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Molecular identification of *Klebsiella* species from pneumonic goats, Iraq

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ABSTRACT

Background: In goats, acute and chronic respiratory infections are often characterized by a rapidly progressing clinical course with little opportunity to develop an effective antibiotic therapy.

Aim: This study aimed to identify *Klebsiella* spp. in pneumonic goats, assess its antibiotic susceptibility, and confirm the molecular phylogenetics of *Klebsiella* spp.

Methods: A total of 80 pneumonic goats were selected from the slaughterhouses located in Basra province (Iraq) from June to November 2023, and each animal was subjected to obtaining only one sample. The studied samples were included 30 nasal swabs obtained from the lived goats, while 30 lung samples in addition to 20 tracheal swabs were collected from slaughtered goats. All study samples were inoculated onto MacConkey agar and tested biochemically. Eleven types of antibiotics were served in the Kirby-Bauer disc diffusion method to identify the susceptibility of *Klebsiella* spp. Positive culture isolates were tested molecularly using the polymerase chain reaction (PCR) and then sequenced for phylogenetic analysis of study isolates.

Results: The findings indicated that 35% of samples were positive to *Klebsiella* spp. including 30% in trachea, 33.33% in nasal swabs, and 40% in lungs. *Klebsiella* colonies appeared on MacConkey agar as bright pink mucoid texture; while on blood agar, they were large, glossy, mucoid, whitish-grey, spherical, and free of hemolysis. Biochemically, all isolates were exhibited a negative reactivity to motility, oxidase, indole, and methyl red, but positives to urease, citrate utilization, catalase, and Voges-Proskauer, acid and gas production. Antibiotic susceptibility testing revealed the high susceptibility of *Klebsiella* isolates to meropenem (71.43%), and intermediate susceptibility to ciprofloxacin (28.57%), but high resistance to imipenem (60.71%). Targeting the *16S rRNA* gene, PCR results confirmed all tested isolates as *Klebsiella* spp. Finally, phylogenetic analysis of 9 positive isolates demonstrated the identity of local *Klebsiella* isolates to *Klebsiella aerogenes* (no = 4), *Klebsiella pneumoniae* (no = 3), *Klebsiella quasivariicola* (no = 1), and *Klebsiella quasipneumoniae* (no = 1).

Conclusion: Our study confirms the presence of *K. aerogenes*, *K. quasivariicola*, and *K. quasipneumoniae* in pneumonic goats, highlighting the importance of molecular phylogeny in the detection of new *Klebsiella* species. However, furthermore studies are necessary to investigate various *Klebsiella* species/strains in goats and other domestic animals.

Keywords: Caprine pneumonia, *K. aerogenes*, *K. quasipneumoniae*, *K. quasivariicola*, *K. pneumoniae.*

Introduction

Klebsiella is a Gram-negative, rod-shaped, capsulated bacterium belonging to the Enterobacteriaceae family. This bacterium causes various infections in both animals and humans with different outcomes that range from mild to moderate and severe even death (Sathyavathy and Madhusudhan 2020; Wu *et al.*, 2021). Respiratory infections due to *Klebsiella* spp. are often characterized clinically by a rapidly progressing course and complicated by the occurrence of lung abscesses and multi-lobular involvement, which leave little chance of effective antibiotic therapy. Moreover, the development of antibiotic resistance among nosocomial isolates of *Klebsiella pneumoniae* has limited the therapeutic activity in the treatment of these infections (Khalid *et al.*, 2022).

Pneumonia is an inflammation of lung tissues that infects additionally either bronchioles to result in bronchopneumonia or pleura leading to pleuropneumonia (Emikpe *et al.*, 2019). However, almost all pneumonic infections have been attributed to multifactorial etiologies such as parasites, bacteria, and viruses, alongside with environmental factors like extreme weather (cold or hot), and management

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practices that increase the strength of infection (Al-Ammiri *et al.*, 2016).

In goats, acute respiratory infections lead to poor weight gain, mortality, and great economic losses due to morbidities and mortalities, medical cares, and preventive measures (Saleh and Allam 2014). Whilst, chronic infections may cause endotoxemia, septicemia, and even death due to insufficiency and severe systemic illness (Mekibib *et al.*, 2019). During respiratory outbreaks, the selection of effective antibiotics is of great importance due to the rapid progression of lung injury and endotoxin release (Nejibanand Al-Amery 2018). Therefore, this study was performed to isolate *Klebsiella* spp. from different samples of pneumonic goats, with the assessment of antibiotic susceptibility, and confirmation of different *Klebsiella* species by the molecular phylogenetic analysis.

