

Molecular and Phylogenetic Study of (Tox A) Virulence Gene of *Pseudomonas - Aeruginosa* Isolated from Respiratory Infections of Cats in Baghdad city

Teba Ahmed Jaralla¹, Amanee Mohammed Radhy²

Email : tiba.jar2301m@covm.uobaghdad.edu.iq

^{1,2} Department of Internal and Preventive Veterinary Medicine, College of Veterinary Medicine, University of Baghdad

Abstract

Background *Pseudomonas aeruginosa* is an opportunistic Gram-negative pathogen whose virulence strongly associated with exotoxin A encoded by the *ToxA* gene. Aim the study To detect and molecularly characterize the (*ToxA*) gene of *Pseudomonas aeruginosa* isolated from respiratory infections in cats in Baghdad city, and determine the phylogenetic relationship of local isolates with global strains. Methodology A total of 30 bacterial isolates previously identified as *Pseudomonas aeruginosa* based on cultural characteristics, biochemical identification using the VITEK-2 system with molecular detection, Genomic DNA was extracted using a commercial kit. the DNA purity and concentration were measured using a Nanodrop. polymerase chain reaction amplification of the *ToxA* gene. positive polymerase chain reaction products were selected for sequencing and sent to for Sanger sequencing. Results polymerase chain reaction findings revealed that 29 *Pseudomonas aeruginosa* isolates were positive for the *ToxA* gene, producing a clear amplification band at 511 bp. These results confirmed the widespread presence of this virulence gene among isolates obtained from feline respiratory infections. The phylogenetic analysis of partial *ToxA* gene sequences demonstrated that Iraqi isolates clustered closely with reference strains from several countries. The short branch lengths indicated a high degree of genetic similarity and limited divergence among global isolates. Additionally, some Iraqi isolates formed closely related sub-clusters, suggesting possible local adaptation or common sources of infection. Conclusions The present study confirms that the *ToxA* gene is highly prevalent among *Pseudomonas aeruginosa* isolates recovered from respiratory infections in cats and highlights its critical role in bacterial pathogenicity and disease severity. Phylogenetic analysis revealed a close genetic relationship between local Iraqi isolates and global reference strains, indicating limited genetic variation in the *ToxA* gene.

Key words: PCR, *ToxA* gene, *Pseudomonas aeruginosa*, cats, Baghdad.

المخلص

الخلفية: الزائفة الزنجارية هي بكتيريا انتهازية سالبة الغرام، ترتبط ضرورتها ارتباطاً وثيقاً بالذيفان الخارجي A المشفر بواسطة جين *ToxA*. الهدف: الكشف عن جين *ToxA* في الزائفة الزنجارية المعزولة من عدوى الجهاز التنفسي لدى القطط في مدينة بغداد، وتحديد خصائصه الجزيئية، وتحديد العلاقة التطورية بين العزلات المحلية والسلالات العالمية. المنهجية: تم جمع 30 عزلة بكتيرية سبق تحديدها على أنها زائفة زنجارية بناءً على خصائصها المزرعية، وتم التحقق من هويتها الكيميائية الحيوية باستخدام نظام VITEK-2 مع الكشف الجزيئي. استُخلص الحمض النووي الجينومي باستخدام مجموعة تجارية، وقُيس نقاء الحمض النووي وتركيزه باستخدام جهاز Nanodrop. تم تضخيم جين *ToxA* باستخدام تفاعل البوليميراز المتسلسل (PCR). تم اختيار نواتج تفاعل البوليميراز المتسلسل الإيجابية للتسلسل، وأُرسلت لإجراء تسلسل سانجر. أظهرت نتائج تفاعل البوليميراز المتسلسل أن 29 عزلة من بكتيريا الزائفة الزنجارية كانت إيجابية لجين *ToxA*، حيث أنتجت حزمة تضخيم واضحة عند 511 زوجاً قاعدياً. أكدت هذه النتائج الانتشار الواسع لهذا الجين المسبب للضرارة بين العزلات المأخوذة من التهابات الجهاز التنفسي لدى القطط. أظهر التحليل التطوري لتسلسلات جين *ToxA* الجزيئية أن العزلات العراقية تجمعت بشكل وثيق مع السلالات المرجعية من عدة دول. يشير قصر أطوال الفروع إلى درجة عالية من التشابه الجيني وتباين محدود بين العزلات العالمية. بالإضافة إلى ذلك، شكلت بعض العزلات العراقية مجموعات فرعية وثيقة الصلة، مما يشير إلى احتمال وجود تكيف محلي أو مصادر عدوى مشتركة. الخلاصة: تؤكد هذه الدراسة أن جين *ToxA* منتشر بكثرة بين عزلات الزائفة الزنجارية المعزولة من التهابات الجهاز التنفسي لدى القطط، وتسلط الضوء على دوره الحاسم في إمراضية البكتيريا وشدة المرض. كشف التحليل الوراثي عن وجود علاقة وراثية وثيقة بين العزلات العراقية المحلية والسلالات المرجعية العالمية، مما يشير إلى وجود تباين وراثي محدود في جين *ToxA*.

