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Evaluation of the Genetic Diversity of Antibiotic-resistant *Klebsiella pneumoniae* Isolated from Diarrheal Humans and Poultry using Multilocus Sequence Typing

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Abstract

Background: *Klebsiella pneumoniae* is an opportunistic pathogen usually responsible for healthcare-associated infections. Multilocus sequence typing (MLST) analysis utilized four housekeeping genes among *Klebsiella pneumonia* isolated from diarrheal humans and poultry to determine the extent of genetic diversity.

Material and methods: Seventy-five fecal samples from both human and poultry sources were cultured and diagnosed with the bacterial isolates by the VITEK-2 system as well as an Antibiotic sensitivity test (AST).

Results: Antibiotic susceptibility profile of Human's *K. pneumoniae* revealed all isolates bacteria were absolute resistant against Ampicillin and highly resistant toward Cefazolin 86%. Poultry's *K. pneumoniae* was highly resistant to Ampicillin at 100% and to Cefazolin at 46.6%. MLST results of all four housekeeping genes together revealed that humans *K. pneumoniae* (K1-K6) and poultry *K. pneumoniae* (K7-K11) were similar, specifically isolates K6 and K11, and different in (K1, K5, and K7).

Conclusion: The present study is considered the first in Iraq to determine the genetic relationship between *K. pneumonia* isolates from humans and poultry. *K. pneumonia* has a high rate of antibiotic resistance and high genetic diversity as a result of the sequencing of (*rpoB*, *gapA*, *phoE* and *tonB* genes) of human and poultry isolates. The genetic association (similarity) of antibiotic resistance *K. pneumoniae* strain between both sources where all isolates are resistant to most antibiotic agents in significant differences is evidence of the transmission of isolates from an animal source (poultry) to humans which poses a public health threat.

Keywords: Antibiotic resistance, Diarrheal poultry, Genetic diversity, *Klebsiella pneumoniae*, Multilocus sequence typing.

1. Introduction

K. pneumoniae is an important opportunistic bacteria, causing many diseases such as septicemia, liver abscesses, diarrhea, pneumonia and a few infectious diseases in humans (Riley, 2020).A variety of virulence factors are displayed by *K. pneumoniae*, including capsules, endotoxins, ferrous ferrites, iron removal mechanisms, binders, and antibiotic resistance that have been found to play major roles in pathogenesis (Zhang *et al*., 2018).

K. pneumonia is frequently found in food, including raw vegetables, milk powder, fish, and meat. It is reported to have a significant increase in foodborne outbreaks among different countries (Davis *et al*., 2015, Abu-Zaid *et al*., 2016, Hajikarim *et al*., 2020).

K. pneumoniae develops antibiotic resistance more easily than most bacteria by producing enzymes such as Extended Spectrum β-lactamase (ESBLs) and Carbapenemase (Padmini *et al.*, 2017). These bacteria develop resistance to antibiotics largely due to the evolution of bacterial enzymes of the superfamilies as a result of the diversity of genes (SHV-1., TEM-1, TEM-2, TEM-12) that they encode (Egorov et al., 2018, Sukhum et al., 2019).

Antimicrobial resistance genes are passed from environmental bacteria through *K. pneumonia* (Wyres and Holt, 2018). Food consumption is one of the most common ways that transmit antibiotic-resistant bacteria and genes into the human digestive system (Verraes *et al.,* 2013). Antibiotics are mainly used in the poultry industry for treatment, prevention, and growth promotion to boost farm animal productivity (Kiambi *et al*., 2021). In many countries of the world, antibiotics are given daily to food-producing animals to make them grow faster and prevent sickness (Milanović *et al*., 2017).

Multifocal sequencing (MLST) has emerged as an effective new DNA typing tool for assessing genetic relatedness between species (Robles *et al*., 2004). MLST is a molecular method that relies upon comparing sequenced portions of several housekeeping genes (genes that encode basic metabolic proteins processes functions), and identification of the "type of sequence" (ST) for each strain based on the existing alleles, phylogenetic analysis using the analyzed sites DNA sequence (Maiden *et al*., 1998). MLST scheme for *K. pneumonia* was developed by Laure

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Diancourt; using inner fragments housekeeping genes. These genes encode proteins essential for cellular metabolisms, such as RNA polymerase beta-subunit (*rpoB*), glyceraldehyde 3-phosphate dehydrogenase (*gapA*), phosphorine E (*phoE*), initiation, and periplasmic energy transducer (*tonB*). These were selected because they show a low level of mutation (Diancourt *et al*., 2005).

