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Isolation and identification of *Klebsiella pneumoniae* from respiratory disease in chicken

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Abstract

The objective of the current study was to identify *Klebsiella Pneumoniae* from respiratory illness in chickens and to look into specific virulence characteristics associated with antibiotic resistance. Between April and August of 2023, fifty respiratory tract samples (trachea and lung) were obtained from hens with respiratory infections from various farms in the Nineveh Governorate. After being deposited in the sterile container, all samples were quickly sent for microbiological processing to the Department of Microbiology, College of Veterinary Medicine, University of Mosul, Iraq. The results showed that *K. pneumoniae* isolates were recovered in chicken samples at a percentage of 30% (15/50), which was diagnosed by culture on selective media, staining, and biochemical tests. Six selected isolates were randomly confirmed by polymerase chain reaction (PCR) and appeared to have SHV and CTM-X genes responsible for the resistance of this bacteria toward the antibiotics (ESBLs group), while they did not have the TEM gene. Also, an antibiotics sensitivity test was done on these six isolates of *K. pneumoniae*; the results show resistance to most of the antibiotics used in this study. In conclusion, *K. pneumoniae* is one of the leading causes of respiratory infections and has a property of the genetic resistance of beta-lactam antibiotics.

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Introduction

The respiratory illnesses that affect domestic poultry species are primarily caused by bacterial infections (1). Klebsiella bacteria are a genus that belongs to the Enterobacteriaceae family. These bacteria are gram-negative coccobacilli, non-motile, non-polar, lactose fermented, facultatively anaerobic, and have capsules made from polysaccharides (2-4). Klebsiella bacteria is a global and live saprophytic in water, soil, and vegetables, and also typically inhibits the respiratory system and digestive tract for humans and animals and causes airsacculitis in poultry and yolk sac infection in chicks (5-7). *Klebsiella pneumoniae* has appeared as a significant pathogen causing several illnesses (8). After a first viral or environmental infection, bacterial infections frequently settle in the respiratory system as a secondary invasion. Birds have been known to have *K. pneumoniae*, a major pathogen associated with respiratory tract diseases (9). Additionally, young chickens infected with *K. pneumoniae* had a higher risk of respiratory illness (10). Furthermore, *K. pneumoniae* has long been suspected of contributing to community-acquired pneumoniae in North America, Europe, and Australia. During the last two epochs, *K. pneumoniae* has been an oddly uncommon source of community-acquired pneumoniae (11-13). Antibiotics are often used in cattle and poultry as a growth stimulant and to prevent the spread of illness (14). Antibiotic resistance is one of the detrimental effects of routine antibiotic usage in the livestock business, particularly in hens. The Klebsiella species is one of the bacteria that are aware of drug resistance. Gram-negative Klebsiella bacteria are typical oral, cutaneous, and intestinal flora, but they can sometimes

harm people and animals. Large animals and poultry are susceptible to bacterial illnesses that this particular bacterium can cause. Numerous papers claim antibiotic resistance has been observed in Klebsiella species (15). *K. pneumoniae* which produces extended-spectrum B-lactamases (ESBL) is a growing issue in human and animal medicine. We were preoccupied with looking into potential transmission along the broiler production chain and evaluating their potential impact on human health since SHV-2, which encodes *K. pneumoniae*, was recently discovered in broiler production (16). Most ESBLs possess mutations from the traditional TEM and SHV genes, enabling them to hydrolyze antibiotics with a broader range. There have been reports of over 150 TEM enzymes and over 50 distinct SHV mutations. Several countries worldwide have seen the appearance of a recently identified family of plasmid-mediated ESBLs known as CTX-Ms, which hydrolyze cefotaxime in diverse ways (17,18). Currently, there are known to be over 40 distinct CTX-M enzyme variations (19,20).

The current study aims to isolate and identify *K. pneumoniae* as one of the essential causes of respiratory diseases in poultry and investigate the SHV, CTM-X, and TEM genes responsible for antibiotic resistance.