الكلمات المفتاحية: تفاعل البوليميراز المتسلسل، جين *ToxA*، الزائفة الزنجارية، القطط، بغداد.

Introduction

Cats are widely kept as companion animals around the world, and they play a notable role in the spread of infectious agents including: Virus, bacteria and parasites particularly of zoonotic importance [1] Cats are kept around the world , and could

represent domestic reservoirs of zoonotic agents such as bacterial pathogens that can cause respiratory tract infections (RTIs). *Pseudomonas aeruginosa* is an opportunistic Gram-negative bacterium that can cause respiratory tract infections in cats, particularly in immunocompromised

animals. It has been isolated from both the upper and lower respiratory tract and is associated with severe respiratory diseases (pneumonia) due to its multiple virulence factors [2]. It is known to be a pathogen with intrinsic resistance to many antimicrobials, possessing virulence determinants like exotoxins, proteolytic activity, secretion system components and biofilm-associated genes that enhance bacterial persistence and colonization in host tissues [3] [4]. Its isolation from feline clinical specimens, including respiratory tract infections and otitis externa, has now been confirmed in recent veterinary studies [5], [6], [7] highlighting its opportunistic pathogen profile in small animal medicine. *P.aeruginosa* is known to display a significant virulent factor, *Exotoxin A*, which inhibits protein synthesis through ADP-ribosylation of elongation factor-2 (EF-2). This process can be performed as cell death and tissue damage that is encoded by the *ToxA* gene. The toxin A (*ToxA*) gene is known to be an important molecular marker for determining pathogenic potential and virulence heterogeneity in isolates [8], [4]. Polymerase chain reaction detection of virulence gene is a one the most sensitive and specific molecular methods. Virulence genes crucial for its pathogenicity and epidemiology (including *ToxA*, *exoS*, *exoU* and *lasB*) have been documented in molecular studies on *P.aeruginosa* from cats as well as other companion animals [5]. This includes genes such as exotoxin A (*ToxA*), exoenzyme S (*ExoS*), Efflux Pump Inhibitor gene [9] and several others involved in antibiotic resistance [10], [11], [12]. tree helps in studying genetic diversity and evolution of *P.aeruginosa* isolates from cats Therefore, the molecular

detection and [13] characterization of *P.aeruginosa* isolates from cats and of the *ToxA* gene could improve diagnostic accuracy as well as our understanding of virulence mechanisms in veterinary microbiology [14]. The *ToxA* gene is commonly detected in both clinical and environmental strains of *Pseudomonas aeruginosa*, which confirms its utility as a molecular indicator for species identification as well as for assessing virulence potential. Detection of *ToxA* using PCR techniques is extensively used because of its accuracy, high sensitivity and speed. Moreover, analysis of *ToxA* gene sequences contributes to a better understanding of genetic relatedness among isolates, variation between strains, and potential patterns of geographic adaptation [15].

Material and methods

Ethical approved

The ethical and research committee of the College of Veterinary Medicine at the University of Baghdad, Ministry of High Education and Scientific Research, approved this investigation. Iraq project (No.: P-G 3114, date 28/12/2025) nasal swabs were collected from cats during routine clinical visits. Animals included in the study had clinically evident respiratory tract infections. The cat's owners gave their verbal agreement before samples were taken and all procedures complied with ethical and professional standards.