This study aims to establish the extent of genetic diversity among *K. pneumonia* isolates that were isolated from diarrheal human and poultry using MLST analysis. This study, for the first time, determined the genetic diversity among *K. pneumonia (rpoB),* (gapA), *(phoE)* and *(tonB)* and provide evidence of antibiotic-resistant transmission between poultry and human.

2. Materials and Methods

2.1. Identification of K. pneumoniae from Fecal Samples

Seventy-five *K. pneumoniae* isolates were isolated and identified from fecal diarrheal humans and poultry samples and according to (Obaid and Hasson, 2021)**.**

2.2. Antibiotic Resistance testing by VITEK-2 Compact

Antibiotic resistance testing (AST) was carried out using the VITEK-2 compact system based on the identification of Minimum inhibitory concentration technology using the AST-N222 card (Biomerieux/ France). This card contained the following Ampicillin, Piperacillin/tazobactam, Cefazolin, Cefoxitin, Ceftazidime, Ceftriaxone, Cefepime, Ertapenem, Imipenem, Amikacin, Gentamicin, Ciprofloxacin, Levofloxacin, Tigecycline, Nitrofurantoin, Trimethoprim/sulfamethoxazole**.** The results were interpreted according to (Wayne, 2011).

2.3. DNA extraction

Genomic DNA of *K. pneumoniae* isolates was extracted using Presto™ Mini gDNA Bacteria Kit supplied from Geneaid Company as per manufacturer's instructions (Geneaid, USA). The extracted Chromosomal DNAs were used as DNA templates for MLST.

2.4. PCR amplification

PCR reactions were carried out according to Diancourt *et al.* 2005 using the following cycling conditions 94°C for 30 s; followed by 30 cycles of 95°C for 30 s; 45°C, or 50°C, or 60 °C for 30 s (annealing Tm was different to each gene according to (Diancourt *et al*., 2005) and 72°C for 30 s, with a final extension of 72°C for 10 min followed by the hold at 4°C. A 5 µl of the PCR products were loaded into 2% agarose gels with loading dye in 1 X TAE, and runs at 1X TAE for 7V/cm, 45min. The gels Images were captured using a gel documentation system (Genosens 2000 series, Japan).

Table 1. Primers used in the study according to (Diancourt *et al*., 2005).

Gene name (Locus)	Primer sequence (5'-3')	Size (bp)
gapA	F:gapA173: TGAAATATGACTCCACTCACGG R:gapA181: CTTCAGAAGCGGCTTTGATGGCTT	450
phoE	F:phoE604.1: ACCTACCGCAACACCGACTTCTTCGG R:phoE604.2: TGATCAGAACTGGTAGGTGAT	420
rpoB	F: Vic3: GGCGAAATGGCWGAGAACCA R: Vic2: GAGTCTTCGAAGTTGTAACC	501
ton B	F:tonB1F: CTTTATACCTCGGTACATCAGGTT R:tonB2R: ATTCGCCGGCTGRGCRGAGAG	414

F: forward R: reveres

2.5. PCR product sequencing

All the PCR products were cleaned and sequenced as follows: The Gel/PCR DNA Fragments extraction kit (Geneaid, USA) was used to remove the amplification primer from the PCR product, as directed by the manufacturer. Purified DNA was sequenced at Macrogen (Korea) using sequencing primers for each gene as described by Diancourt *et al*., (2005), (Applied Biosystems, Foster City, CA, USA.

2.6. Multilocus sequence typing (MLST) analysis

The data from the raw sequences were edited and linked to the sequence of control. The Multi Locus Sequence Typing database provided the standard sequences for alignment. Multiple alignments were performed using ClustalW (Thompson *et al*., 1994) of Geneious Prime Software V2021.1 (Biomatters, Inc., North America). Identification of ST and allele profile was carried out by interrogation of gene sequences against the international MLST database at https://pubmlst.org/ and http://www.genomicepidemiology.org/. Regarding the identification of phylogenetic relationships among *K. pneumoniae* isolates, the merged edited sequences were used to generate phylogenetic tree using the PhyML maximum likelihood and the unweighted pair group method with arithmetic averages (UPGMA) (Kumar *et al*., 2018).

