Open Veterinary Journal, (2024), Vol. 14(9): 2325-2333
ISSN: 2226-4485 (Print)
ISSN: 2218-6050 (Online)
Research Article
DOI: *10.5455/OVJ.2024.v14.i9.21*

Submitted: 22/05/2024 Accepted: 05/08/2024 Published: 30/09/2024

Phylogenetic analysis of *Klebsiella pneumoniae* isolates of respiratory tract infections in humans and sheep

Khadeeja Sami Madhi[1], Alyaa Sabti Jasim[2], Hiba Ali Nasear[3], Hanaa Khaleel Ibraheim[2] and Hasanain A.J. Gharban[4*]

[1]*Department of Microbiology, College of Medicine, University of Basrah, Basra, Iraq*
[2]*Department of Microbiology, College of Veterinary Medicine, University of Basrah, Basra, Iraq*
[3]*Department of Veterinary Public Health, College of Veterinary Medicine, University of Basrah, Basra, Iraq*
[4]*Department of Internal and Preventive Veterinary Medicine, College of Veterinary Medicine, University of Wasit, Wasit, Iraq*

Abstract

Background: *Klebsiella pneumoniae* is an important opportunistic pathogen, which is capable of colonizing the respiratory system in both humans and animals causing mild to severe infections.

Aim: This study aims to isolate *K. pneumoniae* from the nasal discharges of human and sheep as well as identify the antibiotic resistance and molecular phylogeny of local isolates.

Methods: A total of 100; 50 humans and 50 sheep, positive nasal swab isolates were selected, and confirmed biochemically and by the VITEK-2 system. Molecular testing using the polymerase chain reaction (PCR) and phylogeny was conducted.

Results: On MacConkey agar, *Klebsiella* isolates appeared as large, pinkish, and mucoid colonies; while microscopically, it appeared as Gram-negative rods. Traditional biochemical tests revealed that 62% and 78% of human and sheep isolates were positive *Klebsiella* isolates, whereas respectively, 54.84% and 71.8% of these isolates were positive by VITEK-2. Antibiotic susceptibility tests showed that the human isolates were sensitive to aztreonam, piperacillin-tazobactam, ciprofloxacin, and cefuroxime. Subsequently, sheep isolates were sensitive to cefuroxime, ciprofloxacin, piperacillin-tazobactam, ampicillin, cefoxitin, and tetracycline. Targeting *16S rRNA* gene, a total of 17 human and 28 sheep isolates were molecularly positive *K. pneumoniae*. Phylogenetic analysis of study human and sheep isolates showed their identity to NCBI Indian (LC747146.1) and Iraqi (LC711141.1) isolates, respectively. Comparative analysis between the local human and sheep isolates revealed a significant identity that ranged from 99.82% to 99.88% with a percentage of mutation ranging from 0.008% to 0.002%.

Conclusion: *Klebsiella pneumoniae* is a highly prevalent bacterium in both human and sheep with an observable resistance to antibiotics. Molecular phylogeny of study isolates demonstrated their close relation, suggesting the possible direct or indirect transmission of the bacterium from sheep to human or *vice versa*. Moreover, studies are greatly important to estimate the routes of bacterial transmission. Also, extensive hygiene practices could be lowered the spreading of *K. pnuemoniae* to farm workers.

Keywords: *16S rRNA* gene, Antimicrobial susceptibility, Iraq, Polymerase chain reaction, Upper respiratory tract infections.

Introduction

Klebsiella genus is a group of non-motile, rod-shaped, Gram-negative bacteria that typically expresses two antigens on its cell surface: lipopolysaccharide (O antigen) and capsular polysaccharide (K antigen), (Choi *et al*., 2020; Gujarati *et al.*, 2020). In both human and animals, *K. pneumoniae* is the major pathogenic member of the *Klebsiella* genus, which colonized mainly in intestinal and respiratory tracts causing different diseases such as meningitis, arthritis, urinary tract illness, liver abscess, and bacteremia (Liao *et al.,* 2016; Wareth and Neubauer, 2021). In the respiratory system, asthma and pneumonia are the most common respiratory diseases which are complicated by other factors such as interactions with other infectious respiratory pathogens, low immunity of the host, poor environmental conditions, and stress (Trueba and Ritz, 2013; Lou *et al.,* 2018).

