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Antimicrobial Resistance and Molecular detection of Class 1 and 2 Integrons in *Klebsiella pneumoniae* Isolated from Human Urine and Bovine Milk Samples

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Abstract

This study aimed to isolate, identify, and compare *Klebsiella pneumoniae* from cow milk and human urine samples, and to evaluate their antimicrobial resistance patterns and the presence of integron genes associated with multidrug resistance. A total of 200 samples were examined, including 100 milk samples from cows and 100 human urine samples. Following culturing on selective and differential media, *Klebsiella spp*. were successfully isolated. On MacConkey and Eosin Methylene Blue (EMB) agar, 25% and 30% of suspected isolates were obtained from milk and urine samples, respectively. On Klebsiella Chrome agar, 48% of milk isolates and 46.66% of urine isolates were confirmed as *Klebsiella pneumoniae* based on characteristic blue mucoid colonies. For molecular identification, a total of 24 isolates (12 from milk and 12 from urine) were subjected to conventional PCR targeting the rpoB gene. All selected isolates (100%) were confirmed as *K. pneumoniae*, producing a PCR product size of 108 bp. Antimicrobial susceptibility testing using the Kirby–Bauer disk diffusion method revealed varying levels of resistance to five classes of antibiotics among all isolates. Similar resistance patterns were observed against penicillins, tetracyclines, and aminoglycosides, while urine isolates appeared to exhibit higher resistance rates to carbapenems and ciprofloxacin. All isolates were identified as multidrug-resistant (MDR), showing resistance to more than three classes of antibiotics. Detection of integron genes demonstrated that class 1 integron (int1) was present in 100% of all isolates, producing a PCR product size of 280 bp, whereas class 2 integron (int2) was detected in 75% of milk isolates and 33.33% of urine isolates, with a PCR product size of 233 bp.In conclusion, the findings highlight the widespread presence of multidrug-resistant Klebsiella pneumoniae in both animal and human sources, with integrons potentially contributing to the dissemination of antimicrobial resistance.

Keywords: *Klebsiella pneumoniae* , antimicrobial resistance , integrons

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Introduction

A Gram-negative bacterium, *Klebsiella pneumoniae*, can be found in various environmental niches, such as water, soil, plants, and mucosal surfaces of animals and humans (1). A notable increase in morbidity and mortality has been noticed in recent years, associated with *K. pneumoniae* infection, which has a significant negative impact on livestock, poultry, and wildlife (2). Among animals, K. pneumoniae infections manifest in various ways, such as pneumonia, mastitis, and septicemia in swine, and pneumonia and upper respiratory infections in dairy cows. Hospitalized patients are at an increased risk of infection caused by *K. pneumoniae* (4). Antibiotics are widely used without restriction in agricultural practices, animal husbandry, and human infection prevention, which has led to multidrug resistance in bacteria. Therefore, it is essential to examine environmental bacterial reservoirs in a comprehensive manner to better understand the mechanisms behind the development and dissemination of antimicrobial resistance. Although antimicrobial resistance occurs naturally, the recent increase in multidrug-resistant strains is largely driven by selective pressures resulting from the excessive and unregulated use of antibiotics (8,9). Under such pressure, bacteria can undergo spontaneous genetic mutations within their chromosomes, enabling some offspring to survive, or they may obtain resistance genes from their surroundings via mobile genetic elements (10).Proteins with different modes of action are translated from the open-reading frame of acquired antimicrobial resistance genes (ARGs). Antibiotics are eliminated or directly altered by enzymes such as beta-lactamases and chloramphenicol acetyltransferases. In certain situations, the antibiotic's target site may be changed to stop the medication from effectively binding. The translation of some open reading frames may reduce the drug-permeability of the cell membrane. Others are described as efflux pumps that forcefully remove the medication from the cell (11). Mobile genetic elements spread these ARGs among bacteria in the environment. Therefore, DNA components, including integrons, transposons, and plasmids, play a significant role in the dissemination of ARGs(12).Multi-drug resistant (MDR) isolates *of K. pneumoniae* are becoming more common in clinical and nosocomial settings, and they pose a serious risk to the management of nosocomial infections (13). Antibiotic resistance in bacterial strains develops and spreads due to a variety of processes and conditions. Among these, the primary cause of the widespread prevalence of antimicrobial resistance is thought to be the acquisition of resistance genes, particularly through mobile genetic elements (14). The spread of these MDR strains is thought to be facilitated by integrons, unique mobile genomic elements (15).

Materials and methods

Sample Collection (Cattle Samples):

One hundred milk samples (10–20 mL) were collected from the Veterinary Hospital in Basrah Province between January 2025 and December 2025 using sterile containers. The samples were

transported as soon as possible in a cooling box to the Microbiology Laboratory at the College of Veterinary Medicine, University of Basrah.