2.7. Statistical analysis

The Differences in data percentage values were analyzed by chi-square (X^2) test with the SPSS Statistics 25 software. The $P < 0.05$ Values were considered a statistical significance.

3. Results

3.1. Profile of Antibiotic Susceptibility of K. pneumoniae Isolates from diarrheal Human and Poultry

Human *K pneumoniae* were resistant to Ampicillin by 100%. The results revealed a high resistance rate (86%) to Cefazolin and, (60%) to each Ceftriaxone, Ceftazidime, Cefepime, and Trimethoprim/Sulfamethoxazole, but they showed a low level of resistance to Nitrofurantoin, Cefoxitin, Gentamicin (46.6%), (33.3%), and (20%) respectively (Table 1).

Poultry *K pneumoniae* were resistant to Ampicillin (100%) and highly resistant to Cefazolin (46.6%) as well as it revealed resistance to Ciprofloxacin, Levofloxacin, and Trimethoprim/ Sulfamethoxazole (40%), followed by Ceftriaxone, Ceftazidime, Cefepime (33.3%).

Table (2) showed all these results, where the antibiotics resistance rate against *K. pneumoniae* isolates appeared as highly significantly different with a p-value (< 0.001).

Table 2: Antibiotic resistance of ***K. pneumonia*** isolated from diarrheal humans and poultry.

Classes	Members	Number and % of Resistant rate (Human)	Number and % of Resistant rate (poultry)	X^2	P-value
	Ampicillin	75(100)	75(100)		
	Piperacillin/Tazobactam	0(0)	5 (6.6)		
B-Lactam	Cefazolin	65(86.6)	35(46.6)	27	0.001*
	Ceftriaxone	45(60)	25(33.3)	10.71	0.001*
	Cefoxitin	25(33.3)	10(13.3)	8.38	0.004*
Cephems	Ceftazidime	45(60)	25(33.3)	10.71	0.001*
	Cefepime	45(60)	25(33.3)	10.71	0.001*
	Ertapenem	0(0)	0(0)		
Carbapenems	Imipenem	0(0)	10(13.3)		
Aminoglycoside	Amikacin	0(0)	10(13.3)		
	Gentamicin	15(20)	20(26.6)	0.932	0.001*
Fluor quinolones	Ciprofloxacin	5(6.6)	30(40)	23.29	0.001*
	Levofloxacin	5(6.6)	30(40)	23.29	0.001*
Glycylcycline	Tigecycline	0(0)	0(0)	0	
Nitro furans	Nitrofurantion	35(46.6)	20(26.6)	6.45	0.001*
Sulfonamides	Trimethoprim/Sulfamethoxazole	45(60)	30(40)	6	0.014*

X^2: the test of chi-square, * significant difference (P<0.05).

3.2. Multilocus Sequence Typing Analysis (MLST)

3.2.1. Detection of Housekeeping genes using PCR:

The four *K. pneumonia* housekeeping genes were detected using PCR, sequenced and then analyzed by MLST to determine the extent of genetic diversity and relation among human's *K. pneumoniae* (K1, K2, K3, K5, and K6), (K4 excluded from the study) and poultry's *K. pneumoniae* isolates (K7, K8, K9, K10 and K11).