Materials and Methods

Preparation of culture media and reagents

Following the manufacturer's instructions (HiMedia, India), media (MacConkey and blood agars), brain-heart infusion broth, reagents of chemical tests (oxidase, catalase, and Kovac's), and Gram stain were prepared.

Samples

A total of 80 pneumonic goats were selected from slaughterhouses located in Basra province (Iraq) from June to November 2023, and each animal was subjected to obtaining only one sample. The studied samples were included 30 nasal swabs obtained from the lived goats, while 30 lung samples in addition to 20 tracheal swabs were collected from slaughtered goats. The collected samples were transported to the Microbiology Lab (College of Veterinary Medicine, University of Basrah) using the brain-heart infusion broth.

Bacterial isolation

Different samples were inoculated aseptically on MacConkey and blood agars at 37°C for 24 hours, and subsequently, the suspected colonies were purified to identify their morphological characteristics (Quinn *et al.*, 2011). Additional biochemical tests including oxidase test, catalase test, urease activity test, coagulase test, sugar fermentation, indole production test, and gas production test were done.

Antibiotic susceptibility test

The Kirby-Bauer disc diffusion method was performed on Muller Hinton agar (Oxoid, UK) to identify the susceptibility of *Klebsiella* isolates toward 11 types of ready-to-use antibiotic discs based on the inhibition zone developed around each antibiotic.

Molecular examination

Presto™ Mini gDNA Bacteria Kit (Geneaid, Taiwan) was used to extract the genomic DNAs from purified *Klebsiella* colonies. Nanodrop spectrophotometer (Thermo Scientific, UK) was served to measurement the purity and concentration of DNAs. Targeting the *16S rRNA* gene, a set of primers was designed [(F: 5' AGA GTT TGA TCC TGG C-3') and (R: 5'- GGT TAC CTT GTT ACG ACT T-3')] to preparing the MasterMix tubes (Bioneer/South Korea) at a final volume of 50 µl (Munaff and Chmagh 2014). Thermocycler for Mastermix tubes was done as follows: 1 cycle for initial denaturation (92°C/2 minutes); 30 cycles for denaturation (94°C/30 seconds), annealing (52°C/45 seconds), and extension (72°C/1 minute); and 1 cycle final extension (72°C/5 minutes). Electrophoresis of PCR products was performed in 3% agarose gel stained with 3 µl Ethidium Bromide at 100V and 80A for 1 hour. Positive samples were indicated under the UV transilluminator (Clinx Science, China) at approximately 1,500 bp.

Phylogeny

The DNAs of 9 molecularly positive samples were sent for sequencing in the Macrogen Company (South Korea) following the Modified Sanger dideoxynucleotide sequencing method. The sequence data were received via private email, and the local *Klebsiella* isolates were named, submitted to NCBI-GenBank database, and analyzed phylogenetically through the MEGA-11 Software.

Statistical analysis

The *t*-test and One-Way Analysis of Variance in the GraphPad Prism Software (*version 6.0.1*) to estimate significant differences between study results at $p \leq 0.05$ (Wahab *et al.*, 2024).

Ethical approval

This study follows the ethics guidelines of the College of Veterinary Medicine (University of Wasit, Iraq) under the awarded access number (WU/CVM: 138-9-2023).

Results

Culture and biochemical testing

The total findings revealed that 35% (28/80) of samples were positive *Klebsiella* spp., including 30% (6/20) in trachea, 33.33% (10/30) in nasal swabs, and 40% (12/30) in lung (Fig. 1). *Klebsiella* grown on MacConkey agar appeared as bright pink and mucoid colonies; while on blood agar, the colonies shown large, glossy, mucoid, whitish-grey, spherical and free-hemolysis appearance.

Biochemically, all isolates exhibited a negative reaction to motility, oxidase, indole, and methyl red. A positive reaction was observed to urease, citrate utilization, catalase, and Voges-Proskauer with the generation of acid and gas in the glucose fermentation test.