Human Samples

One hundred urine samples (10–20 mL) were collected from Al-Sadr Teaching Hospital and Basrah Republican Hospital between January 2025 and December 2025 using sterile containers. The samples were transported promptly in a cooling box to the Microbiology Laboratory at the College of Veterinary Medicine, University of Basrah.

Isolation and identification of Bacteria

A small amount of the sample is streaked onto selective and differential agar plates, such as MacConkey agar, Eosin Methylene Blue (EMB) agar, and *Klebsiella* Blue Chrome agar, which aid in the isolation and differentiation of enteric bacteria, particularly *Klebsiella* species.The inoculated agar plates are then incubated at the appropriate temperature (usually 37°C) to allow bacterial growth. The plates are monitored regularly for the appearance of colonies.After incubation, bacterial colonies exhibiting different morphologies (size, shape, color, and texture) were observed. On *Klebsiella* Blue Chrome agar, *Klebsiella* species typically produce characteristic-colored colonies, facilitating their identification among other enteric bacteria.

Molecular diagnosis of *Klebsiella pneumoniae*

The boiling method was used to extract DNA from bacteria in the stored broth. Traditional PCR was done to determine the presence of the rpoB gene, the 2 nd subunit of RNA polymerase. To amplify the rpoB gene, a pair of primers were utilized with the following sequences (F: 5′-R : CAACGGTGTGGTTACTGACG-3): and (R: 5′-TCTACGAAGTGGCCGTTTTC-3): The PCR reaction mixture was comprised of 12.5 µl of hot-start premix, 1 µl of each primer (10 pmol) and 4 µl of template DNA. Nuclease-free water was added to bring the total volume up to 25 µl. The amplification was performed on the basis of a PCR system, which included an initial denaturation stage of 95 o C over 5 minutes, 35 cycles of 94 o C over 1 minute, 55 o C over 1 minute and 72 o C over 1 minute. One more extension with 72 °C was conducted for 5 minutes (16). The agarose gel electrophoresis (2%) was used to verify the size of the amplified products with RedSafe DNA staining solution.

Antimicrobial susceptibility testing — multi-drug resistance assessment

All presumptive purified *Klebsiella pneumoniae* were tested for their susceptibilities to the selected antibiotics by the standard Kirby-Bauer disc diffusion method as described in the Clinical and Laboratory Standards Institute (CLSI) guidelines. The bacterial isolates were screened for their antibiotic resistance against 5 antibiotics of 5 different classes, as shown in table 1.

Table (1) types of antibiotics and its classes

Class of antibiotic	antibiotic	Symbol	Concentration
Carbapenems	Imipenem	IPM	10 µg
cephalosporins	Ceftriaxone	CRO	30 µg
tetracyclines	tetracycline	TE	30 µg
Aminoglycosides	gentamicin	CN	10 µg
fluoroquinolones	Ciprofloxacin	CIP	5 µg

Molecular Detection of Class 1 and Class 2 Integron Genes in Multidrug-Resistant *Klebsiella pneumoniae*

To detect integron genes (*intI1* and *intI2*), conventional polymerase chain reaction (PCR) was performed using the primers listed in Table 2. PCR amplification was carried out in a thermocycler under the following conditions: initial denaturation at 94°C for 4 min, followed by 35 cycles of denaturation at 94°C for 40 s, annealing at 58°C for *intI1* and 51°C for *intI2* for 30 s, and extension at 72°C for 40 s. A final extension step was performed at 72°C for 5 min. The amplified PCR products were separated by electrophoresis on 1.5% agarose gel stained with 0.5 µg/ml ethidium bromide and visualized under ultraviolet (UV) illumination.

Table 2 primers for detection of Integrons *intI1*, *intI2*

Target Gene	Primer Sequence (5′→3′)	Expected Size (bp)	Reference
intI1	**F: CCTCCCGCACGATGATCR: TCCACGCATCGTCAGGC**	**280**	**17**
intI2	**F: TTATTGCTGGGATTAGGCR: ACGGCTACCCTCTGTTATC**	**233**	**18**

Results

A total of 200 samples were examined in this study, including 100 milk samples and 100 human urine samples. After culturing on selective and differential media, bacterial growth of *the Klebsiella* species was observed. On MacConkey agar, suspected *Klebsiella* isolates appeared as large, smooth, moist, mucoid colonies with a pink color. The mucoid appearance was prominent due to capsule production. From the human urine samples, 30 suspected isolates were obtained, while 25 suspected isolates were recovered from milk samples based on colony morphology.