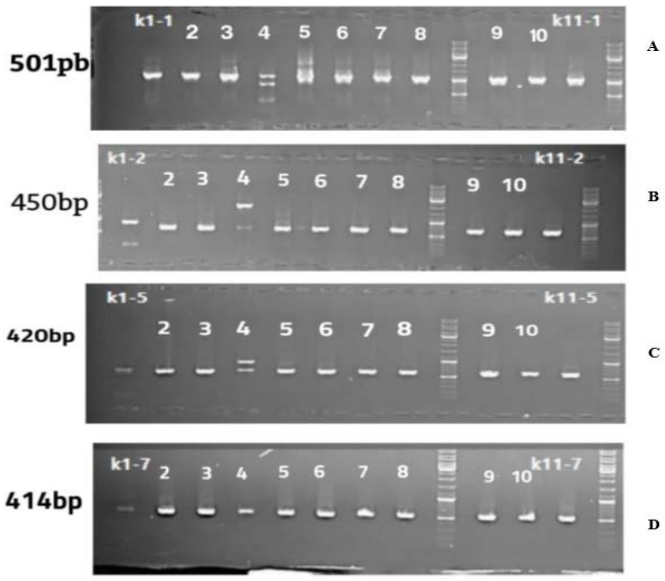

Figure 2. Electrophoresis the PCR product for A:*rpoB* (501 bp), B*: gapA* gene (450 bp), C*:phoE* gene (420 bp), and D*:tonB* gene (414 bp) of *K. pneumoniae* isolated from diarrheal human and poultry.

3.2.2. rpoB gene

Using UPGMA, the sequenced *rpoB* gene shows the phylogenetic Cladogram figures (2,3). There was no similarity detected in human *K. pneumoniae* isolates, but one group resemblance was found in poultry *K. pneumoniae* isolates (K9 and K10), while the link between *K. pneumoniae* in humans and poultry indicated two groups of commonalities (K6 with K7) samples and (K2 with K8) isolates.

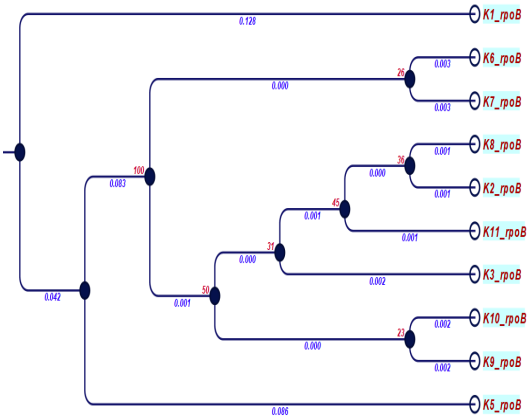

Figure 3. Phylogenetic Cladogram for *rpoB* gene of *K. pneumoniae* isolates from diarrhea human and poultry by using UPGMA, the blue text represents branch length and red text represents bootstrap values. (K1, K2, K3, K5, and K6) represent human *K. pneumonia* and (K7, K8, K9, K10, and K11) represent poultry *K. pneumoniae*.

3.2.3. The gap A gene

The outcomes of *gap A* sequence using UPGMA method showed phylogenetic cladogram figures (2,4). Except for one group, there are no similarities among all *K pneumoniae* isolates of human and poultry. The association between humans and poultry is reflected by similarities between isolates (K6 with K9).

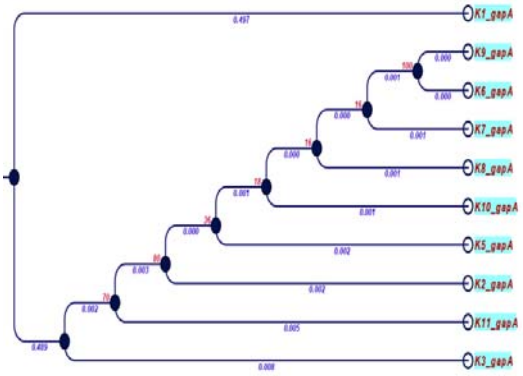

Figure 4. Phylogenetic Cladogram for *gapA* gene of isolates *K. pneumoniae* by using UPGMA, the blue text represents branch length and red text represents bootstrap values. (K1, K2, K3, K5, and K6) represent human *K. pneumonia* while (K7, K8, K9, K10, and K11) represent poultry *K. pneumoniae.*

3.2.4. The phoE gene

The phylogenetic tree of the *phoE* gene was shown in Figures 2 and 5. All human *K pneumoniae* isolates were different, while one group of poultry *K pneumoniae* isolates had similarities to one human K pneumoniae isolate (K7 with K9). At the same time, two parallels between humans and poultry emerged in (K5-K11) and (K5-K12) (K6-K10).