In many countries, sheep represent the most important growing animals in the livestock sector due to their ability to convert different types of forage into valuable

***Corresponding Author:** Hasanain A.J. Gharban. Department of Internal and Preventive Veterinary Medicine, College of Veterinary Medicine, University of Wasit, Wasit, Iraq. Email: *hghirban@uowasit.edu.iq*

products such as wool, milk, and meat (Gowane *et al.,* 2017). However, severe ovine infections could cause a financial impact on the farming industry due to decreasing milk production and body weight with increasing morbidities and mortalities in addition to the costs of therapeutic and control programs (Al-Dawood, 2017; Hu *et al.,* 2020). Therefore, it is essential to solve or limit the effects of pathogens that affect sheep in order to enhance and sustain productivity and meet the demand of human's consumption (Saleh and Allam, 2014; Hernández-Castellano *et al.,* 2019).

In recent years, there has been a gradual awareness of the spreading *K. pneumoniae* in both human and animals throughout the world, considerable interest for nosocomial infection, and a trend toward multiple antibiotic resistance (Yang *et al.,* 2019; Iskandar *et al.,* 2021; Jian *et al.,* 2021). In Iraq, *K. pneumoniae* has been studied in humans (Al-Nakeeb *et al.,* 2018; Alyassari *et al.,* 2019; Hashim, 2021) and sheep (Jasim *et al.,* 2020; Hasan *et al.,* 2021; Aldabbagh, 2022); however, no available studies have referred to a possible association between the human and sheep respiratory infections caused by *K. pneumoniae*. Hence, this study was conducted to fulfill this aim through isolation and molecular phylogenetic analysis of human and sheep K. *pneumoniae* isolates.

Materials and Methods

Samples

Several nasal swab samples were collected from human and sheep populations showing respiratory illness in different areas in Basra province (Iraq) from October–December (2022). All collected samples were initially cultured on MacConkey agar as described by Rawy *et al*. (2022). However, a total of 100 positive *Klebsiella* isolates by culture (50 from humans and 50 from sheep) were selected based on their morphological characteristics and Gram staining, as mentioned by other studies (Mahesh *et al.,* 2017; Gao *et al.,* 2020).

Biochemical and VITEK-2 analysis

They included the Simmon citrate, urease tests, methyl red, indole, motility, and triple sugar iron tests (TSITs) performed (Hansen *et al.,* 2004). The VITEK-2 compact system was done using the GN card that consists of 41 tests: 18 for sugar incorporation, 18 for sugar fermentation, 2 for decarboxylase and tryptophan deaminase, and 3 for urease and malonate utilization (Rave *et al.,* 2018).

Antimicrobial susceptibility test

The Kirby-Bauer disc diffusion susceptibility test was used to determine the sensitivity or resistance of *Klebsiella* isolates against various antimicrobial compounds on the Muller Hinton agar (Oxoid, UK). Ready to use antibiotic discs (Oxoid, UK) involved amoxicillin clavulanic acid (AMC), ampicillin (AMP), aztreonam (ATM), cefotaxime (CTX), cefoxitin (FOX), ceftazidime (CAZ), cefuroxime (CXM), chloramphenicol (CHL), ciprofloxacin (CIP), gentamicin (GEN), mecillinam (MEL), meropenem (MEM), nitrofurantoin (NIT), piperacillin-tazobactam (TZP), tetracycline (TET), tigecycline (TGC), and trimethoprim-sulfamethoxazole (SXT) were served to indicate antibiotic resistance.

Molecular examination

In this study, genomic DNAs were extracted from pure isolates of *Klebsiella* spp. following the manufacturer instructions of Presto™ Mini gDNA Bacteria Kit (Geneaid, Taiwan), and tested by the NanoDrop spectrophotometer (Thermo Scientific, UK). Targeting *16S rRNA* gene, one set of primers [(F: 5′-CCT GAT GGA GGG GGA TAA CT-3′) and (R: 5′-CGT AAG GGC CAT GAT GAC TT-3′)] was designed for this study based on an Indian isolate (GenBank Accession No HM007813), and then used to prepare the MasterMix tubes (Bioneer/South Korea) at a final volume of 20 µl. The conditions of the PCR reaction performed using a Thermocycler were 1 cycle for initial denaturation (95°C for 5 minutes), 30 cycles for denaturation (95°C for 40 seconds), annealing (57°C for 40 seconds), and extension (72°C for 1 minute), and 1 cycle for final extension (72°C for 7 minutes). Electrophoresis of PCR products was done in 1.5% agarose gel stained with Ethidium Bromide, at 100 V and 80 Am for 1 hour. Positive PCR products were detected at a product size of 1,081 bp under the UV transilluminator (Clinx Science, China) and photographed by the digital camera (Nikon, Japan).