Antibiotic susceptibility testing

Klebsiella spp. isolates were shown a high susceptibility to meropenem (71.43%), and intermediate susceptibility to ciprofloxacin (28.57%) but a high resistance to imipenem (60.71%). Other findings revealed variable rates of susceptibility as following: cefazolin (50%), amikacin (64.29%), amoxicillin-clavulanic acid (42.86%), and piperacillin-tazobactam (57.14%). However, resistance to ampicillin (50%), gentamicin

(53.57%), tobramycin (46.43%), ceftriaxone (53.57%), ciprofloxacin (46.49%), levofloxacin (39.29%), and trimethoprim-sulfamethoxazole (50%) was detected (Table 1).

Molecular examination and phylogeny

Targeting the *16S rRNA* gene, PCR assay confirmed that all tested isolates were *Klebsiella* (Table 2, Fig. 2). Phylogenetic analysis of 9 positive isolates demonstrated significant identity between the local *Klebsiella* spp. isolates and various *Klebsiella* species existed in the NCBI-GenBank as follows: 4 local isolates identical to *Klebsiella aerogenes* of Chinese (BQ.1), Ethiopian (BQ.2 and BQ4), and Mexican (BQ6) origin; 3 isolates identical to *K. pneumoniae* of Iraqi (BQ.5 and BQ9) and Chinese (BQ.8) origins; 1 isolate identical to *Klebsiella quasipneumoniae* of South Korean origin (BQ.7); and 1 isolate identical to *Klebsiella quasivariicola* of Chinese (BQ.3) origin. Analysis of homology sequence identity revealed a range of similarity (*) and substitution mutation between the local and the NCBI-BLAST *Klebsiella* species at 99.78%–100% and 0.0002%–0.01%, respectively (Table 3, Figs. 3 and 4).

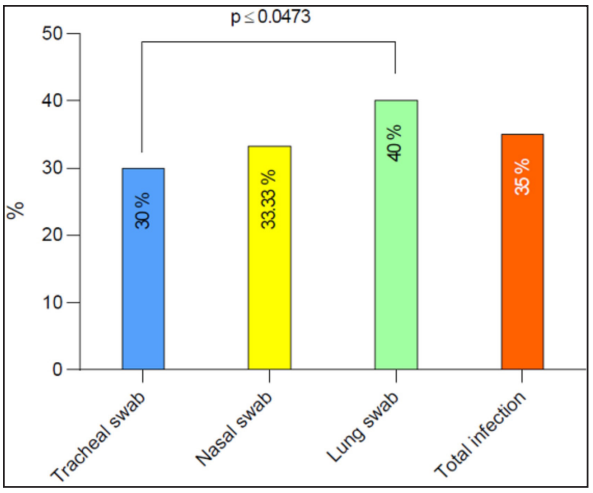

Fig. 1. Total positive isolates of *Klebsiella* spp. by MacConkey agar, blood agar and biochemical tests.

Discussion

Goat is an important domestic animal which known which adaptable to diverse environmental conditions ranging from arid deserts to lush mountainous regions; however, several respiratory infections remain of serious concern (Zhou *et al.*, 2017). In the current study, 35% of study animals were infected with *Klebsiella* spp. In comparison to other national studies, the prevalence rate of *Klebsiella* infection was 22% in Saudi Arabia (Mansour *et al.*, 2014), 17% (Yaseen *et al.*, 2019), 13.83% (Ahmed and Abdullah, 2022), and 6% (Mohammed, 2023); whereas internationally, it was 5.6% in India (Aher *et al.*, 2012), 36% in Egypt (Ali and Abu-Zaid, 2019), and 51.72% in Nigeria (Adam *et al.*, 2023). However, variations in the prevalence of respiratory bacteria may be reflected by the sampling scheme, sample size, diagnostic methods serve, and pathogenicity of

Table 1. Antibiotic susceptibility testing of *Klebsiella* isolates using the Kirby-Bauer disc diffusion method (Total No: 28).

