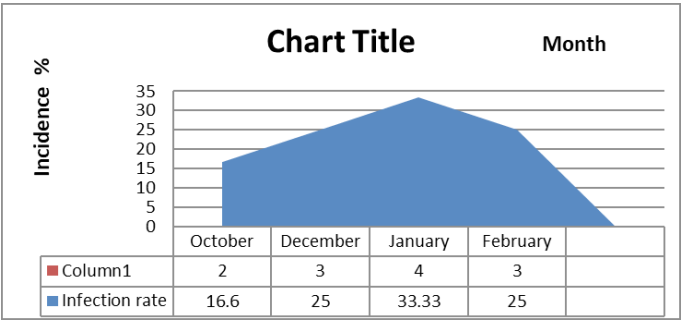


Figure 2: The incidence of *P. aeruginosa* isolated from dogs ear samples according to date.

CONFIRMATION OF *P. AERUGINOSA* ISOLATES

Bacteriologic culture of ear samples was recovered on selective media. Smooth round colonies with pale yellow color on MaCconkey agar occurred by utilizing of non-lactose in the agar, while cetrimed agar was considered as a rapid and accurate method for distinguishing *P. aeruginosa* from other Gram-negative bacteria. The visible colonies appeared blue or yellow green, which indicated non lactose fermentation by producing a pyocyanin (blue green), fluorescein (yellow green) pigments as noticed by Forbes et al. (2002).

Bacterial colonies were observed to be blue colored on nutrient agar. On nutrient agar, several odors ranging from a sweet to earth smell were observed. The colonies were large, low, oval, convex and rough, sometimes surrounded by serrated growth (Pitt and Simpson, 2006). On the cetrimide agar, the bacterial colonies appeared as greenish yellow, most of which produce pyocyanin, which is green blue and pyoverdine, as reported earlier (Sudhakar et al., 2015).

DETERMINATION OF SOME VIRULENCE FACTORS PRODUCE BY *P. AEUROGINOSA*

Distribution of virulence factors in clinical isolates of *P. aeruginosa* showed all isolates were β -hemolysis bacteria where colonies appeared on blood agar, and evidence of hemolysin enzyme production. The results are in agreement with Selim et al. (2015) which showed 100% positivity for lecithinase. On the egg yolk agar, the bacterial colonies showed an opaque zone around the colonies indicating phospholipase production. It has been identified previously that most of the *P. aeruginosa* form brown opaque zones around growing colonies (Atlas,1995; Mahmood, 2015) (Table 6).

Table 6: The production of some virulence factor by *P. aeuroginosa*.

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

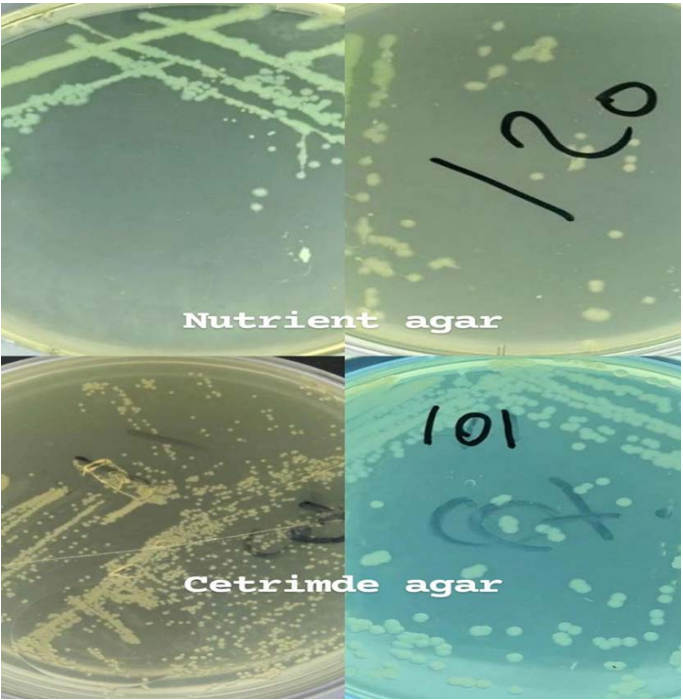


Figure 3: *P. aeruginosa* isolates on selective and differential media.