Phylogeny

The DNAs of 10 positive *K. pneumoniae* isolates, five human and five sheep, were selected and sent to the Macrogen Company Ltd. (South Korea) to be sequenced using the Modified Sanger dideoxynucleotide sequencing method based on the forward and reverse primers of the study. The received data were reported in the Genbank-NCBI, analyzed phylogenetically by the MEGA-11 Software, and identity was identified between the local human and sheep *K. pneumoniae* isolates as well as with the global NCBI isolates.

Statistical analysis

The *t*-test, one- and two-way ANOVA in the GraphPad Prism Software version 6.01 (GraphPad Software, Inc, USA) were served to detect significant differences between the obtained values at $p < 0.05$ (Gharban, 2023).

Ethical approval

This study was licensed by the Scientific Committees of the College of Veterinary Medicine and College of Medicine at the University of Basrah (Basra, Iraq) and the College of Veterinary Medicine at the University of Wasit (Wasit, Iraq), [Access No. 348 (25-September-2022)].

Results

Culture, biochemical, and VITEK-2 findings

On MacConky agar, *Klebsiella* appeared as large, pinkish, and mucoid colonies. Microscopic examination

of prepared slides has shown Gram-negative non-spore rods. Biochemical tests reported that 62% (31/50) of human and 78% (39/50) of sheep isolates were positive to *Klebsiella* showing a positive reaction to Simmon citrate and urease but a negative reaction to methyl red indole and motility tests (Fig. 1). Yellow bottom/yellow slope reactions with gas production were seen by the TSIT.

Among the biochemically positive human and sheep isolates, the VITEK-2 analysis reported that 54.84% (17/31) and 71.8% (28/39) of human and sheep isolates, respectively, were positive for *K. pneumoniae* (< 0.0294), (Table 1).

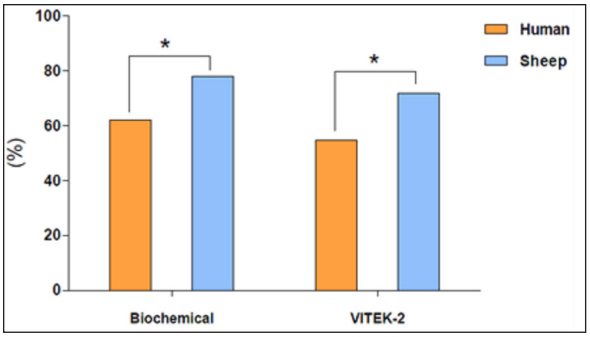

Fig. 1. Positive rate of biochemical tests and VITEK-2 system, * ($p < 0.05$).

Antibiotic susceptibility

In the present study, the positive human *K. pneumoniae* isolates were reported a significantly higher sensitivity to ATM (94.12%), TZP (94.12%), CIP (88.24%), and CXM (88.24%); whereas sheep isolates were more susceptible to CXM (85.71%), CIP (28.14%), TZP (82.14%), AMP (78.57%), FOX (78.57%), and TET (78.57%) than other antibiotics. Comparatively, *K. pneumoniae* human isolates were highly sensitive to ATM, CAZ, CTX, GEN, MEM, and TGC than *K. pneumoniae* sheep isolates that were susceptible to AMC, CHL, FOX, and SXT. Both human and sheep *K. pneumoniae* isolates showed an insignificant variation ($p > 0.05$) in their susceptibility or resistance to AMP, CIP, CXM, MEL, NIT, and TZP (Table 1).

Molecular examination

Targeting the *16S rRNA* gene, molecular examination of a total of 17 human and 28 sheep isolates revealed that all samples were *K. pneumoniae* (Fig. 2).