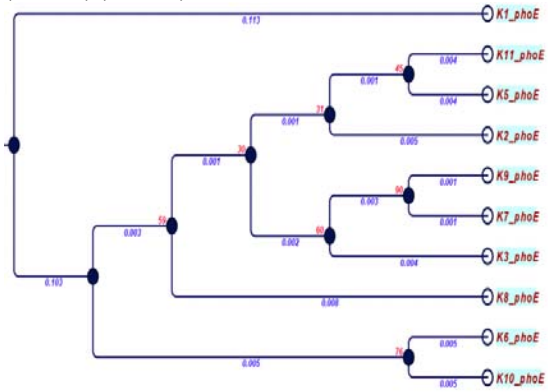

Figure 5. Phylogenetic Cladogram for *phoE* gene of isolates *K. pneumoniae* by using UPGMA, the blue text represents branch length and red text represents bootstrap values. (K1, K2, K3, K5, and K6) represent human *K. pneumonia* while (K7, K8, K9, K10, and K11) represent poultry *K. pneumoniae*.

3.2.5. The tonB gene

Phylogenetic tree of *tonB* gene (Fig 2 and 6) revealed there are differences in all humans and poultry *K. pneumoniae*. Three groups showed similarities between humans and poultry in (K2-K9), (K3-K10), and (K5-K8).

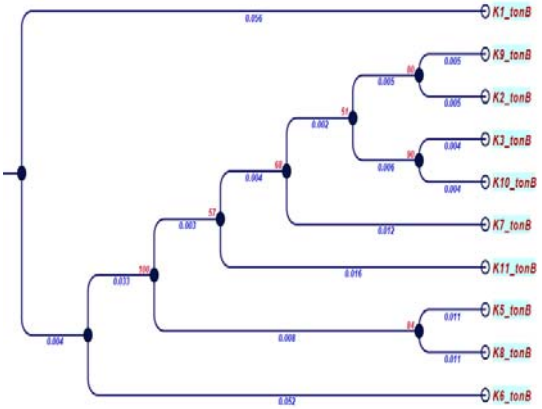

Figure 6. Phylogenetic Cladogram for *tonB* gene of isolates *K. pneumoniae* by using UPGMA, the blue text represents branch length and red text represents bootstrap values. (K1, K2, K3, K5, and K6) represent human *K. pneumonia* while (K7, K8, K9, K10, and K11) represent poultry *K. pneumoniae*.

MLST result of Diarrheal Human and Poultry based on locus gene and Allelic profile

Using the housekeeping genes, multilocus sequence typing was used to analyze the genetic diversity of *K. pneumoniae* isolates from diarrheal humans and poultry. Multiple sequence alignment of the four gene sequences from ten isolates revealed nucleotide sequence similarities and variations, as well as unique alleles (Table 3).

Multilocus sequence typing with four housekeeping genes and allelic profiles of 10 *K. pneumoniae* isolates from human and poultry feces were new sequence types of all isolates from both sources for all studied housekeeping genes as was generating many unique and novel alleles for the genes utilized in this investigation, particularly *tonB* gene alleles.

Table 3. MLST analysis to Identify MLST sequence types (STs), nearest ST, and allelic profiles of ten *K. pneumoniae* isolates from the fecal specimen of humans and poultry with four housekeeping genes.

Sample	Sours	ST	Nearest ST	Allelic profile			
				rpoB	gap A	pho E	ton B
K. pneumoniae 1	Human	New	1877	24	1*	72*	38*
K. pneumoniae 2	Human	New	857	24	2	56	19*
K. pneumoniae 3	Human	New	1300	1	4*	7	12*
K. pneumoniae 5	Human	New	2419 Or 4370	258*	10*	12	38*
K. pneumoniae 6	Human	New	1352	1*	2	191*	664*
K. pneumoniae 7	Poultry	New	3050 Or 1701	260	10*	275*	39*
K. pneumoniae 8	Poultry	New	15	1	1	1*	1*
K. pneumoniae 9	Poultry	New	3044 Or 4465	45*	2	275*	65*
K. pneumoniae 10	Poultry	New	3127	45	204*	334*	12*
K. pneumoniae 11	Poultry	New	3403 Or 1537	1	2	12	408

Note: * represent Novel allele.

