AL-KUFA JOURNAL FOR BIOLOGY
ISSN (Print): 2073-8854 **ISSN (online):** 2311-6544

Original Research Paper

Molecular and Phylogenetic Identification of Some Virulence Genes of Klebsiella Spp. Isolated from Ear Infection in dogs.

Mass Ameer Hasan, Ibtisam Q. Abdul-Kareem

Department of Veterinary Public Health, Collage of Veterinary Medicine, University of Baghdad, Baghdad, Iraq

Article history
Received: 18/03/2026
Revised: 01/04/2026
Accepted: 13/04/2026

*Corresponding Author: Mass Ameer Hasan
Department of Veterinary Public Health, Collage of Veterinary Medicine, University of Baghdad, Baghdad, Iraq
mas.aamer2309m@covm.uobaghdad.edu.iq; Tel: +9647730108506

Abstract: *Klebsiella* species such as (*Klebsiella pneumoniae*, *Klebsiella aerogenes*), opportunistic pathogens belong to the family *Enterobacteriaceae* are gram-negative bacteria, especially in immunocompromised dogs. **Methods:** A total of No. 50 ear swab samples of dogs, samples were cultured on MacConkey agar, Blood agar, and Chromogenic agar to isolate *Klebsiella spp.* followed using Molecular method to confirm the bacterial identity, by PCR amplification *16S rRNA gene*, phylogentic analysis and detection of some virulence gene (blaCTX-M, magA, and mrkA), and susceptibility test. **Results:** Bacteriological analysis revealed that Klebsiella spp. was accounting for a 14% in dogs (85.71%*K. pneumoniae*, *Klebsiella aerogenes* 14.28%), they were detected in dogs isolates blaCTX-M 1(14.3%), magA3(42.9%) and mrkA1(14.3%). Sequencing and Phylogenetic analysis confirmed that the majority of isolates belonged to *Klebsiella Spp*. (*K. pneumoniae*, *Klebsiella aerogenes*), Phylogenetic analysis revealed high identity (99–100%) with reference strains deposited in GenBank (ID: PV490247.1, ID: PV490248.1, ID: PV490249.1, ID: PV490250.1, ID: PV490251.1, ID: PV490252.1, ID: PV490253.1). **Conclusion:** *Klebsiella spp.* isolates showed complete resistance (100%) to Amoxicillin/Clavulanic acid and Imipenem, with high resistance to both Amikacin and Cefotaxime (71.4%), while showing complete sensitivity to Levofloxacin, Co-trimoxazole, and Chloramphenicol. In feline isolates, complete resistance (100%) was recorded to Co-trimoxazole, Amoxicillin/Clavulanic acid, and Imipenem, in addition to high resistance to Amikacin (66.6%), while all isolates showed complete sensitivity to Levofloxacin, Chloramphenicol, Trimethoprim, and Cefotaxime. Most of these results were highly significant ($P \leq 0.01$).

Keywords: *Klebsiella spp.*, Molecular identification, *16S rRNA*, Virulence genes, Phylogenetic analysis.

1.Introduction

Otitis media (external otitis) is one of the most common conditions in veterinary practice, particularly in dogs, and is a frequent cause of clinical reviews and antibiotic use. The anatomical structure of the ear canal in dogs makes them more susceptible to ear infections than other animals, as well as overlapping environmental and behavioral factors. The ear canal in dogs is long and curved like L-shaped, which impedes natural ventilation and facilitates moisture retention and secretions. This medium is an ideal environment for the growth of negative bacteria such as *klebsiella* Species. [1]. In addition to the factor of the shape of the outer ear, dogs with ears also suffer from an increase in the temperature

inside the ear and poor ventilation, and this represents an environment for the growth of negative bacteria and other factors that may be the natural microbial fluora that turns it into a nurse[2].

Klebsiella spp. is non-motile, rod-shaped bacteria belonging to the family *Enterobacteriaceae*. The genus includes several clinically important species such as *K. pneumoniae*, *K. aerogenes* and other speaces [3]. *Klebsiella* species possess characteristics that make them capsular pathogens, preventing phagocytosis and protecting the bacteria from the immune system [4]. They also form biofilms that aid in adhesion and increase antibiotic resistance [5], and their iron acquisition systems are essential for bacterial survival within the host [6] Furthermore, they exhibit high antibiotic resistance, produce the ESBL carbapenemase enzyme, and possess the ability to exchange genes via plasmids, which accelerates the spread of resistance [7].