Phylogenetic analysis

The sequence data of 10 positive DNAs were deposited in the GenBank database under the access numbers OR484852, OR484853, OR484854, OR484855, OR484856, OR484857, OR484858, OR484859, OR484860, and OR484861. Multiple sequence alignment analysis identified the presence of similarity and substitutions in local human (Fig. 3) and sheep (Fig. 4) isolates. Phylogenetic tree analysis detected

Table 1. Antibiotic sensitivity test for positive *K. pneumoniae* isolates by VITEK-2.

Antibiotic	Concentration (µg)	Human (Total no: 17)		Sheep (Total no: 28)		*p-value*
		No.	%	No.	%	
AMC	20–10	10	58.82	20	71.43	0.0286 S
AMP	10	14	82.35	22	78.57 *	0.0559 NS
ATM	30	16	94.12*	20	71.43	0.0351 S
CAZ	10	3	17.65	3	10.71	0.0463 S
CHL	30	1	5.88	4	14.29	0.0469 S
CIP	5	15	88.24*	23	82.14*	0.0506 NS
CTX	5	14	82.35	20	71.43	0.0445 S
CXM	30	15	88.24*	24	85.71*	0.0557 NS
FOX	30	12	70.59	22	78.57*	0.0478 S
GEN	10	14	82.35	18	64.29	0.0219 S
MEL	10	2	11.77	5	17.86	0.0511 NS
MEM	10	4	23.53	3	10.71	0.041 S
NIT	100	6	35.29	9	32.14	0.0506 NS
SXT	1.25–23.75	5	29.41	15	53.57	0.0284 S
TET	15	14	82.35	22	78.57*	0.0551 NS
TGC	30–6	12	70.59	17	60.71	0.0486 S
TZP	30	16	94.12*	23	82.14*	0.0437 S
p-value		0.0157		0.0162		-

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

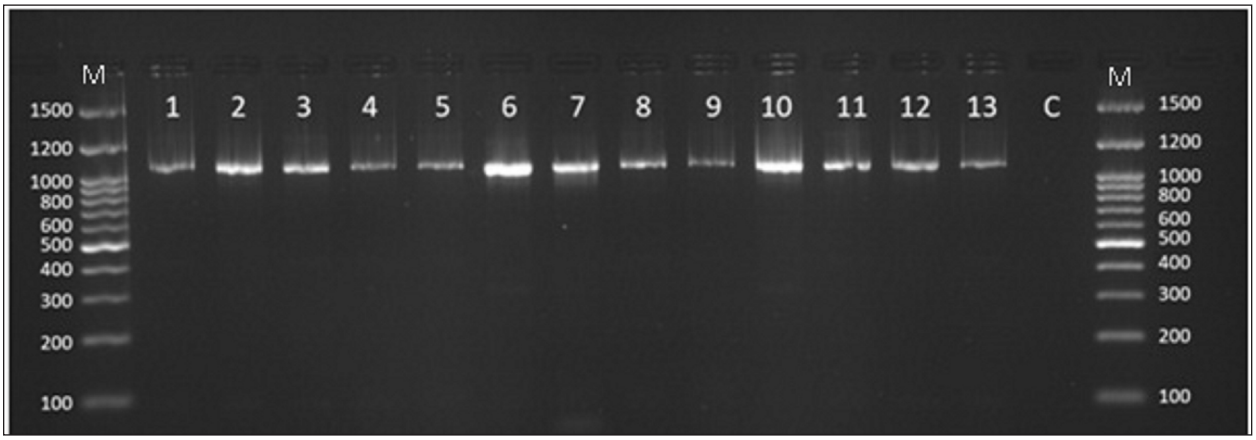

Fig. 2. Agarose gel electrophoresis of some positive human and sheep PCR products. Lane (M): Ladder marker (1,500-100 bp); Lanes (1, 2, 3, 4, 5, 7, 8, 9, 10, 11, 12, and 13): Positive samples at 1,081 bp; Lane (6): Positive control; Lane (C): Negative control, the PCR products run at 100 V and 80 Am for 1 hour.