3.3. Genetic Relatedness of K. pneumoniae by MLST analysis among Diarrheal Humans and Poultry

The Multi- drug resistance B- Lactamase *K. pneumoniae* sequences of the four housekeeping genes revealed strong links between humans and poultry *K pneumoniae* (Figure 7). A tight association was discovered between K6 (human *K. pneumoniae*) and K11 (poultry *K. pneumoniae*), whereas a distance relationship was discovered (K1, K5, and K7) revision. A comprehensive tree based on a cladogram is presented as a method to determine the efficiency of *K. pneumoniae* isolated from fecal samples of people and poultry using the high potential of these genetically determined sections to give a realistic picture of the possibility of human infection with bacteria of animal origin such as chicken through identical or close genetic linkage.

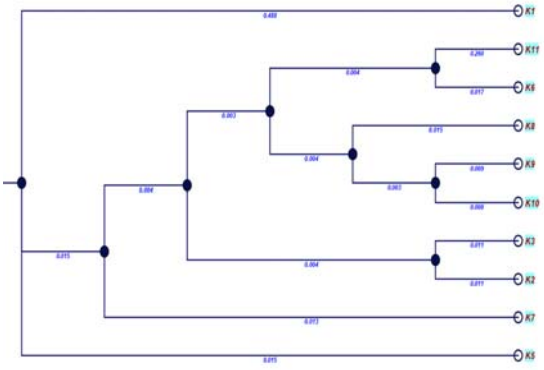

Figure 7. Cladogram Phylogenetic analysis of *K. pneumoniae* isolates from diarrheal human and poultry serotypes using PhyML maximum likelihood. (K1, K2, K3, K5, and K6) represent Human *K. pneumonia* also (K7, K8, K9, K10, and K11) represent Poultry *K. pneumoniae* .

4. Discussion

K. pneumoniae is a gastrointestinal commensal bacteria that occasionally causes diarrhea in humans. Some of the diarrhea traces carried the thermostable or thermolabile toxin (Forsythe *et al*., 2015). Identifying illness infection pathogenic mechanisms of *Klebsiella* can explain the interaction between bacterial cells and the host (Bengoechea and Sa Pessoa, 2019). *K. pneumoniae* pathology has been attributed to many virulence genes that allow it to evade the host's innate defense responses such as *rpoB* (Beta-subunit of RNA polymerase B) *gapA* (Glyceraldehyde 3- Phosphate dehydrogenase), *tonB* (Periplasmic energy transducer) and *phoE* (Phosphoporine E) were responsible for the essential metabolic process in addition to pathogenicity and virulence activity related to antibiotic resistance (Alcántar-Curiel *et al*., 2013, Blin *et al*., 2017).

The production of a wide range of beta-lactamase or changing the permeability barrier or at the target site represented by the penicillin-binding protein, or a change in the outer membrane protein, are the most important problems of increasing infection in hospitals and the main causes of *K. pneumoniae* antibiotic resistance (beta-lactamase) (Aghamohammad *et al*., 2020).

K. pneumoniae possesses many pumps that circulate and eject the antibiotic, especially Beta-lactam antibiotics including penicillin with cephalosporin, Alcarpinimat, and Monobactam among others (Smith and Kendall, 2021). Antimicrobial resistance is a global danger to food security, animal welfare, long-term therapy, and public health. Many variables contribute to the irrational use of antibiotics, including policymakers' perceptions of their expertise, manufacturers' prescriptions, consumers' perceptions of their knowledge, and dispensers' perceptions of their knowledge (Wall, 2019). *rpoB* gene, which codes for the RNA polymerase ß-subunit, has emerged as a core gene candidate for phylogenetic analysis, allowing for the separation of closely related isolates (Michodigni *et al*., 2021). *K. pneumoniae* and other *Klebsiella* species can be correctly identified and differentiated using phylogenetic analysis of the *rpoB* gene. Urbaniak *et al*., (2018) recommended using the *rpoB* gene rather than the 16S rRNA gene to categorize *Klebsiella*. Both NAD-dependent oxidative phosphorylation and NAD-dependent conversion of D-erythrose 4-phosphate are encoded by the *gapA* gene. NAD-dependent oxidative phosphorylation of glyceraldehyde 3-phosphate to 1,3-bisphosphoglycerate is catalyzed by glyceraldehyde-3-phosphate dehydrogenase, while the NAD-dependent conversion of D-erythrose 4-phosphate to 4-phosphoerythronate is catalyzed by erythrose-4-phosphate dehydrogenase. The periplasmic energy transducer is encoded by the *tonB* gene. *phoE* gene

(phosphoporine E), is a controller ion transmembrane transporter that regulates the expression of porins were present in the outer membrane of *K. pneumoniae* which responsible for antibiotic entry and actually associated with antibiotic resistance (Kaczmarek *et al*., 2006).