Evidence of realistic sensitivity indicates that middle ear infections are a leading cause of medical clinic visits and show high infection rates, especially in developing countries. In recent years, the problem of antibiotic resistance has emerged as a serious issue, with multidrug-resistant (MDR) and even extensively drug-resistant (XDR) *Klebsiella* species strains posing a global health threat. This is linked to the presence of resistance genes and genetic elements that contribute to their spread among bacteria [8,9].

Consequently, the importance of molecular studies to isolate and diagnose *Klebsiella* species from ear infections has emerged, focusing on identifying virulence genes to understand pathogenicity mechanisms, develop effective treatment strategies, and limit the spread of resistant strains.

2. Methodology

Sample collection

Clinical samples (50 No.) of ear infections from dogs, the samples were collected by Trans Media from different locations of Baghdad city including the privet clinic for different age, between October 2024 and February 2025. The samples were labeled and placed in an ice box and then transported to the Microbiology Laboratory, Unit of Zoonotic Disease, College of Veterinary Medicine, University of Baghdad.

Isolation and Identification of klebsiella Spp.

Primer	Sequence	Primer sequence	Tm (°C)	GC%	Size of Product (bp
16s RNA	27F	AGAGTTTGATCCTGGCTCAG	56.92	50.00	1250bp
	1492R	GGTTACCTTGTTACGACTT	52.20	42.11	
bla CTX-M	F	ACCGCCGATAATTCGCAGAT	59.97	50.00	585bp
	R	GATATCGTTGGTGGTGCCATAA	58.53	45.45	
Mag A	F	TAGGACCGTTAATTTGCTTTGT	56.27	36.36	797bp
	R	GAATATTCCCACTCCCTCTCC	56.94	52.38	
Mrk A	F	GGCAGTTTTATTTTCTGACGG	55.78	42.86	358bp
	R	GCACTAAACAGGATGACGTAA	56.01	42.86	

Table.1: The sequence of primers that used in the study

Samples from dog's ear swabs were inoculated by the sterile standard loop method. The isolates under examination were first identified on the Blood agar - Himedia, India, MacConkey agar -Oxoid, UK and selective media Klebsiella chromogenic agar. Pure isolates were produced by subculturing distinct colonies with morphological traits suggestive of Gram-negative bacteria on nutrient agar. and incubated aerobically at 37 °C for (18 to 24) hours [10,11]. The isolation and identification methods used in this study were consistent with those described in [12,13], who reported similar bacterial isolates from ear infections in dogs. following Gram stain and biochemical tests like (IMViC, Oxidase, Catalase, Motility test, TSI and urease), were used to validate the bacterial diagnosis. Bacteria using the gene for ribosomal (16s r rRNA) confirmed the diagnosis [14].

DOI: 10.36320/ajb/v18.i2.23787

Molecular Analysis

Genomic DNA was extracted from the Klebsiella spp. isolates using the FavorPrep Bacterial/Cultured Cells Genomic DNA Extraction Mini Kit, Macrogen® Company (Korea) by the companies instructions than concentration and purity of the extracted DNA were measured using a NanoDrop spectrophotometer.

Specific primers were employed to amplify target genes, including *16S rRNA* for molecular identification (Primers were synthesized by a commercial supplier (sequence details in Table 2), and prepared as stock and working solutions according to the manufacturer's instructions), *bla CTX-M* for extended-spectrum β-lactamase (ESBL) resistance [15,16], and two virulence-associated genes, *mrkA* (fimbrial adhesin involved in biofilm formation) and *magA* (capsule formation)[17,18].

Table 2. The optimum PCR conditions for *(16s RNA*, virulence *magA*, *mrkA*, and resistance *CTX-M* genes.