Fig. 3. Multiple sequence alignment analysis of *16S rRNA* gene in local and NCBI-GenBank *K. pneumoniae* human isolates.

that identity ranged from 99.46%–99.56% and mutation at 0.02–0.01 when compared to the NCBI-Blast Indian *K. pneumoniae* isolates (Table 2 and Fig. 5); while in sheep, identity was 99.44%–99.50% and mutation was 0.0%–0.01% (Table 3 and Fig. 6). Comparative analysis between the local human and sheep *K. pneumoniae* isolates reported a significant high identity (99.82% to 99.88%) and low level of mutation (0.008% to 0.002%), (Fig. 7).

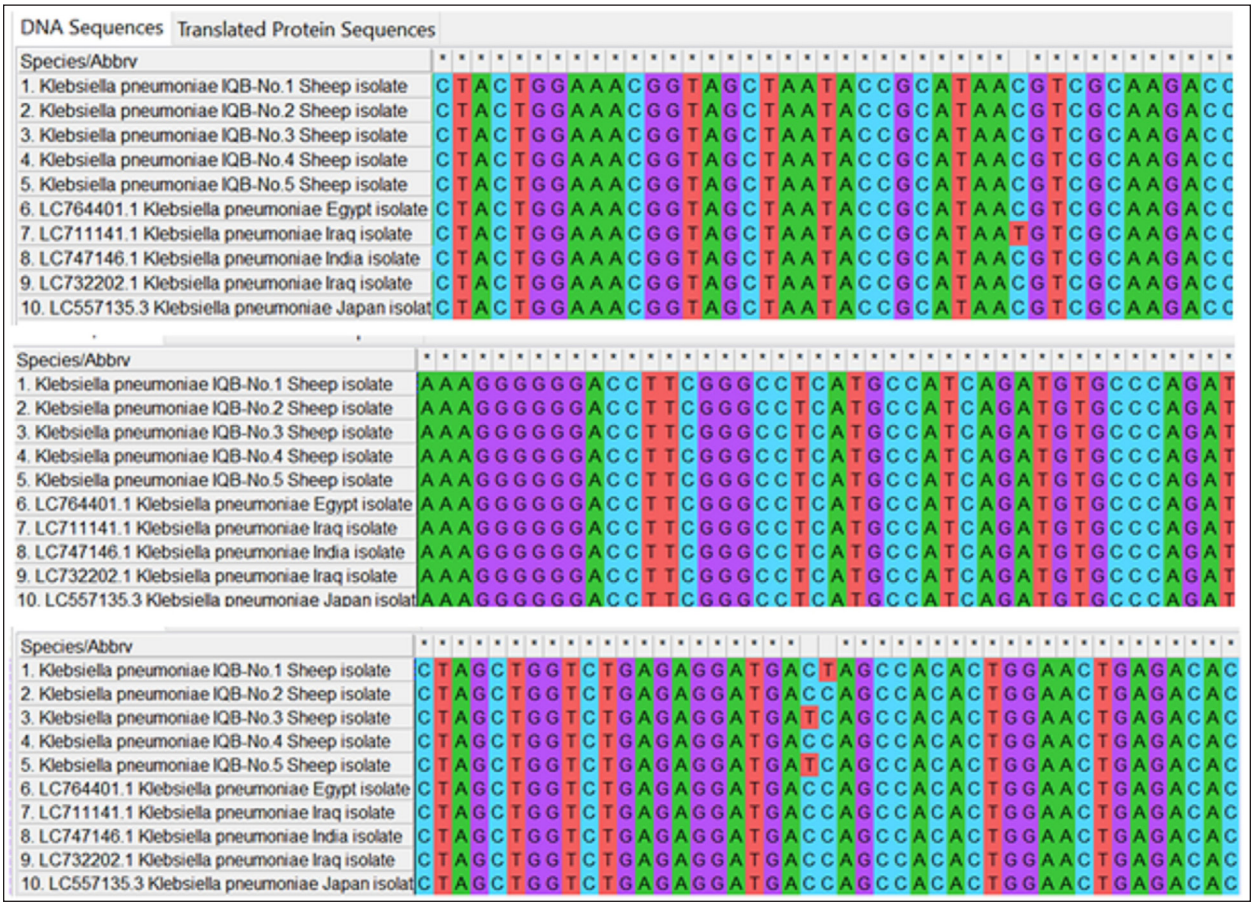

Fig. 4. Multiple sequence alignment analysis of *16S rRNA* gene in local and NCBI-GenBank *K. pneumoniae* sheep isolates.