Genotyping of *rpoB*. *gapA, tonB* and *phoE* is critical for identifying *K. pneumoniae* infections and determining the source and incidence of infections. MLST is a useful method for determining genetic diversity and population organization in epidemiological settings (Guo *et al*., 2016). The isolates K6 and K11 (which reflect the human-poultry interaction) were comparable in that they lacked ESBL and were resistant to Ampicillin, which was a novel finding in this investigation. The formation of extended spectrum beta-lactamase (ESBL), which is one of the most severe concerns of rising infection in hospitals, or modifying the permeability barrier or at the target site represented by a binding protein were the major causes of antibiotic resistance in *K. pneumoniae* (Tilahun *et al.,* 2021). ESBL product due to continuous exposure of *K. pneumoniae* to b-lactam antibiotic resulted in a dynamic and persistent production and mutation of b-lactamases, which expanded their resistance activity (Shaikh *et al*., 2015).

In addition, new data in K6 and K11 (which represent the human-poultry interaction) show the same rate of resistance to tested antibiotics. As a result, the strain of antibiotic-resistant bacteria can be transmitted from animals to humans directly, such as through direct contact with farmers or veterinarians, or indirectly, through the consumption of contaminated animal feed, contaminated groundwater or surface water, and methods of animal waste treatment (Daniel *et al*., 2015). Antibiotic-resistant or vulnerable persons can be infected not just via direct contact but also through animal-derived food items. (Lekshmi *et al*., 2017). Furthermore, antibiotic-resistant bacteria in food are a major public health concern because they can transmit antibiotic resistance features to pathogenic bacteria, making it difficult to treat bacterial infections in the medical environment (Imran *et al*., 2019). However, when compared to gene-based phylogenetic methods, the multilocus sequence typing (MLST) analysis demonstrated a high detection specificity. As a result, the currently available PCR sequencing procedures, as well as phylogenetic tools, revealed surprisingly close relationships between some of the isolated *K. pneumoniae* studied. MLST is thought to be a good method for characterizing the genetic links between bacterial isolates as well as identifying and tracking the global spread of drug-resistant strains (Adwan *et al*., 2020). In commercial cultures, antibiotics are used extensively in chickens, even with modest quantities of hormones, which may be one of the contributors to generating germ resistance in some people (Waters *et al*., 2022). It has a major influence and may increase the risk of cancer and premature puberty (Pérez-Rodríguez and Mercanoglu, 2019). The similarities of K11, K6, and differences between K1, K5, and K7 among the isolates categorized by MLST suggested that *K. pneumoniae* might be transmitted in the community from animal sources throughout time. Isolated *K. pneumoniae* could produce a significant signal of drug resistance among a considerable signal of drug resistance in the community. One of the most essential strategies for the rapid deployment of a substantial multi-drug resistance among bacteria is the horizontal transfer of drug resistance genes (Blin *et al*., 2017).

5. Conclusions

The current study is considered the first in Iraq and the world on the genetic relationship between *K. pneumoniae* isolates from humans and poultry. *K. pneumoniae* has a high rate of antibiotic resistance and high genetic diversity as a result of the sequencing of (*rpoB*, *gapA*, *phoE* and *tonB* genes) of human and poultry isolates. The genetic association (similarity) of antibiotic resistance *K. pneumoniae* strain between both sources where all isolates are resistant to most antibiotic agents in significant differences is evidence of the transmission of isolates from an animal source (poultry) to humans which poses a public health threat. As a result, control measures for K. pneumoniae transmission between humans and poultry, as well as cautious antibiotic use in human therapeutic strategies and poultry production, are required.

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Conflict of Interest

"The authors declare that there are no conflicts of interest regarding the publication of this manuscript."

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