No.	Phase	Tm (°C) 16sRNA	Tm (°C) blaCTX-M	Tm (°C) MagA	Tm (°C) MrkA	Time	No of Cycle
1	Initial Denaturation	95°C	95°C	95°C	95°C	5 min	1 cycle
2	Denaturation -2	95°C	95°C	95°C	95°C	sec 45	35 cycle
3	Annealing	58°C	52°C	54°C	56°C	sec 45	
4	Extension-1	72°C	72°C	72°C	72°C	1min	
5	Extensi on -2	72°C	72°C	72°C	72°C	5 min	1 cycle

The PCR products of the 16S *rRNA* gene (1250 bp), were separated by 0.5% agarose gel electrophoresis, the rest of the genes under study were separated by 1.5% agarose gel electrophoresis.in (1× TBE buffer), stained with Red safe DNA stain, and visualized under a UV transilluminator to confirm product amplification of the target genes.

Interpreting Susceptibility Tests of Klebsiella Spp.

Based on the primary reference for Antibiotic susceptibility testing, Clinical and Laboratory Standards Institute CLSI. On controlling antibiotic resistance, using standard agar disc diffusion tests (Kirby-Bauer disc diffusion method)- CLSI 2024- and interpreting results as resistant, intermediate, or susceptible [19,21]. On the following antibiotics: Co_trimoxazole (25mg), Levofloxacin (5mg), Gentamicin (10mg)[20], Amoxicillin/Clavulanic Acid (20/10 mg), Chloramphenicol (10mg), Trimethoprim(10mg), Oxytetracycline (30 mg), Penicillin G (10mg), Ciprofloxacin(5mg), imipenem (10mg), (Cefotaxime 10mg) [22,23].

Statistical Analysis

The Statistical Packages of Social Sciences -SPSS (2019) program was used to detect the effect of difference factors in study parameters (percentage distribution). Chi-square test was used to significant compare between percentage (0.05 and 0.01 probability) in this study.

3. Results and discussion

The colonization appearance of *Klebsiella spp.* in Figure 1 shows isolates on different culture media. Pink, sticky, large and their ability to ferment lactose, colonies appeared on MacConkey agar, a characteristic of *Klebsiella* bacteria. On Blood agar showed raised, non-hemolytic colonies that were shiny. The colonies on Chromogenic agar, were characterized by a purple, that helped in the initial differentiation of *Klebsiella spp*. from the *Enterobacteriaceae* family.

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Klebsiella pneumoniae that show in the Table 3 and Figure 1 was the most common bacterial isolate causing ear infections in dogs, accounting for 85.71%, followed by *Klebsiella aerogenes* for 14.28%. These results indicate that *K. pneumoniae* is the main causative agent of these infections, the chi-square test also showed that the differences between the species were statistically significant (P< 0.05).

(a) (b) (c)

Fig 1: Colonies characteristic on MacConkey agar, Blood agar and chromogenic agar (a) Klebsiella Spp. on MacConkey agar (lactose fermenter), (b) on Blood agar (non-hemolytic) , and (c) on chromogenic agar (selective media).

Table 3: Distribution of *Klebsiella spp.* isolated from ear infections in dogs according to bacterial species.

Bacterial Species	No. of Isolates	Percentage (%)	Chi-square (P-value)
Klebsiella pneumoniae	6	85.71%	4.819 * (0.0398)
Klebsiella aerogenes	1	14.28%	
Total	7	100%	

* (P≤0.05)- Significant.

The isolation rate of *Klebsiella spp.* from ear infections in dogs was 14% that shows in Table 4, with a slight increase in males (57.14%) compared to females (42.85%). However, this difference was not significant (P < 0.05).

Klebsiella spp. that shows in Table 5, The highest isolation rate was in the age group from 2 year to 4 year, with (5) isolates out of (50) samples (71.42%), while the isolation rate in the age group from 2 month to 2 year (2) isolates out of (50) samples (28.57%).Despite the higher rate in the age group from 2 year to 4 year,, statistical analysis showed that the differences were not significant (χ^2 = N.S, P < 0.05) between gender, while significant according to the age (P≤0.05)- Significant.

Table 4: Prevalence and percentage of *klebsiella spp.* from ear infections in dogs according to age and gender. Total No. of Sample (50).