The optimum growth of *P. aeruginosa* in the biochemical testes occurred at 37 °C. The growth on triple sugar iron agar (TSI) with a (K/K/g-H2S-) profile, (Alkaline-Alkaline) on both the slant and bottom with appear no gas bubbles but no H2S production. We also noticed that IMVIC was negative for *P. aeruginosa*, as well as indole negative, methyl red-negative and VP negative. However, we observed that the citrate test was positive. It also gave positive results in the motility test. The results of catalase tests was positive as well as for oxidase (Todar, 2011).

BIOCHEMICAL CONFIRMATION BY VITIK 2 COMPACT SYSTEM

THE SUSPECTED ISOLATES WERE CONFIRMED BY VITIK 2 COMPACT SYSTEM

The VITEK2 small system was used (Biomérieux-France), which offers an advanced approach to bacterial inspection by delivering more dependable technology, high speed, and high sensitivity for bacterial identification, with 99 % accuracy findings. As shown in Figure 5, the study’s conclusion was that isolates were indeed (99%) *P. aeruginosa*.

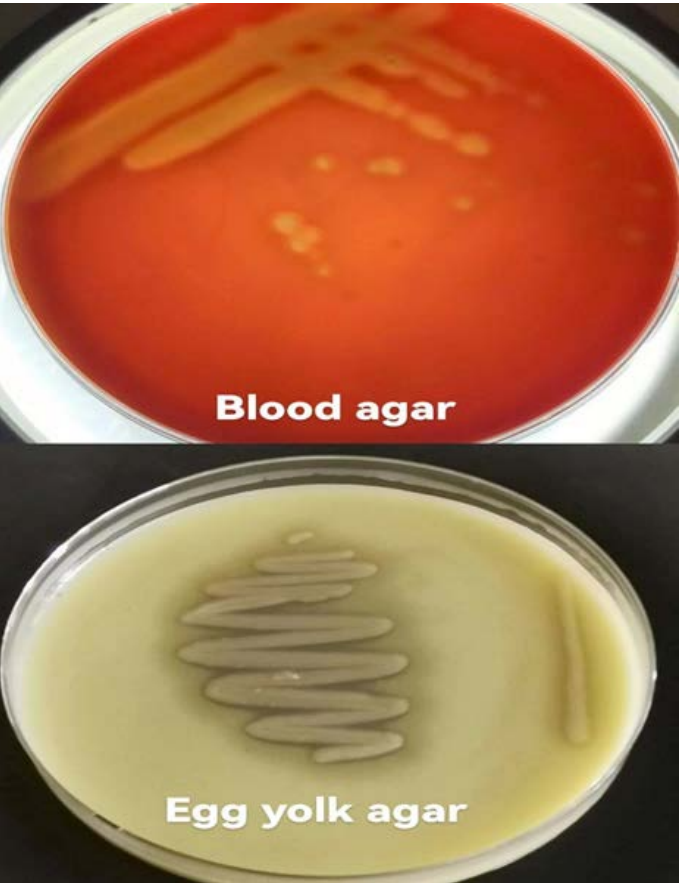


Figure 4: Phospholipase enzyme and hemolysin produce by *P. aeruginosa* on blood and egg yolk agar.

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Figure 5: Result sheet of VITEK2system for *P. aeruginosa*.

MOLECULAR TECHNIQUES

PCR was applied to confirm genomic DNA for *P. aeruginosa*. The invention of polymerase chain reaction (PCR) and automated DNA sequencing has allowed the characterization of bacteria thoroughly and economically. A comparison of the genomic sequences of bacterial species showed that the 16S ribosomal RNA (16S rRNA) gene is highly conserved within a species and among species of the same genus and, hence, can be used as the new gold standard for the specification of bacteria (Woo et al.,2000).