Antibiotic	Antibiotic susceptibility test			*p-value*
	Susceptible	Intermediate	Resistant	
Ampicillin	11 (39.29%)	3 (10.71%)	14 (50%) *	0.0239 S
Cefazolin	14 (50%) *	2 (7.14%)	12 (42.86%)	0.0175 S
Gentamicin	10 (35.71%)	3 (10.71%)	15 (53.57%) *	0.0256 S
Meropenem	20 (71.43%) *	2 (7.14%)	6 (21.43%)	0.0089 S
Tobramycin	10 (35.71%)	5 (17.86%)	13 (46.43%) *	0.0403 S
Amikacin	18 (64.29%) *	3 (10.71%)	7 (25%)	0.0189 S
Amoxicillin-clavulanic acid	12 (42.86%) *	6 (21.43%)	10 (35.71%)	0.0359 S
Piperacillin-tazobactam	16 (57.14%) *	5 (17.86%)	7 (25%)	0.0416 S
Cefuroxime	12 (42.86%)	2 (7.14%)	14 (50%)	0.0135 S
Imipenem	11 (39.29%)	0 (0%)	17 (60.71%) *	0.0197 S
Ceftriaxone	9 (32.14%)	4 (14.29%)	15 (53.57%) *	0.0402 S
Ciprofloxacin	7 (25%)	8 (28.57%)	13 (46.49%) *	0.0396 S
Levofloxacin	7 (25%)	0 (0%)	11 (39.29%) *	0.0213 S
Trimethoprim-sulfamethoxazole	10 (35.71%)	4 (14.29%)	14 (50%) *	0.0199 S
p-value	0.0104 S	0.0471 S	0.0113 S	-

S: Significance * ($p < 0.05$); NS: Non-Significance ($p > 0.05$).

Table 2. Molecular PCR results for testing a total of 28 suspected *Klebsiella* isolates.

Source of *Klebsiella* spp. isolate	Total No.	Positive No. (%)
Trachea swab	6	6 (100%)
Nasal swab	10	10 (100%)
Lung swab	12	12 (100%)
Total	28	28 (100%)

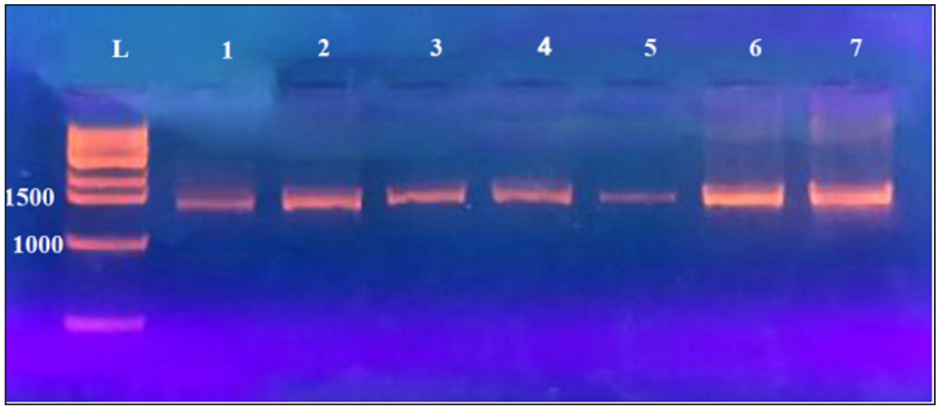

Fig. 2. Electrophoresis of 3% agarose gel stained with Ethidium Bromide to amplify PCR products. Lane L: Ladder marker at 3,000 bp; Lanes 1–7: Some positive PCR products to *Klebsiella* spp. at 1,500 bp.

Table 3. NCBI-BLAST Homology sequence identity of local BQ *Klebsiella* strains in goats comparing the NCBI-GenBank *Klebsiella* species targeting the *16S rRNA* gene.

Local isolate			NCBI Isolate		
Name	**Access No.**	***Klebsiella* species**	**Access No.**	**Country**	**Identity (%)**
BQ.1	OR484045.1	*K. aerogenes*	OQ421704.1	China	100
BQ.2	OR484046.1	*K. aerogenes*	OQ132851.1	Ethiopia	100
BQ.3	OR484047.1	*K. quasivariicola*	OQ405576.1	China	99.96
BQ.4	OR484048.1	*K. aerogenes*	OQ132851.1	Ethiopia	99.94
BQ.5	OR484049.1	*K. pneumoniae*	OP136157.1	Iraq	99.78
BQ.6	OR484050.1	*K. aerogenes*	OR426158.1	Mexico	99.85
BQ.7	OR484051.1	*K. quasipneumoniae*	OP491987.1	South Korea	99.89
BQ.8	OR484052.1	*K. pneumoniae*	KX636139.1	China	99.92
BQ.9	OR484053.1	*K. pneumoniae*	OP136157.1	Iraq	100