Positive identified bacteria	Total No.of isolation	Total No.isolated from 2 month to 2 year	%	Total No.isolated from 2 year to 4 year	%	Total No. Gender Male	%	Total No.Gender Female	%
Klebsiella spp	7	2	28.57 %	5	71.42 %	4	57.14 %	3	42.85 %

(P≤0.05)- Significant (age) &(χ^2 = N.S, P < 0.05)(gender)

Molecular Analysis

Klebsiella spp. detection by using specific (*16S rRNA* gene), the findings of this study show that 7 isolated of ear infections in dogs of the bacteria *klebsiella spp*. the bacterium's diagnostic gene. As show in Figure 3, the results of the polymerase chain reaction (PCR) for the *16S rRNA* gene appeared in all positive isolates (100%) *Klebsiella spp*.

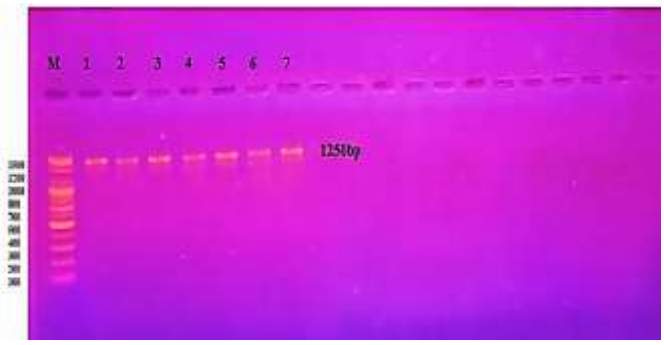

Fig 2: PCR product size of *16S ribosomal RNA* gene of *klebsiella spp.* for dogs positive at 1250 bp PCR product size. Lan 1, 2, 3, 4, 5, 6,7 positives. 0.5% agarose at 5

DOI: 10.36320/ajb/v18.i2.23787

volt/cm2. 1x TBE buffer for 30 min. M: DNA ladder (100).

All isolates of *Klebsiella spp*. were tested to identify the virulence genes. The primers that were reported in Table 1 were used to ascertain the availability of the genes result (*blaCTX-M* 1(14.3%), *magA* 3(42.9%) and mrkA1(14.3%)), respectively as show in Table 5. The *MagA* gene with a resulting size of 585 base pairs (bp) was detected in 4 out of 7 isolates (42.9%), As shown in Figure 4.

Table 5: Frequency of virulence genes of *Klebsiella Spp.* detected in dogs isolates.

Virulence genes	No. of isolates	Detected genes n (%)	Chi-square (P-value)
CTX-M	**7**	**1(14.3%)**	**3.571 (0.050) ***
MrkA	**7**	**1(14.3%)**	**3.571 (0.050) ***
MagA	**7**	**3(42.9%)**	**0.143NS (0.706)**

*** (P≤0.05), NS: Non-Significant**.

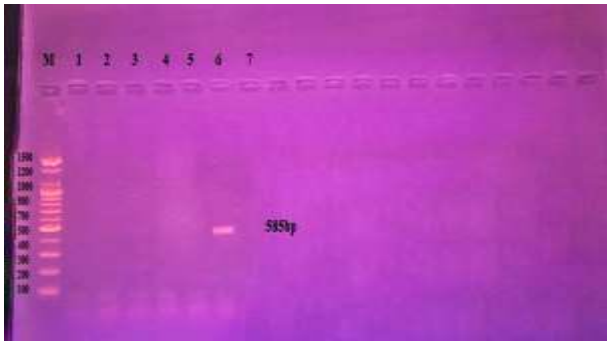

Fig 3: PCR product the band size *bta CTX-M* gene of *klebsiella spp.* in dogs positive at 585 bp PCR product size. Lan 6 positive. 1.5% agarose at 5 volt/cm2. 1 x TBE buffer for 1:30 hours. M: DNA ladder (100).

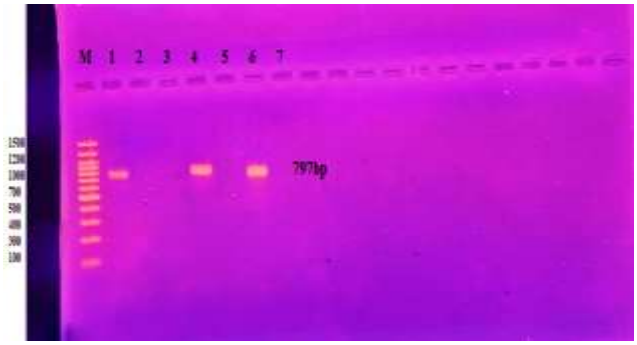

Fig 4: PCR product the band size *MagA* gene of *klebsiella spp.* in dogs positive at 797 bp PCR product size. Lan1,4,6 positive. The product was electrophoresis on 1.5% agarose at 5 volt/cm2. 1 x TBE buffer for 1:30 hours. M: DNA ladder (100).