The G-spin DNA extraction kit (Intron Biotechnology, Korea) was used for isolation of genomic DNA from bacteria. All six isolates, detected positive for bacteriological characteristics, were also detected positive for genomic DNA amplification on agarose gel electrophoresis. The amplification of bacterial 16s rRNA gene showed an expected 1500 bp (Figure 6).

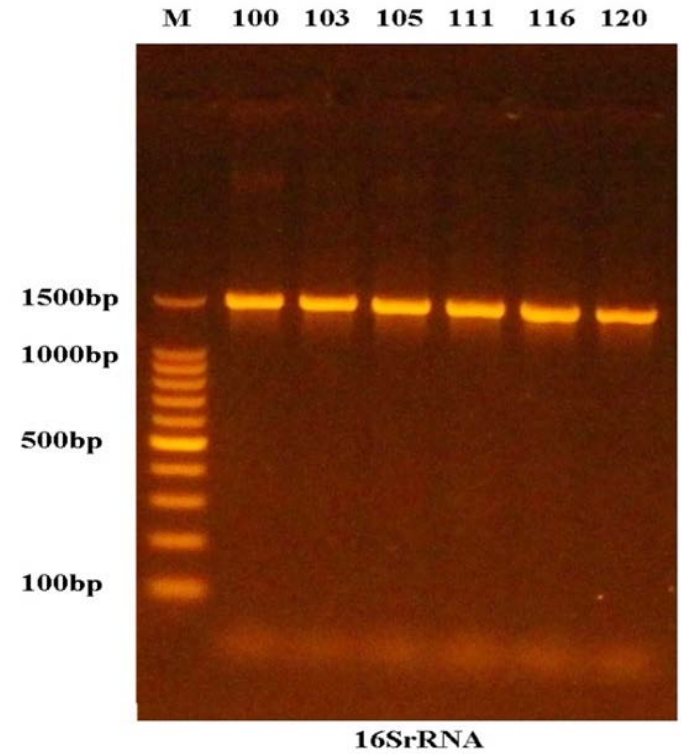


Figure 6: The amplification results of 16SrRNA gene of Unknown bacterial species fractionated on 1.5% agarose gel electrophoresis stained with Eth.Br. M: 100bp ladder marker. Lanes 1-6 resemble 1500bp PCR products.

Four of the twelve *P. aeruginosa* isolates found in dog’s otitis were selected for virulence genes identification. Each of the three DNA samples was examined for four genes (oprI, exo T andphz+M) as showed in Figure 7.

Out of four of *P. aeruginosa* isolates, three (27.27%) isolates were *P. aeruginosa* carried exo T genes, four (36.36%) isolates carried oprI genes, and three (27.27%) isolates were positive for phz+M genes Table 7.

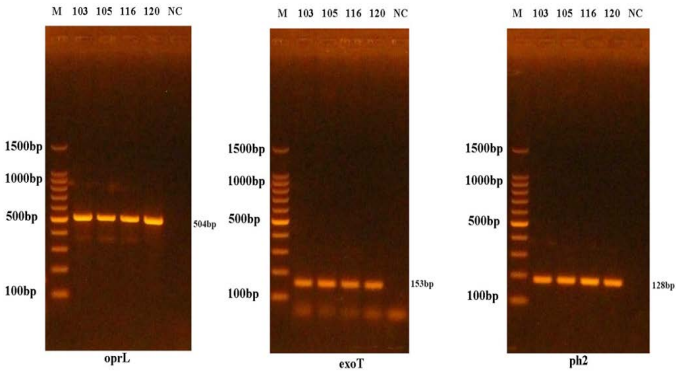


Figure 7: Agarose gel electrophoresis shows amplification product of 504 bp,153 bp,128 bp fragments of (exoT, oprI, phz+M) gene by PCR. (1.5%) agarose gel (5 volt\ cm2\ 1.5 hours). Lane M: DNA ladder (1000 plus) bp.