Sample Collection

A total of 150 swabs from nasal swab samples with respiratory tract infections by the researcher obtained from cats from different areas of Baghdad city, including

the Veterinary Hospital and Pet Clinics from six regions (Baghdad hospital, Al-Jadriyah, Al-Adamiya, Hayu AL-Jamieih, Ghazaliya and Al-Saidiy) from October 2024 to September 2025 ranged in age from 3 months to 5 years. The nasal swabs were taken from different breeds of cats including (local,Himalayan,shirazi, chinchilla,scottish). Case histories, when available, were obtained from the records of participating veterinary clinics. All samples were transported to Laboratory of the Internal and Preventive Medicine Branch, College of Veterinary Medicine, University of Baghdad, and the bacteria were identified by culture, cultured on MacConkey's agar,

Cetrimide agar[16] . Suspected colonies were examined by Gram staining [17] and identified biochemically using vitek-2 system[18] . Genomic DNA was then extracted from the confirmed *Pseudomonas aeruginosa* isolates using a commercial extraction kit (Geneaid, Taiwan). The ToxA gene was subsequently detected by PCR using specific primers.(Table 1). The 30 isolates of *Pseudomonas aeruginosa* were identified in a previous study using conventional methods in addition to molecular diagnosis using (16S rRNA) from the respiratory infections of 150 cats with respiratory signs in Baghdad province[19] .

Table (1). Primers used in the current study.

Gene	Forward 3' 5' →	Reverse 3' 5' →	Product Size	Reference
ToxA	GTGCTGCACTACT CCATGGT	GCTGGGCGAGGT AGTTGTAG	511	Hassan and Saeed . [20]

Table (2): Conditions of PCR cycle for primers of ToxA gene

Steps	Temperature	Time	Cycles
Initial denaturation	94C°	5:00 min.	1
Denaturation	94C°	45sec.	35
Annealing	62C°	45sec.	
Extension	72C°	45sec.	
Final extension	72C°	7:00 min.	1
Hold	4C°	-	-

Sequencing

The PCR products for ten samples of each *Pseudomonas aeruginosa* was sent to MacroGen-Korea for sequencing. The sequences was analyzed by using the UPGMA method [21] . The phylogenetic tree was done by evolutionary distances were computed using the Maximum Composite Likelihood method and used substitutions per site as units for sequencing polymorphism [22] . The Evolutionary analyses were conducted in MEGA12[23] . Statistical analysis

Data were analyzed using SAS program (2010) [24] software version 20.0 (MEGA) and NCBI program [22], [25] . The McNemar test was used to compare between two diagnostic methods. While the levels of agreement between tests was using Kappa test. The value $p < 0.05$ was represented a statistically significant.

Results The present results revealed to a successful amplification of (*ToxA*) gene of 29 isolates Out of 30 samples, sample number 30 is negative of *P.aeruginosa* by PCR at 511bp (Figure 1). These results were explained in the Table (3),