Fig 5: PCR product the band size *Mrk A* gene of klebsiella spp. in dogs positive at 358 bp PCR product size. Lan 4 only positive. The product was electrophoresis on 1.5% agarose at 5 volt/cm2.1x TBE buffer for 1:30 hours. M: DNA ladder (100).

Using Phylogenetic Analysis the local *K. pneumoniae* isolates were clearly clustered with global strains from China, Brazil, Bulgaria, and Singapore, all of which shared 99% similarity, according to the phylogenetic tree created using the UPGMA method. This highlights the zoonotic potential of the *K. pneumoniae* strains isolated from dog ears, the *K. aerogenes* isolate exhibited 100% identity with strains isolated from aquatic environments and river water in Poland and Vietnam, indicating a possible environmental origin or cross-contamination pathway that warrants more research. The phylogenetic tree (UPGMA) showed that most of the local *K. pneumoniae* isolates clustered closely with strains from China(CP180482.1), Taiwan(CP176896.1), Brazil (CP186946.1) and Bulgaria (CP186465.1). While *K. aerogenes* formed independent branches linked to strains from Nigeria (PQ687474.1), China (ON146438.1) and

Turkey (OR412612.1).

Table 6: Sequence similarity and base substitutions in 16S rRNA gene of Klebsiella isolates in dogs.

16S ribosomal RNA gene							
No. Of sample	Type of substitution	Location	Nucleotide	Source	Sequence ID with compare	Sequence ID with submission	Id it
1	Transvertion	449995	G\C	Klebsiella pneumoniae	ID: CP180482.1	ID: PV490247.1	9
2	Transvertion	449995	G\C	Klebsiella pneumoniae	ID: CP180482.1	ID: PV490248.1	9
3	Transvertion	449995	G\C	Klebsiella pneumoniae	ID: CP180482.1	ID: PV490249.1	9
4	Transvertion	449995	G\C	Klebsiella pneumoniae	ID: CP180482.1	ID: PV490250.1	9
	Transvertion	450305	T\G				
	Transition	450306	A\G				
5	-------	-----	-----	Klebsiella aerogenes	ID: PQ687474.1	ID: PV490251.1	100 %
6	Transvertion	449995	G\C	Klebsiella pneumoniae	ID: CP180482.1	ID: PV490252.1	99%
7	Transvertion	449995	G\C	Klebsiella pneumoniae	ID: CP180482.1	ID: PV490253.1	99%
	Transvertion	450317	T\A				