On Eosin Methylene Blue (EMB) agar, the suspected isolates produced large, mucoid, pink to purple colonies without a metallic green sheen, differentiating them *from Escherichia coli*. The number of suspected isolates was consistent with those observed on MacConkey agar (30from human samples and 25 from milk samples). On Klebsiella Blue Chrome agar (chromogenic medium), a total of 12and 14 confirmed isolates exhibited characteristic blue to metallic blue mucoid colonies, which are typical of *Klebsiella pneumoniae*. The colonies were smooth, convex, glistening, and highly mucoid, clearly distinguishable from other enteric bacteria present on the medium. The chromogenic reaction facilitated accurate differentiation and confirmation of *Klebsiella* isolates fig (1) table (3) .

Table (3) Numbers and percentages of animal, human and environmental that showed growth on MacConkey agar ,Eosin methylene blue agar

Sample Type	Agar Type	Growth (Number)	Growth (%)
Milk samples	MacConkey agar	25 /100	25%
	Eosin-methylene blue	25 /100	25%
	Klebsiella Chrome agar	12/25	48%
Human urine samples	MacConkey agar	30/100	30%
	Eosin-methylene blue	30 /100	30%
	Klebsiella Chrome agar	14/30	46.66%

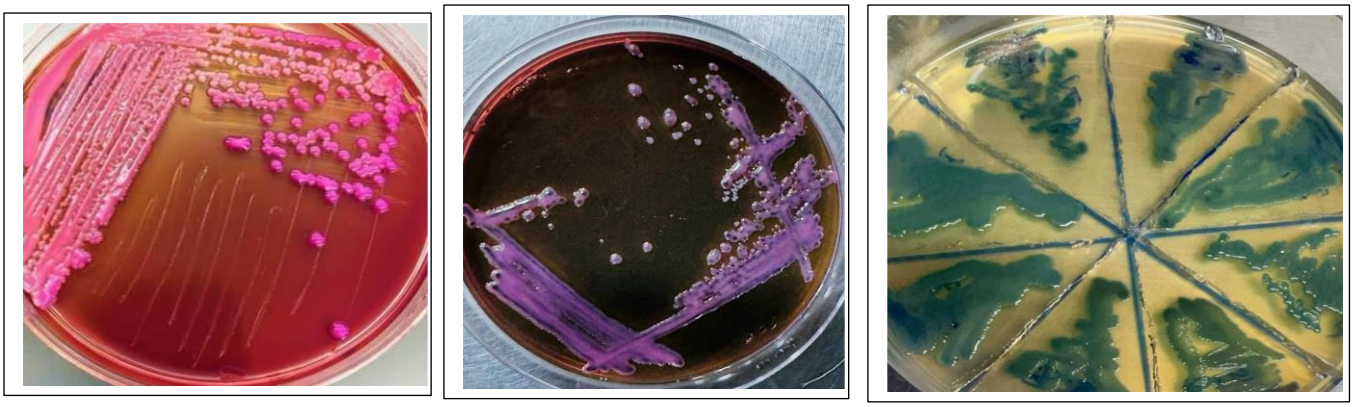


Fig (1) Growth *Klebsiella* isolates on MacConkey agar , Eosin Methylene Blue and Klebsiella Blue Chrome agar

The suspected bacterial isolates were identified by using a conventional PCR assay for the presence of the rpoB gene. 12 isolates were selected from each samples . All the isolates (100%) showed positive results. The size of the rpoB gene band was 108 bp, and the gene was represented by a single band in the corresponding region of the DNA ladder (fig 2).

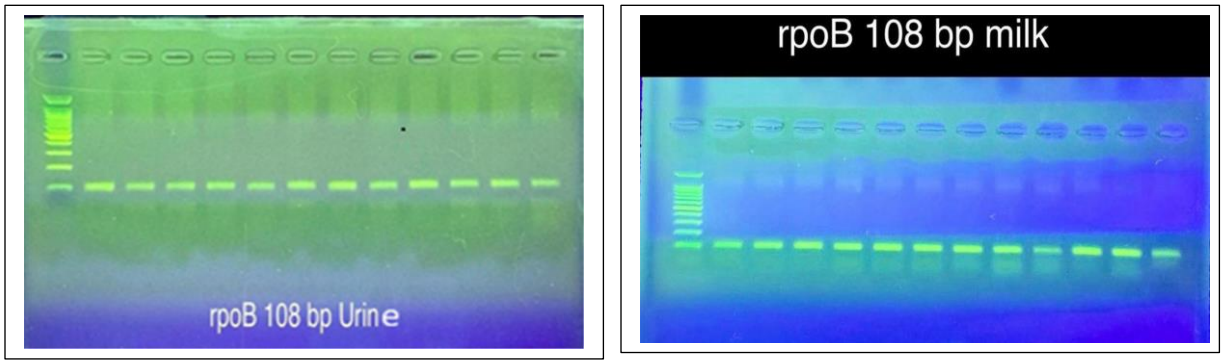


Figure 2: PCR amplification of rpoB gene (108bp) in *Klebsiella pneumoniae* isolates. M: DNA ladder (100–1500 bp).