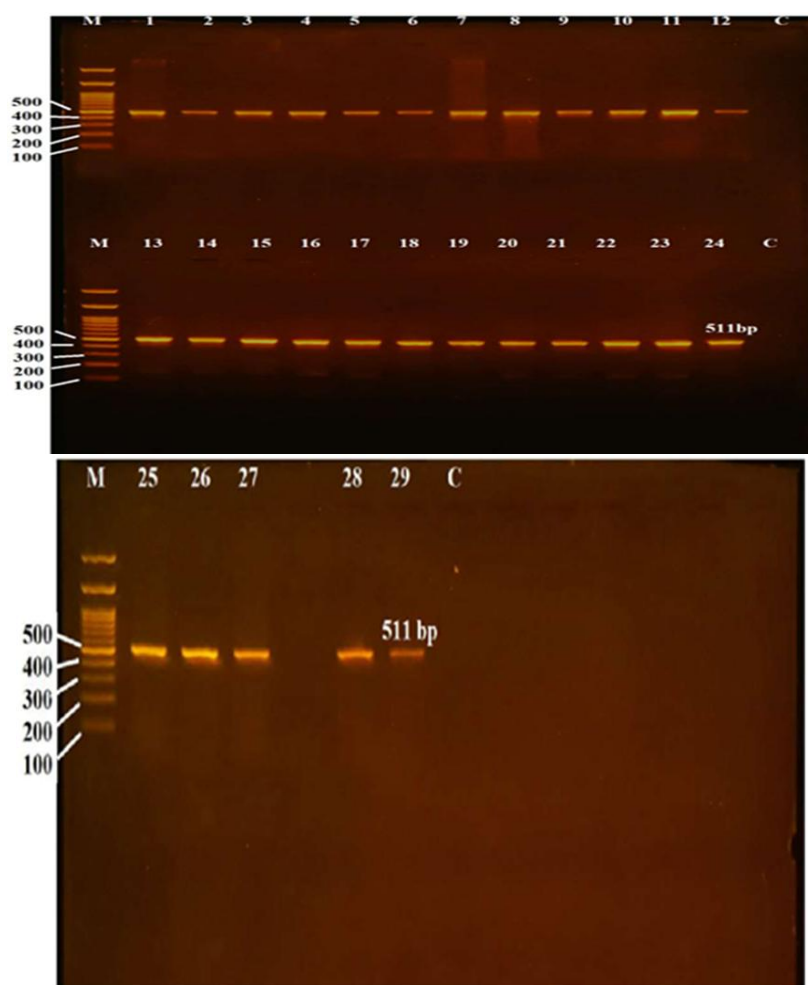


Figure (1). The positive result of (29) isolates of *Pseudomonas aeruginosa* for virulence gene (*ToxA*) gene. Gel electrophoresis (1.2%) 80 mAm, 100 vol. with red safe stain show the result of amplification of 511 bp of *P.aeruginosa ToxA* gen of *Pseudomonas aeruginosa*

Table (3). Prevalence and Statistical Analysis of ToxA Gene in *Pseudomonas aeruginosa* Isolates from Cats

Parameter	Number (n)	Percentage (%)	95% Confidence Interval	P-value
Total samples collected	150	—	—	—
<i>P.aeruginosa</i> isolates	30	20.0%	—	—
ToxA positive isolates	29	96.7%	83.3 – 99.4	<0.0001
ToxA negative isolates	1	3.3%	—	—

150 respiratory samples collected from cats, 30 (20.0%) isolates were identified as *Pseudomonas aeruginosa*. Among these isolates, 29 (96.7%) were positive for the *ToxA* virulence gene, while only one isolate (3.3%) tested negative. Statistical analysis revealed that the prevalence of the *ToxA* gene was significantly high ($p < 0.0001$). The 95% confidence interval ranged from 83.3% to 99.4%, indicating a consistently high distribution of this virulence determinant among the isolates.

Sequencing Analysis

The PCR amplicons for ten *P.aeruginosa* isolates were sequenced by Macro gen (South Korea) the obtain nucleotide sequences were submitted to the NCBI GenBank database under accession numbers and conducted phylogenetic analysis using Unweighted Pair Group Method with Arithmetic Mean (UPGMA), as described by (Sneath & Sokal et al, 21). Also,

Evolutionary distances were calculated using the Maximum Composite Likelihood (MCL) method and nucleotide substitutions per site. All evolutionary and phylogenetic analyses were done using Molecular Evolutionary Genetics Analysis software (MEGA), version 12.

The phylogenetic analysis of ToxA

The phylogenetic tree of the Exotoxin A gene sequences of *P.aeruginosa* (Figure2) demonstrated that the Iraqi isolates in cats clustered within the main clade of reference strains from different countries, including the USA, Russia, Portugal, Spain, India, and Turkey. The branch lengths are short approximately 0.003–0.004, this indicated that a low degree of genetic relationships between Exotoxin A gene of isolate among difference countries and origins, these findings confirmed that Iraqi isolates of *P.aeruginosa* had minor genetic divergence in this virulence gene, and these isolates

have global importance. Whereas, several Iraqi isolates in cats formed closely related sub-clusters with limited local

genetic variation due to host adaptation or environmental effects.