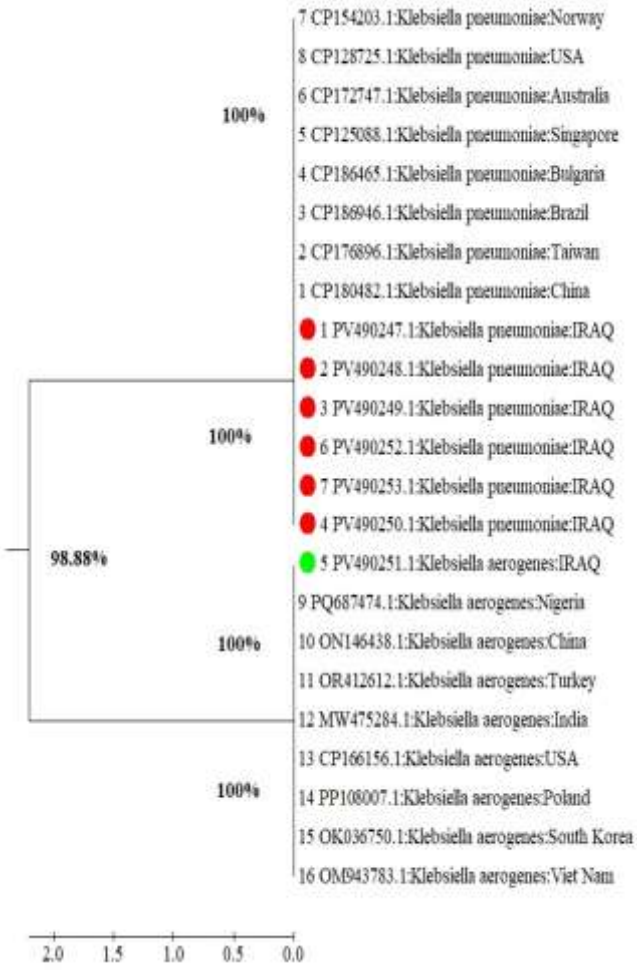

Fig 6: Evolutionary relationships of *Klebsiella spp.* taxa based on *16S rRNA* gene using UPGMA method. The analysis involved 23 sequences with 838 conserved positions. The tree was generated using MEGA6 software.

Interpreting susceptibility tests of klebsiella Spp.

The results showed that *Klebsiella spp.* isolates from dogs exhibited very high sensitivity (100%) to several antibiotics, including Levofloxacin, Co-trimoxazole, Chloramphenicol , high sensitivity to Gentamicin (85.7%), Trimethoprim(85.7%) and Ciprofloxacin (85.7), while high resistance(100%) to Amoxicillin/Clavulanic acid and Imipenem, while Amikacin (71.4%), and Cefotaxime (71.4%), as in the table (7).

Table 7: Results of *Klebsiella spp.* antibiotic sensitivity in dogs.

DOI: 10.36320/ajb/v18.i2.23787

No.	Antibiotics	Dosage	Symbol	Sensitive	Intermediate	Resistance	P-value
1.	Levofloxacin	5 mg	LEV-5	7(100%)	0	0	0.0042 **
2-	Co_trimoxazole	10 mg	COT-10	7(100%)	0	0	0.0041 **
3.	Gentamicin	10 mg	CN-10	6(85.7%)	0	1(14.2%)	0.0056 **
4.	Amoxicillin/Clavulanic Acid	20/10 mg	AMC-30	0	0	7(100%)	0.0042 **
5.	Chloramphenicol	10 mg	C-10	7(100%)	0	0	0.0042 **
6.	Amikacin	10 mg	AK-10	2(28.5%)	0	5(71.4%)	0.0094 **
7.	Oxytetracycline	30 mg	T-30	2(28.5%)	3(420.8%)	2(28.5%)	0.871 NS
8.	Trimethoprim	10 mg	TMP-10	6(85.7%)	0	1(14.2%)	0.0056 **
9.	Ciprofloxacin	5 mg	CIP-5	6 (85.7%)	1(14.2%)	0	0.0056 **
10.	Penicillin G	10 mg	P-10	0	0	0	0 NS
11.	Cefotaxime	10 mg	CTX10	2 (28.54%)	0	5(71.4%)	0.0094 **
12.	Imipenem	10mg	IpM10	0	0	7(100%)	0.0042 **
** (P≤0.01), NS: Non-Significant.							

The isolation rate of *Klebsiella spp.* from ear infections in dogs was recorded at 14% accordind to the table 3, This makes it a biased disease and is consistent with what was indicated in the [24] study, *Klebsiella spp*.bacteria were found to be present in 13.8% percent of otitis media in dogs, These results are also partially consistent with what was reported in the study by [25] Canine bacteria are not among the gram-negative bacteria isolated from cases of external otitis in dogs, but their prevalence is lower compared to *Staphylococcus* species and *Pseudomonas arginosa.*

These results according to the table 4 are consistent with the epidemiological evidence cited by [26], which confirmed that both age and gender are predisposing factors for otitis external in dogs. Higher or similar incidence rates were observed in middle-aged males 56% from (4 to 5) years represents the peak of the infection and female 44%. This explains the higher isolation rates among older males. This finding also aligns with [27] observation of higher isolate rates in adult dogs (>2 years). The percentage of males was higher than that of females (males 55-57%, females 43-45%) the main Gram-negative pathogens linked to dogs otitis externa are *Pseudomonas spp*. and *Proteus* was 25-30% as the study [28].