Table 7: The result of virulence genes in *P. aeruginosa* isolate.

Percent-ages	No. of isolates carrying gene	No. of total isolates	Name of genes
27.27%	3	11	<i>ExoT</i>
36.36%	4	11	<i>OprI</i>
27.27%	3	11	<i>Phz+M</i>
0.903 NS	---	---	Chi-Square (χ^2)

** ($P \leq 0.01$).

All of *P. aeruginosa* strains tested here contained the *oprI* genes (sensitivity= 100%, specificity= 80%). Similar in this study, all of the 268 isolates were remarkably positive for both *oprI* genes (Lavenir et al., 2007). In this study, we observed a lower prevalence of *exoT* gene (5%, respectively) (Berthelot et al., 2003). Six of the *P. aeruginosa* isolates were selected and sent to Korean laboratory for the identification of the sequences and comparison with similar sequence of the 16S rRNA gene of the reference strains of *P. aeruginosa* in GenBank. These isolates were confirmed by using small subunit ribosomal PCR gene (*16s rRNA* gene) amplified by a specific primer. Sequencing and analysis for similarities across GenBank sequences were conducted. The full gene sequences of the strains were compared automatically using the Basic Local Alignment Search Tool (BLAST) in the NCBI against the sequences of bacteria available in databanks. The aim of the sequence analysis for *P. aeruginosa* was to analyze the novel sequence of the *16S rRNA* gene of strains isolated in this study in order to understand the phylogenetic relationships between these sequences and those between the sequences of bacteria available in databanks. Six isolates were confirmed by using small subunit ribosomal PCR gene (*16s rRNA* gene) amplified by a specific primer. Based on the sequencing and analysis for similarities across GenBank, it appeared that six isolates showed a 100% nucleotide sequence identity (Table 8).

Table 8: The isolates of *P. aeruginosa* by 16s rRNA gene sequencing ID in gene bank and nucleotide sequence identity from (NCBI).

Gene: 16S ribosomal RNA gene				
Iden-tities	Sequence ID with compare	Accession numbers	Source	Isolate No.
100%	ID: MT646431.1	OM918231.1	<i>P. aeruginosa</i>	1
100%	ID: MN889007.1	OM918232.1	<i>P. aeruginosa</i>	2
100%	ID: MT180543.1	OM918233.1	<i>P. aeruginosa</i>	3
100%	ID: MT448952.1	OM918234.1	<i>P. aeruginosa</i>	4
100%	ID: CP053747.1	OM918235.1	<i>P. aeruginosa</i>	5
100%	ID: MT598026.1	OM918236.1	<i>P. aeruginosa</i>	6

CONCLUSIONS AND RECOMMENDATIONS

The findings of this study clearly show that dogs with otitis serve as an ecological reservoir for strains of *Pseudomonas aeruginosa* that are resistant to antibiotics, which may be transmissible and dangerous to cats, cattle, and people. The comparatively low prevalence of *Pseudomonas aeruginosa* in ear samples would suggest that domestic dogs do not have a significant impact on the epidemiology of this bacteria. But in the case of multidrug-resistant *Pseudomonas aeruginos*, dogs can be crucial emerging host. It appears that using the *oprI*, *exoT*, and *phzM* genes simultaneously increases the reliability of *P. aeruginosa* detection based on the PCR. The identification of many virulence genes in *P. aeruginosa* isolates led to believe that these genes are linked to various degrees of intrinsic virulence and pathogenicity, which warrant future investigations.

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NOVELTY STATEMENT

Molecular identification of *Pseudomonas aeruginosa* virulence genes in Baghdad region add novel finding in this domain which warrant future investigations.

AUTHOR’S CONTRIBUTION

Authors of manuscript Baraa Qasim Mohammed, Asmaa Hamoody Abdullah, and Ahmed Raheem Rayshan had similar participation in this study.

CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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