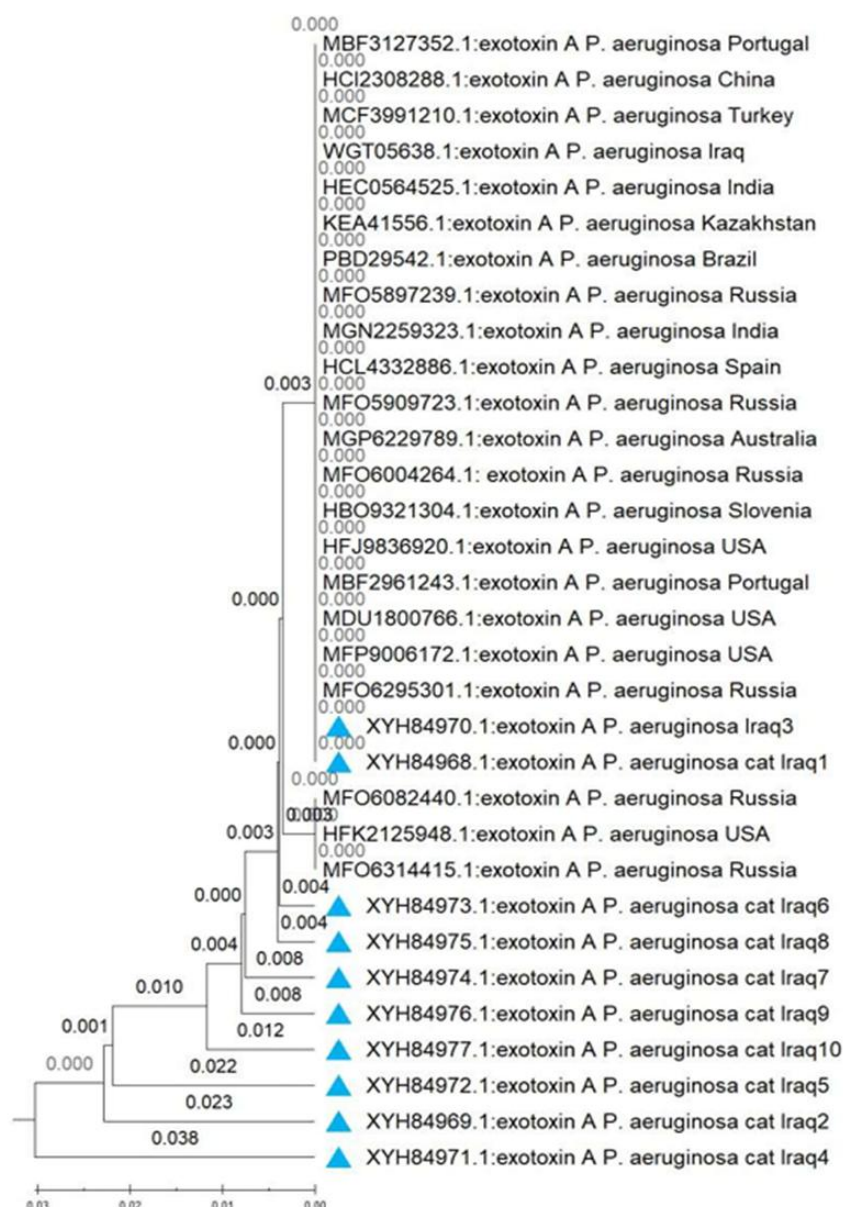


Figure (2): phylogenetic tree of the *ToxA* of Iraqi *Pseudomonas aeruginosa* isolates compared with different global isolat