According to the table 5, these pattern is consistent with [27] findings that Klebsiella spp. isolates from the ear of dogs were magA positive in 40% of cases, while mrkA was less frequent (30%). He attributed this to the fact that chronic ear infections in animals often rely more on capsular protection against host defenses than on mechanical adhesion to epithelial cells. The results are consistent with who demonstrated that the *magA* gene plays a key role in the development of the hypermucoviscosity phenotype, conferring high resistance to phagocytosis and immunosera within the moist otitis media [29], additionally, the low *CTX-M* content (14.3%) in Egypt, where limited resistance was found in animal ear isolates (<20%). This was attributed to the less intensive topical use of antibiotics in treating ear infections compared to systemic therapy, thus reducing the opportunity for the acquisition of resistance genes, is consistent with the findings of [30].

Therapeutically, The results of this study are consistent with those reported in a Bulgarian review [31], where *Klebsiella* isolated from canine otitis showed sensitivity to cefotaxime (88.88%) and complete resistance to

penicillin and amikacin (100%).also aligns with the findings of [32], where 12% of *Klebsiella pneumoniae* isolates from dogs exhibited complete susceptibility to imipenem and fluoroquinolones, and high resistance to amoxicillin and aminoglycosides, supporting the pharmacological pattern observed in this study. The current results contradict some studies that reported higher re-sistance to fluoroquinolones and aminoglycosides in canine ear isolates, [33] reported that *Klebsiella spp.* from dogs exhibited re-sistance to ciprofloxacin (42.8%) and amikacin (57.1%), which are higher than the resistance rates recorded in our study (14.2% and 71.4%), also reported that some *Klebsiella pneumoniae* isolates from Caribbean canine ear were 20% resistant to imipenem.

Conclusions

The results of this study show that *Klebsiella spp*. is a significant bacterial causative agent of ear infections in dogs, with an isolation rate of 14%. *Klebsiella spp.* was the most prevalent species, confirming its role as a common opportunistic pathogen in these cases.

In dogs, *Klebsiella spp*. was the most common, with significant differences in some isolates by sex and age ($P \leq 0.05$), but no significant difference ($P > 0.05$) in the overall age distributionMolecular analysis using *16S rRNA* gene sequencing demonstrated high diagnostic accuracy, with all isolates showing genetic similarity (99–100%) to global strains registered in GenBank. This reflects genetic stability and close evolutionary relatedness to isolates from multiple countries.

The importance of virulence and resistance genes was also highlighted. The *blaCTX-M* gene, responsible for producing broad-spectrum beta-lactamase, was detected in 1(14.3%) of the isolates, which explains the levels of resistance to certain antibiotics. The presence of *magA3(42.9%)* and *mrkA* 1(14.3%) genes was also recorded in varying proportions, indicating that some isolates possess a high capacity for capsule formation, adhesion, and biofilm formation—factors that increase the severity and persistence of infection.

The *Klebsiella spp.* isolates from dogs showed complete resistance (100%) to Amoxicillin/Clavulanic acid and Imipenem, with high resistance to both Amikacin and Cefotaxime (71.4%), while showing complete sensitivity to Levofloxacin, Co-trimoxazole, and Chloramphenicol. In feline isolates, complete resistance (100%) was recorded to Co-trimoxazole, Amoxicillin/Clavulanic acid, and Imipenem, in addition to high resistance to Amikacin (66.6%), while all isolates showed complete sensitivity to Levofloxacin, Chloramphenicol, Trimethoprim, and Cefotaxime. Most of these results were highly significant ($P \leq 0.01$).

These findings collectively underscore the importance of integrating molecular diagnostics with traditional examinations in the evaluation of otitis media and the necessity of monitoring resistance patterns and associated genes within the population. The results also underscore the need for further large-scale studies to track the genetic and epidemiological spread of *Klebsiella spp.* isolates, and to assess the impact of therapeutic and environmental pressures on enhancing their bacterial diversity and pathogenicity.

Acknowledgement

The authors would like to thank Collage of Veterinary Medicine, University of Baghdad, Baghdad, Iraq for its support in the present work

Funding Information

None

Author's Contributions

Masa Ameer Hassan was responsible for all aspects of the study, including study design, laboratory work, data analysis, and manuscript preparation.

Ethics

This study was approved by the College of Veterinary Medicine at the University of Baghdad (permission no. PG2673 dated 21/10/2025) for animals used. All procedures were carried out in accordance with ethical and legal standards.

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