Materials and methods

Ethical approve

All samples were collected from broiler farms after getting approval from their owner; all birds were euthanized ethically, according to the Animal Welfare Committee in Mosul University- College of Veterinary Medicine No. UM.VET.2023.025 in 10 /3 /2023.

Samples

Between April and August of 2023, 50 lung and tracheal samples were taken from broilers with respiratory illnesses housed in several poultry farms in the Nineveh Governorate. After being placed in a sterile container, all samples were quickly sent to the microbiological lab in the Department of Microbiology, College of Veterinary Medicine, University of Mosul, Iraq.

Bacterial isolation

All lung and tracheal samples were cultured in the Brain heart infusion broth for primary enrichment steps; after that, an inoculum from enriched media was subculture on both McConkey agar and Blood agar (Oxoid/UK). All cultures were incubated at 37°C aerobically for 18 hours. The suspected colonies that grow in blood and McConkey agar, similar to phenotypic to *K. pneumoniae*, were stained with Gram stain. Additional biochemical diagnostic tests included indole, methyl red, Vocus-Proskauer, and Simon citrate tests for all isolates, and the VITEK2® Compact system was performed for final conformation; six phenotypically comparable positive *K. pneumoniae* isolates were used randomly to validate the presence of antibiotic resistance and virulence genes by molecular method (PCR).

Antibiotic sensitivity test

The test was done according to the method described by Bauer-Kirby. A standard bacterial inoculation of $1.5x10^8$ cfu/ml was used to perform the test (21). Six commonly used antibiotic discs were used; the antibiotic concentrations are listed in table 1. According to the World Health Organization, the inhibition zone's result was estimated by millimeters, and the results were classified into three intervals: resistance, intermediate, and sensitive (22).

Table 1: the antibiotics used for the antibiotic sensitivity test in this study

Antibiotics	Concentration (µg/disc)
Imipenem	10
Trimethoprim Sulphathiazole	25
Ciprofloxacin	10
Penicillin	10
Amoxicillin	10
Cefadroxil	30

DNA extraction and amplification

DNA was extracted from All suspected *K. pneumoniae* isolates, molecular confirmation of species and detection of antibiotic virulence genes; the extraction of DNA was done by using Add Prep Genomic DNA Extraction (Korea) according to company instructions, and all DNA samples were tested to determine its purity by using Nanodrop (Nanophotometer® N50/ Germany) and all stored in -20°C till used (23,24). All extracted bacterial DNA was tested using conventional PCR (Sensoquest, Germany) for the presence of 16S rRNA, SHV, CTX-M, and TEM genes. The PCR reaction mixture (25 µl) included 1µl from each forward and reverse primer, 10 µl of GoTaq® G2 Green Master Mix (Promega, USA), and five µ of *K. pnenomenia* DNA, 8 µl of Danase free water were used to amplify genes. The result amplicons were visualized in 1.5% agarose electrophoresis. All primer sequences and amplification cycles are listed in tables 2 and 3.

Results

Bacteriological isolation

The results showed isolation of 15 positive samples for *K. pneumoniae* isolates from the trachea and lung with a 30% (15/50) isolate rate. All colonies were similar to typical *K. pneumoniae* morphological appearance on McConkey and blood agar (Figure 1). The result of the biochemical test and the VITEK2® Compact system confirmed that all isolates belong to *K. pneumoniae.*

Table 2: Genes and sequences used in this study

Genes name		Primer Sequence	PCR product size	References
16SrRNA	F	ATTTGAAGAGGTTGCAAACGAT	130 bp	25
	R	TTCACTCTGAAGTTTTCTTGTGTTC		
SHV	F	ATGCGTTATATTCGCCTGTG	763 bp	26
	R	TGCTTTGTTATTCGGGCCAA		
CTX-M	F	CGCTTTGCGATGTGCAG	550 bp	26
	R	ACCGCGATATCGTTGGT		
TEM	F	AAACGCTGGTGAAAGTA	822 bp	26
	R	AGCGATCTGTCTAT		