Discussion

The results of the current study showed that out of 30 isolates of *Pseudomonas aeruginosa*, 29 (96.6%) of them carried the *ToxA* virulence gene indicates that it is one of the most prevalent virulence genes in *P. aeruginosa*. This high frequency suggests that Exotoxin A is a major pathogenic determinant and may contribute significantly to the bacterium's virulence in respiratory tract infections. researchers [5] in Poland who detected the isolates of *P.aeruginosa* from nasal swabs and various types of samples of cats by the same gene and reported the presence of the *ToxA* gene in 66.4% of strains of *P.aeruginosa*. Also, the current results are higher than the 91.7% reported by [26] in Italy. In Iraq, the results obtained from a study of Hassan and Saeed . [20] showed that a total of 21 *Tox A* genes were identified in (26) *P.aeruginosa* isolates, whereas the *ToxS* gene was detected in Shaebth et al. [10] of the same isolates. The present findings agreed with Rocha et al. [27] that most *P.aeruginosa* isolates harbored the *ToxA* gene. The availability of this gene is linked with the pathogenicity of the organism because it inhibits the host cell from synthesizing proteins which promotes bacteria to proliferate quickly across every type of tissue [28] . On the other hand, the results of this study disagreed with the findings of Hamad and Abdulgafor [13] who detected the presence of the other types of virulence genes (*gyr b190* and *gyr b222*) in all isolates of *P.aeruginosa* in cats. Also [5] confirmed that all *P.aeruginosa* strains isolated from infected dogs and cats were harboured the *exoT* (97.8%) and *lasB* (93.4%) genes, while the lowest prevalence was found for *exoU* (17.3%) and *plcH* PCR at 511bp. These results is lower than the findings of other (17.3%).

Conclusion

This study showed that the *ToxA* virulence gene is wide spread between *P.aeruginosa* isolate from respiratory tract infections in cats in Baghdad, indicating the important role of

exotoxin A in the pathogenic behavior of this bacterium. The successful amplification of the *ToxA* gene in all examined isolates highlights its value as a reliable molecular marker for confirming virulent *P.aeruginosa* strains in veterinary diagnostic laboratories. The phylogenetic analysis showed a high similarity level of genetic among local Iraqi isolates and reference strains reported from various regions worldwide, suggesting that the *ToxA* gene is genetically conserved and subject to limited evolutionary variation. The close clustering of several local isolates further indicates the possible influence of common infection sources, host-related factors, or shared environmental condition.

Reference

- [1] M. J. Alwan, "Seropathological diagnosis of *Toxoplasma gondii* in stray cats in Baghdad Province," The Iraqi Journal of Veterinary Medicine, vol. 38, no. 1, pp. 92–98, 2014.
- [2] C. E. Greene, *Infectious Diseases of the Dog and Cat*, 4th ed. Elsevier, 2012.
- [3] A. E. LaBauve and M. J. Wargo, "Growth and laboratory maintenance of *Pseudomonas aeruginosa*," Current Protocols in Microbiology, vol. 25, no. 1, pp. 6E.1.1–6E.1.8, 2012.
- [4] G. Elahi, H. R. Goli, M. Shafiei, V. S. Nikbin, and M. Gholami, "Antimicrobial resistance and virulence gene profiling of *Pseudomonas aeruginosa*," BMC Microbiology, vol. 24, p. 546, 2024.
- [5] D. Płókarz, K. Bierowiec, and K. Rypuła, "Screening for antimicrobial resistance and genes of exotoxins in *Pseudomonas aeruginosa* isolates from infected dogs and cats in Poland," Antibiotics, vol. 12, no. 7, p. 1226, 2023.
- [6] A. M. Khudaier et al., "Isolation of *Pseudomonas aeruginosa* from human and feline infections," Current Veterinary Science, vol. 2, no. 2, pp. 7–11, 2025.
- [7] S. F. Bader et al., "Molecular detection of *Pseudomonas aeruginosa* isolated from otitis

externa in cats using PCR,” University of Basrah Repository, 2024.

[8] H. A. Abbas, A. M. Elsherbini, and M. A. Shaldam, “Molecular detection of virulence genes including ToxA in *Pseudomonas aeruginosa*,” *Journal of Infection and Public Health*, vol. 15, no. 3, pp. 350–357, 2022.

[9] D. Sulaiman and G. Abdulhasan, “Curcumin as an efflux pump inhibitor to enhance treatment against multidrug-resistant *Pseudomonas aeruginosa* isolates,” *Iraqi Journal of Science*, vol. 61, no. 1, pp. 59–67, 2020.

[10] L. J. Shaebth, “Molecular identification and sequencing of *Pseudomonas aeruginosa* virulence genes among different isolates in Al-Diwaneyah hospital,” *Iraqi Journal of Veterinary Sciences*, vol. 32, no. 2, pp. 183–188, 2019.

[11] N. N. Yaseen and D. A. Ahmed, “Detection of mexB multidrug efflux gene in some local isolates of *Pseudomonas aeruginosa*,” *Iraqi Journal of Science*, vol. 64, no. 1, pp. 111–118, 2023.