Klebsiella strains. Common opportunistic *Klebsiella* strains significantly affect animals that undergo a compromised or weakened immunity (Hu *et al.*, 2021). In the last decade, many researchers have claimed the increasing rate of *Klebsiella* infection due to to the pathogenic characteristics and virulence factors of this bacterium (Younis *et al.*, 2016; Wang *et al.*, 2020; Zhu *et al.*, 2021). These characteristics could enhance the growth of a colony in a host. Moreover, highly invasive and virulent *Klebsiella* strains could infect healthy animals leading to severe acquired infections such as *pneumonia*, necrotizing fasciitis, meningitis, and pyogenic liver abscess (Marques *et al.*, 2019; Russo and Marr, 2019). In the respiratory tract, the colonized pathogenic *Klebsiella* in pulmonary tissues establish a prominent lesion and causes severe pneumonia through modification resistance to phagocytosis by immune cells (Bengoechea and Sa Pessoa, 2019; Priyanka *et al.*, 2020). Furthermore, *Klebsiella* tends to cause chronic infections due to

Fig. 3. Multiple sequence alignment analysis of local BQ *Klebsiella* strains in goats comparing with the NCBI-GenBank *Klebsiella* species targeting the *16S rRNA* gene. Analysis conducted using the Clustal W alignment tool in MEGA 11 Software showed the presence of nucleotide alignment similarity as (*) and substitution mutations.