Table 2. Sequence identity (%) of *K. pneumoniae* human isolates with sequences in the nucleotide database of GenBank.

Local isolate		Homologous sequences in GenBank		
Name	**Access no.**	**Country**	**Access no.**	**Similarity %**
BQH. 1	OR484852	India	LC747146	99.55
BQH. 2	OR484853	India	LC747146	99.46
BQH. 3	OR484854	India	LC747146	99.55
BQH. 4	OR484855	India	LC747146	99.54
BQH. 5	OR484856	India	LC747146	99.56

Discussion

Klebsiella pneumoniae represents one of the most common important opportunistic pathogens, which leads to healthcare-acquired infection in humans (Al Bshabshe *et al*., 2020) as well as to great economic losses in different animals (Effah *et al.,* 2020). In farm animals, respiratory infection constitutes a serious impact on animal production and husbandry (Chmielowiec-Korzeniowska *et al.,* 2021). In this study, *K. pneumoniae* was identified in both human and sheep samples using traditional and molecular assays. Worldwide, various studies have demonstrated the role of *K. pneumoniae* in different respiratory infections particularly lungs resulting in different symptoms such as cough, breathing distress, flu-like symptoms, pneumonia, and sepsis (Lindblom *et al.,* 2013; El-Radhi, 2018; Chen *et al.,* 2021). Although early diagnosis and the precise choice of antibiotics (e.g., penicillin, amoxicillin, ceftiofur, florfenicol, and tulathromycin) are the best protocol of cure, increases in antibiotic-resistant strains have been noted in the last few years (Sharahi *et al.,* 2021; Ndlovu *et al.,* 2023).

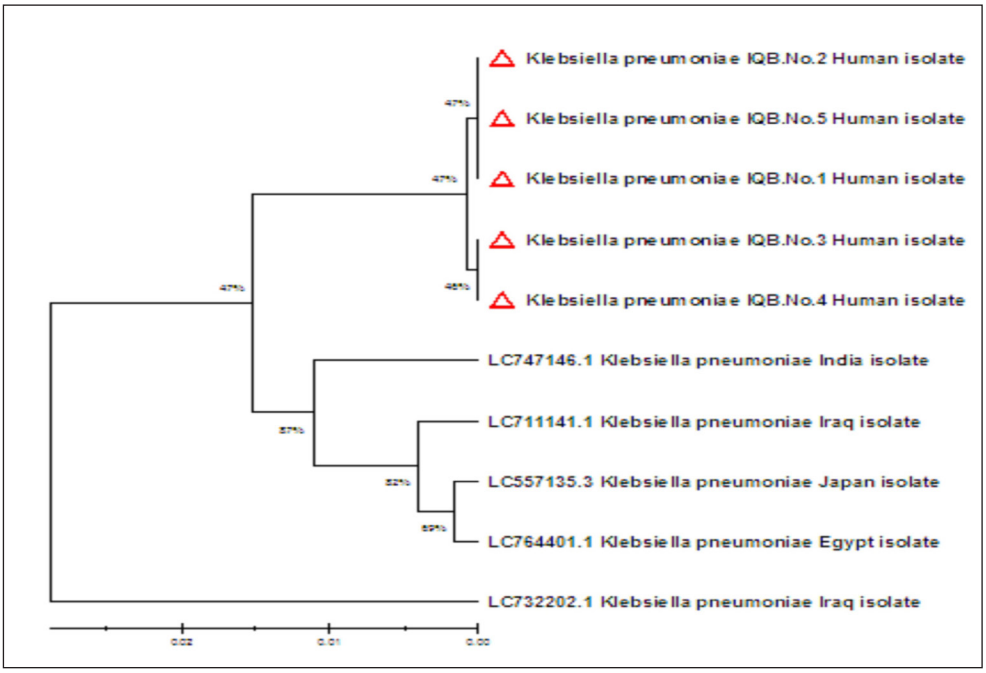

Fig. 5. Phylogenetic tree analysis of the local human *K. pneumoniae* isolates with the NCBI-GenBank isolates.