Table 3: The amplification programs used for the PCR reaction

Genes name	Initial denaturation °c/min	Cycle numbers 35 (°c/min)			Final extension (°c/min)
		Denaturation	Annealing	Extension	
16SrRNA	94/10	94/0.3	57/0.3	72/0.45	72/5
SHV	95/10	94/1	55/1	72/1	72/5
CTX-M	95/10	94/1	55/1	72/1	72/5
TEM	95/10	94/1	50/1	72/1	72/5

Antibiotic sensitivity

the current study revealed that all six *Klebsiella pneumoniae* isolates show complete resistance to most antibiotics used in this study, including penicillin, amoxicillin, cefadroxil, and ciprofloxacin and show sensitivity to imipenem (Figure 2 and Table 4).

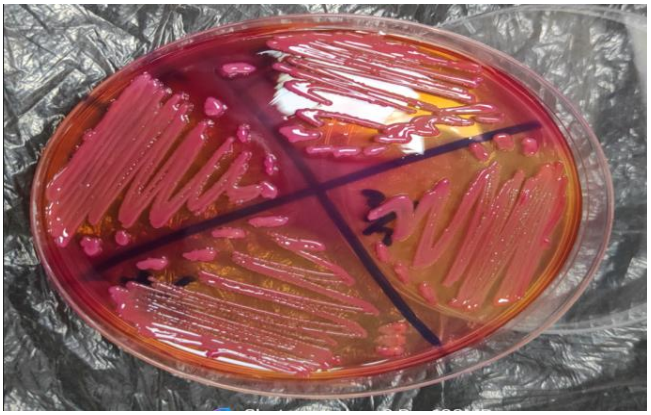

Figure 1: *K. pneumoniae isolates* with mucoid consistency on MacConkey agar.

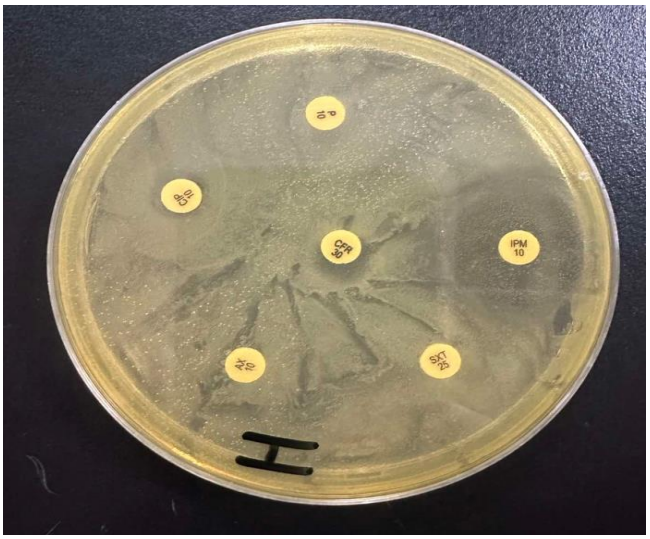

Figure 2: Shows that *K. pneumoniae* e is resistant to most antibiotics used in this study.

Table 4: Shows the results of the antibiotics sensitivity test

Antibiotics	Sensitive (%)	Intermediate (%)	Resistance (%)	Concentration (µg/disc)
Imipenem (IPM)	100	0	0	10
Trimethoprim Sulphathiazole (SXT)	33.3	0	66.6	25
Ciprofloxacin (CIP)	0	0	100	10
Penicillin (P)	0	0	100	10
Amoxicillin (AX)	0	0	100	10
Cefadroxil (CFR)	0	0	100	30

Molecular identification and resistance genes

The outcomes of molecular confirmation of randomly chosen six isolates when using 16S rRNA specific for *K. pneumoniae* revealed that all of them were *K. pneumoniae* (Figure 3) and all these selected isolates had properties of SHV and CTX-M, while no isolate showed the presence of TEM (Figure 4).