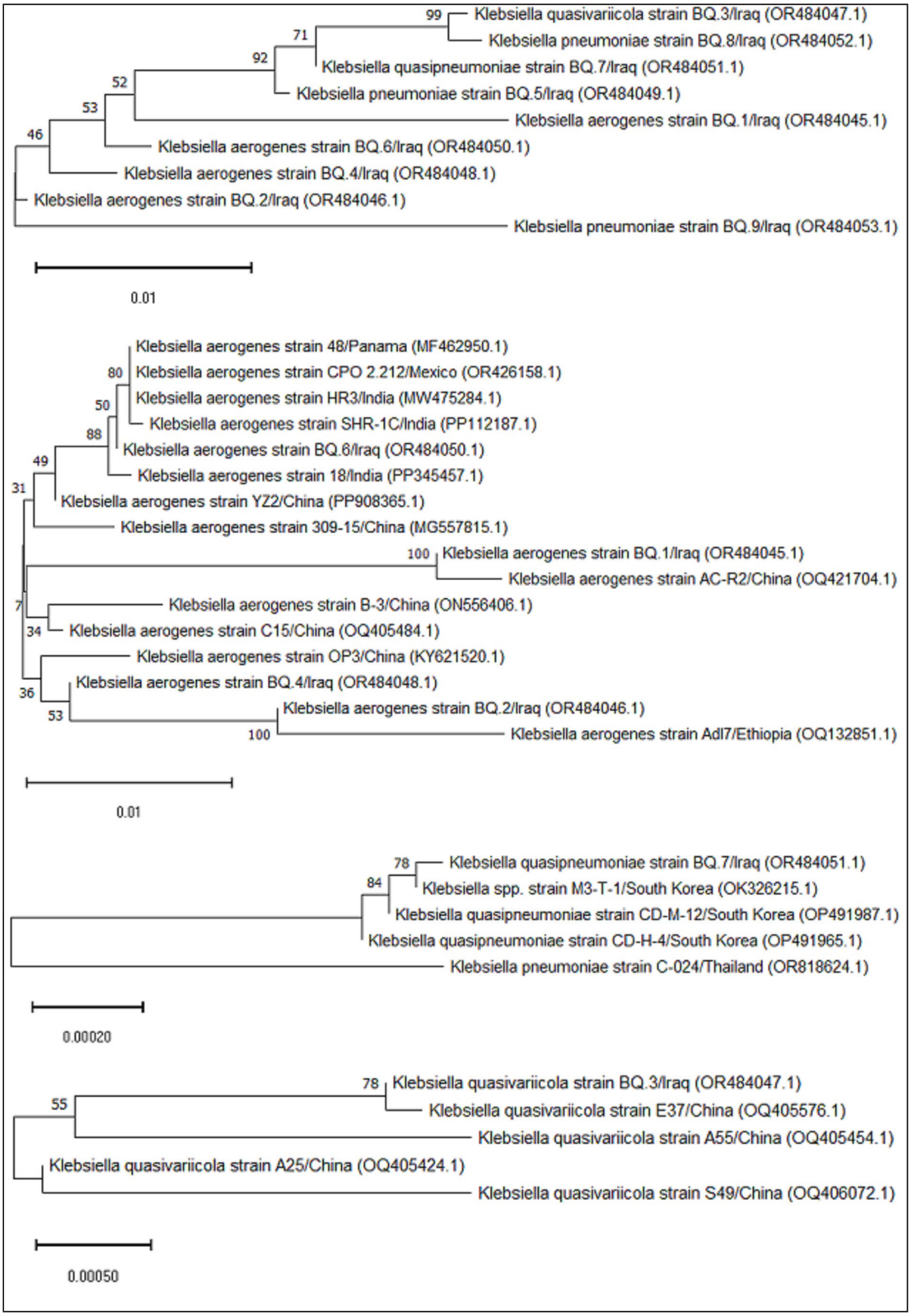

Fig. 4. Phylogenetic tree analysis of the *16S rRNA* gene partial sequence of the local BQ *Klebsiella* strains in goats and identity with the NCBI-GenBank *Klebsiella* species. The analysis was built using the Unweighted Pair Group technique with Arithmetic Mean (UPGMA tree method), and the evolutionary distances were calculated using the Maximum Composite Likelihood method in MEGA 11.

two main factors: the first is the secretion of various enzymes that inhibit certain antibiotics to rendering pathogen resistance, and the second is the development of immune-evading biofilms *in vivo* (Padmini *et al.*, 2017; Abbas *et al.*, 2024).

In this study, results indicated that *Klebsiella* isolates were significantly resistant to several classes of antibiotics, likely due to their frequent use in treating patients (Gao *et al*., 2020a, b). Conversely, the apparent resistance rate of *Klebsiella* isolates to imipenem was higher than reported by others (Hu *et al.*, 2021; Su *et al.*, 2022; Liza *et al.*, 2024). Dapgh *et al.* (2019) mentioned that irregular and excessive administration of antibiotics has increased the prevalence of bacterial resistance and transmission of infections between animals as well as from animals to humans with complicating the treatment strategy. Mukuna *et al.* (2023) studied the antimicrobial susceptibility profile of pathogenic and commensal bacteria recovered from cattle and goats suggesting that both animals could act as reservoirs of multi-drug resistance bacteria. Liza *et al.* (2024) concluded that the presence of extended-spectrum β-lactamase-producing multidrug resistance *K. pneumoniae* isolates that posing a substantial public health threat.

Klebsiella is difficult to detect with frequent misclassification in clinical microbiology laboratories (Shankar *et al.*, 2018). Several investigations have been conducted to determine the efficacy of different strategies to identifying various species of *Klebsiella* (Mukherjee *et al.*, 2020). Molecular PCR assay has proven as more valuable and highly sensitive and specific technique in the diagnosis of *Klebsiella* species when compared to other traditional procedures (Hansen *et al.*, 2020). Phylogenetic analysis of study isolates indicates presence of new *Klebsiella* species in goats including *K. aerogenes*, *K. quasivariicola,* and *K. quasipneumoniae*. Worldwide, several previous and recent studies have shown some of these species in patients with epidemic resistance to antibiotics and phagocytosis (Curie *et al.*, 1978; Williams *et al.*, 1983; Potter *et al.*, 2018; Zhang *et al.*, 2022; Delik *et al.*, 2024). This might explain the prevalence of antibiotic resistance among the study isolates.

Conclusion

This study lies in the first determination of three new species of *Klebsiella* in pneumonic goats in Iraq (*K. aerogenes*, *K. quasivariicola*, and *K. quasipneumoniae*), along with the identification of their distinct species based on NCBI-GenBank database. In addition, the current study clearly identifies the susceptibility and resistance of *Klebsiella* isolates to various antibiotics. However, moreover, studies are necessary to investigate various *Klebsiella* species/strains in goats and other domestic animals based on molecular phylogeny.

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Authors' contribution

HKI: Molecular examination of bacterial isolate. KSM: Bacterial isolation. ASJ: Antimicrobial susceptibility testing. HAJG: Samples collection, phylogeny, and statistical analysis of study results. All authors were approved the final copy of the manuscript.

Conflict of interest

The authors have declared no conflict of interest.

Data availability

All obtained data were included in this manuscript.

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