Table 3. Homology sequence identity (%) of local and NCBI-BLAST closed-related *K. pneumoniae* sheep isolates.

Local isolate		NCBI-GenBank isolate		
Name	**Country**	**Country**	**Access no.**	**%**
IQS. 1	OR484857.1	Iraq	LC711141.1	99.45
IQS. 2	OR484858.1	Iraq	LC711141.1	99.45
IQS. 3	OR484859.1	Iraq	LC711141.1	99.50
IQS. 4	OR484860.1	Iraq	LC711141.1	99.44
IQS. 5	O484861.1	Iraq	LC711141.1	99.46

In the current study, both humans and sheep *K. pneumoniae* isolates showed a significant variation in their sensitivity or resistance to antibiotics. Notably, the high sensitivity was recorded to AMP, CIP, CXM, and TZP but significant resistance to MEL and NIT. In two reports, it showed that the antimicrobial resistance to some antibiotics increased >1% per year (Jones, 2010), and some strains spread throughout the countries including the USA are associated with nearly complete antibiotic resistance (Hirsch and Tam, 2010). The increased resistance might be attributed to pathogen factors (selective pressure, mutation, gene transfer, and societal pressures), inadequate diagnosis, and protocol applied for the selection of antibiotics.

Over the past few decades, various molecular approaches have been described to identify the species of clinically important bacteria (Banin *et al.,* 2017; Janda and Abbott, 2021). Our findings indicated that 54.84% and 71.8% of human and sheep *K. pneumoniae* isolates were positive to *K. pneumonia*. Phylogenetic analysis of sequenced isolates demonstrated the identity of human isolates to Indian *K. pneumoniae* isolate (LC747146.1), and sheep isolates to Iraqi *K. pneumoniae* (LC711141.1). Several studies have been found that sequencing of *16S rRNA* gene can serve as a specific and reliable tool for the accurate identification of scarce pathogens (Culbreath *et al.,* 2019; Zheng *et al.,* 2021). Furthermore, *16S rRNA* is one of the most conserved genes among bacteria, which if sequenced, gives a complete conceive of whether the tested species/strains belong to distant or closed-related lineage (Makharita *et al.,* 2020).

Phylogenetic analysis of study-sequenced isolates observed a high association between human and sheep *K. pneumoniae* isolates. This is in agreement with that reported by other researchers (Alyassari *et al.,*

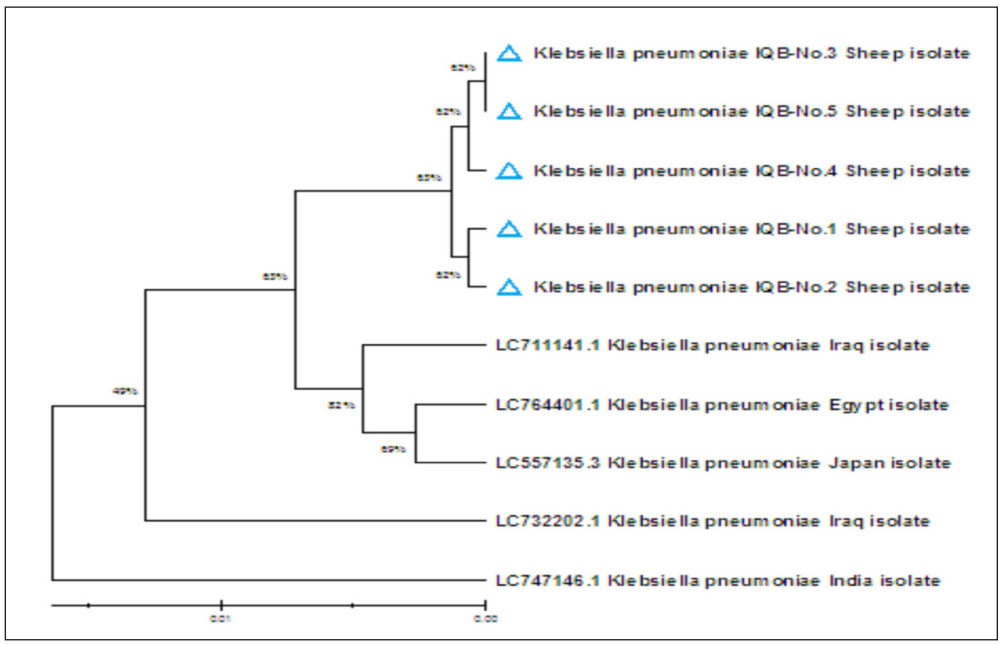

Fig. 6. Phylogenetic tree analysis of the local sheep *K. pneumoniae* isolates with the NCBI-GenBank isolates.