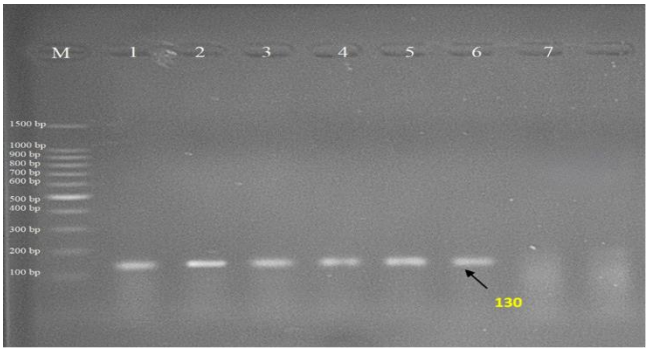

Figure 3: The PCR amplicons of 16SrRNA:M= kilobase Marker,2-6 =positive 130 bp amplicon of 16SrRNA *K. pneumoniae e* isolates 7= negative sample.

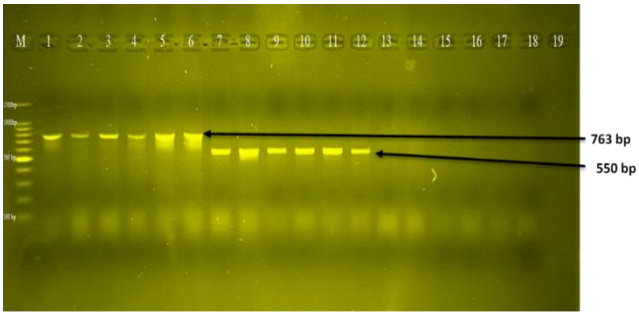

Figure 4: The amplicon of SHV and CTX-M and TEM gene of *K. pneumoniae* isolates: M= kilobase Marker 1-6=SHV gene amplicon 550 bp, 7-13=CTX-M gene amplicon 763 bp, and 13-18= TEM gene amplicon 882bp, 19= negative control.

Discussion

An opportunistic bacterium called *K. pneumoniae e* is responsible for nosocomial and community-acquired infections, including pneumoniae, meningitis, septicemia, wound infections, and urinary tract infections (27); these bacteria are facultatively anaerobic and are members of the Enterobacteriaceae family. This genus has 77 capsular antigens (K antigens), which result in various serogroups (28-30). The results of this study showed by using the culture method that there are 15 isolates from the 50 samples in isolation rate 30% have the properties of *Klebsiella spp* colonies, which grow in McConkey agar and fermented the lactose sugar forming mucoid bright pink color colonies which is the eminent character of klebsiella spp. Also, the microscopic (Gram and Indian ink stain) and the biochemical tests (VITEK2® Compact system) explain that these bacteria are *Klebsiella spp*., and these results agree with many studies that use the conventional methods in general (31,32). The isolation rate in our study 15% agrees with the study done on the broiler chicken by Gorrie *et al.* (27) in al Mansoura, Egypt, and also the study done by Oliveira *et al*. (32) in Portugal. However, this differs from the study on poultry in Bergen, Norway 40.5% (28). This difference may be due to the different regions and the number of samples collected in each study.

The global expansion of MDR strains of *K. pneumoniae e* is another issue since they might result in inappropriate experiential therapy. Extended-spectrum hydrolytic activity b-lactamases mediated by plasmids are typically present in MDR bacteria (33).