[12] H. S. Al-Taee, I. A. Al-Samarrae, and H. I. Al-Ahmed, “Antibiotic susceptibility and molecular detection of *Pseudomonas aeruginosa* isolated from bovine mastitis,” *Iraqi Journal of Veterinary Medicine*, vol. 43, no. 2, pp. 77–85, 2019.

[13] M. A. Hamad and A. B. Abdulgafor, “Molecular detection of *Pseudomonas aeruginosa* in feline otitis externa,” *Al-Qadisiyah Journal of Veterinary Medicine Sciences*, vol. 22, no. 1, Supplement I, pp. 27–33, 2023.

[14] M. Bassiouny, H. Brangsch, I. Stamm, P. Kopp, H. Neubauer, and L. Sprague, “Genomic characterization of multidrug-resistant *Pseudomonas aeruginosa* isolates from companion animals,” *Frontiers in Microbiology*, vol. 17, p. 1754860, 2026.

[15] B. K. Markey et al., *Clinical Veterinary Microbiology*, 2nd ed. Mosby Elsevier, 2015.

[16] P. J. Quinn et al., *Clinical Veterinary Microbiology*, 6th ed. Mosby-Wolfe, 2004.

[17] D. L. Kiska and P. H. Gilligan, “*Pseudomonas*,” in *Manual of Clinical Microbiology*, 8th ed., P. R. Murray, E. J. Baron, J. H. Jorgensen, M. A. Pfaller, and R. H. Tenover, Eds. Washington, DC, USA: American Society for Microbiology Press, 2003, pp. 719–728.

[18] B. K. Markey et al., *Clinical Veterinary Microbiology*, 2nd ed. Elsevier, 2014.

[19] T. A. Jarallah and A. M. Radhy, “Molecular and antimicrobial resistance profile of *Pseudomonas aeruginosa* isolated from feline respiratory infections in Baghdad Province,” *Journal of Animal Health and Production*, vol. 14, no. 2, pp. 621–629, 2026.

[20] B. A. Hassan and A. A. Saeed, “Detection and characterization of virulent *Pseudomonas aeruginosa* genes in fresh fish at Al-Diwaniya city market, Iraq,” *Journal of Veterinary Research*, vol. 17, no. 1, pp. 75–83, 2024.

[21] P. H. A. Sneath and R. R. Sokal, *Numerical Taxonomy*. San Francisco, CA, USA: Freeman, 1973.

[22] K. Tamura, M. Nei, and S. Kumar, “Neighbor-joining method,” *Proceedings of the National Academy of Sciences*, vol. 101, pp. 11030–11035, 2004.

[23] S. Kumar et al., “MEGA12: Molecular evolutionary genetics analysis version 12,” *Molecular Biology and Evolution*, vol. 41, no. 12, p. 263, 2024.

[24] SAS Institute, *SAS/STAT User’s Guide*. Cary, NC, USA: SAS Institute, 2010.

[25] K. Tamura, G. Stecher, D. Peterson, A. Filipski, and S. Kumar, “MEGA6: Molecular evolutionary genetics analysis version 6.0,” *Molecular Biology and Evolution*, vol. 30, pp. 2725–2729, 2013.

[26] J. Hattab, F. Mosca, C. E. Di Francesco, G. Aste, G. Marruchella, P. Guardiani, and P. G. Tiscar, “Occurrence, antimicrobial susceptibility, and pathogenic factors of *Pseudomonas aeruginosa* in canine clinical



samples,” *Veterinary World*, vol. 14, pp. 978–985, 2021.

[27] A. J. Rocha, M. R. Barsottini, R. R. Rocha, M. V. Laurindo, F. L. Moraes, and S. L. Rocha, “*Pseudomonas aeruginosa*: Virulence factors and antibiotic resistance genes,” *Brazilian Archives of Biology and Technology*, vol. 62, p. e19180503, 2019.

[28] I. M. Jawher and M. G. Hassan, “Detection of some virulence genes of *Pseudomonas aeruginosa* isolated from meat at Mosul city,” *Iraqi Journal of Veterinary Medicine*, vol. 46, pp. 101–105, 2022.