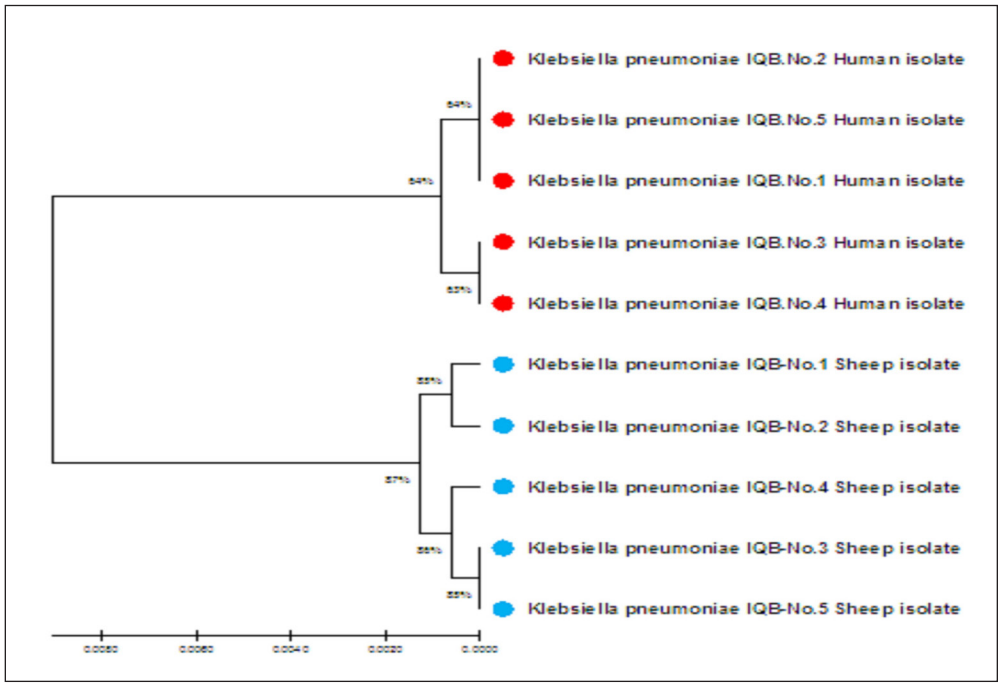

Fig. 7. Phylogenetic tree analysis of the local human and sheep *K. pneumoniae* isolates.

2019; Elmonir *et al.,* 2021; Tietgen *et al.,* 2022). Additionally, Yang *et al*. (2019) concluded that *K. pneumoniae* strains obtained from human and animals such as chickens, cows, and sheep having phenotypic and molecular properties, in addition to its potential transmission from animals to human and *vice versa*.

Conclusion

Based on our data, we suggest that *K. pneumoniae* can potentially transmit between human and animals, with the presence of a significant resistance to different antibiotics in both human and sheep isolates. Therefore, the judicious using of antibiotics in clinical

therapy and control measures is mandatory. Also, the characterization of bacterial resistance and the ability to transmission between animal species and humans can provide a key to understanding the pathogenicity and achieving more efficient therapy.

Acknowledgments

The authors acknowledge the support provided by the College of Veterinary Medicine and College of Medicine staff at the University of Basrah (Basra, Iraq) and the College of Veterinary Medicine at the University of Wasit (Wasit, Iraq).

Authors' contributions

HKI and KSM: Collection of swab samples, bacterial isolation, and characterization. HAN: Biochemical and antibiotic susceptibility testing. HAJG: Molecular, phylogenetic, and statistical analysis. All authors contributed equally in writing and approving the final copy of the manuscript.

Conflict of interest

The authors declared no conflict of interest.

Funding

No external funds were received (private funds).

Data availability

All obtained data were found in this manuscript.

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