Different cephalosporin types, such as ceftazidime, ceftriaxone, cefepime, and cefotaxime, can be hydrolyzed by ESBLs. (34-36). CTX-M β-lactamase was identified as the primary ESBL type, followed by traditional TEM and SHV kinds of β-lactamases. Global reports of it in both human and animal populations have been made by Lee *et al*. (37). ESBLs are a cluster of plasmid-mediated enzymes that belong to the Ambler class A of β-lactamases. They can hydrolyze fourth- and third-generation cephalosporins, critically important antimicrobials, and most penicillin, monobactams, and cephalosporins (31). Plasmids are often used to encode the ESBL. However, plasmids containing ESBL can also transfer other antibiotic resistance genes, severely limiting the treatment options available to bacteria that produce ESBL (38). Ahmed and Roshdi *et al.* suggest that mobile genetic components like integrons, transposons, and plasmids carry a large number of beta-lactamases, which causes a broad spread of resistance factors from one bacterial strain to another (39,40). The current study revealed that six random isolates of Klebsiella spp. PCR confirmed were *Klebsiella pneumoniae*, and all these random isolates had properties of SHV and CTX-M genes, while no isolate showed the presence of the TEM gene. Many studies carried out by Ashraf *et al*., (17), Al-Chalaby (30), Daehre *et al*., (31), Fasciana *et al*., (41) and Lim *et al*. (42) agree with the current study that *Klebsiella pneumoniae* having SHV, CTX-M. *Klebsiella pneumoniae* 's resistance to antibiotics might be attributed to the antibiotics' frequent and haphazard use without a veterinarian's prescription as well as their extensive use as growth promoters until now.

Conclusion

This study confirmed that *Klebsiella pneumoniae isolated* from broiler chickens in the Nineveh governorate is considered one of the leading causes of respiratory diseases and has a high resistance level to almost all β-lactam antibiotics by having SHV and CTX-M genes.

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Conflict of interest

A conflict of interest did not exist.

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العزل وتشخيص لجراثيم الكليبسيلا من حالات الإصابات التنفسية في الدجاج

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الخلاصة

ظهرت الكليبسيلا الرئوية كعامل ممرض مهم لمختلف أنواع الأمراض. هدفت الدراسة الحالية إلى الكشف عن عدوى الكليبسيلا الرئوية من حالات الإصابات التنفسي في الدجاج، والكشف عن بعض عوامل الضراوة المرتبطة بعوامل مقاومة مضادات الميكروبات. تم اخذ خمسين عينة من الجهاز التنفسي (القصبة الهوائية والرئة) من الدجاج المصاب بالتهاب الجهاز التنفسي من حقول مختلفة في محافظة نينوى خلال الفترة ما بين نيسان الى آب ٢٠٢٣. ووضعت جميع العينات في صندوق مبرد ونقلت على الفور الى مختبر الأحياء المجهرية (قسم الأحياء المجهرية، كلية الطب البيطري، جامعة الموصل، العراق) لأجراء المزيد من الاختبارات المكروبيولوجية. أظهرت النتائج أن عازلات الكليبسيلا الرئوية التي تم استخلاصها في عينات الدجاج بنسبة عازلات ٣٠% (١٥/٥٠) والتي تم تشخيصها عن طريق الزرع على الوسائط الانتقائية والصبغ والاختبارات الكيموحيوية. تم اختيار ست عازلات عشوائيا وتم تأكيدها بواسطة تفاعل البلمرة المتسلسل، كما أظهرت هذه العزلات احتواءها على جينات SHV وCTM-X المسؤولة عن مقاومة هذه البكتيريا للمضادات الحيوية) مجموعة البيتا لاكتام، في حين لا تحتوي على جين TEM. كما تم إجراء اختبار الحساسية للمضادات الحيوية على هذه العزلات الستة من بكتيريا الكليبسيلا الرئوية وأظهرت النتائج مقاومة معظم المضادات الحيوية المستخدمة في هذه الدراسة. نستنتج من ذلك أن بكتيريا الكليبسيلا الرئوية كانت أحد الأسباب الرئيسية لالتهابات الجهاز التنفسي ولها خاصية المقاومة الوراثية للمضادات الحيوية بيتا لاكتام.