Antimicrobial susceptibility testing — multi-drug resistance assessment:

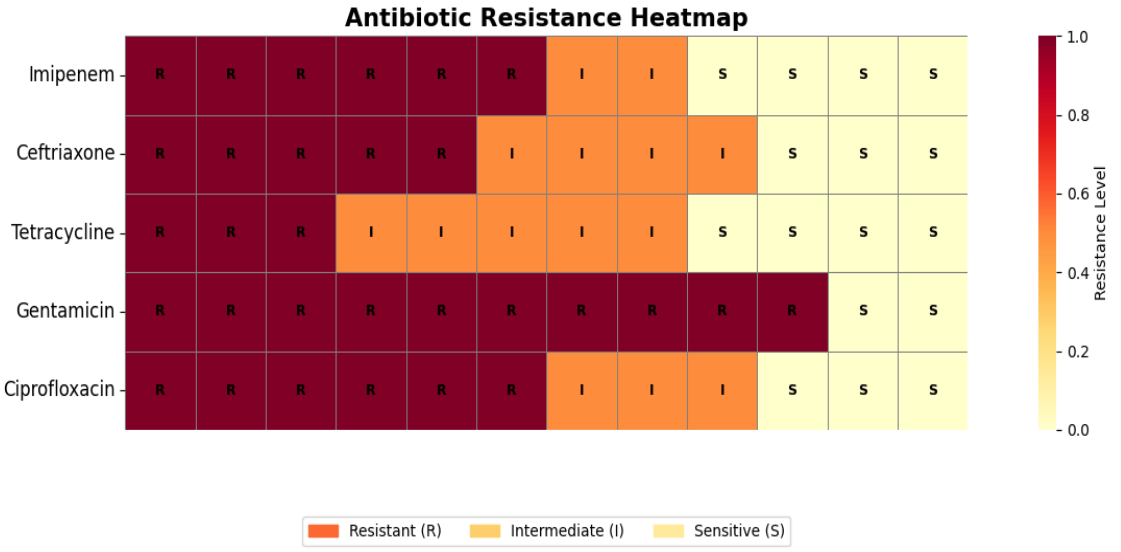
A total of 24 *Klebsiella pneumoniae* (12 from milk , 12 from human urine) were screened for A total of 24 *Klebsiella pneumoniae* isolates (12 from milk samples and 12 from human urine samples) were examined for antimicrobial susceptibility against five antibiotics representing five different antibiotic classes using the Kirby–Bauer disk diffusion method. All isolates exhibited varying levels of resistance to the tested antibiotics, as shown in Tables 4 and 5 and Figures 2 and 3. Isolates recovered from milk and human urine samples demonstrated relatively similar resistance patterns against cephlosporines , tetracyclines, and aminoglycosides. However, human urine isolates appeared to show higher resistance rates to carbapenems and folate pathway inhibitors compared with milk isolates. The findings indicated that all *K. pneumoniae* isolates from both sources were multidrug-resistant (MDR), exhibiting resistance to more than three classes of antibiotics.

Table (4) Milk sample and multidrug resistance

Class of antibiotic	antibiotic	Resistant			intermediate			sensitivities	
Carbapenemases	Imipenem	6	50 %	<12	2	16.66%	11-15	4	33.33%	>16
Cephalosporins	Ceftriaxone	5	41.66%	≤19	4	33.33%	22-20	3	٢٥%	≥23
tetracyclines	tetracycline	3	25%	<11	5	41.66%	12-14	4	33.33%	>15
Aminoglycosides	gentamicin	10	83.33%	≤12	0	0%	12-14	2	16.66%	>15
dihydrofolate	Ciprofloxacin	6	50%	<10	3	25%	11-15	3	25%	>16

Table (5) urine sample and multidrug resistance

Class of antibiotic	antibiotic	Resistant			intermediate			sensitivities		
Carbapenemases	Imipenem	7	58.33%	<12	2	16.66%	11-15	3	%۲۵	>16
Cephalosporins	Ceftriaxone	6	۵۰%	≤19	2	0%	22-20	4	33.33%	≥23
tetracyclines	tetracycline	٤	33.33%	<11	2	16.66%	12-14	6	%۵۰	>15
Aminoglycosides	gentamicin	12	100%	≤12	0	0%	12-14	0	0%	>15
dihydrofolate	Ciprofloxacin	9	75%	<10	0	0%	11-15	3	25%	>16



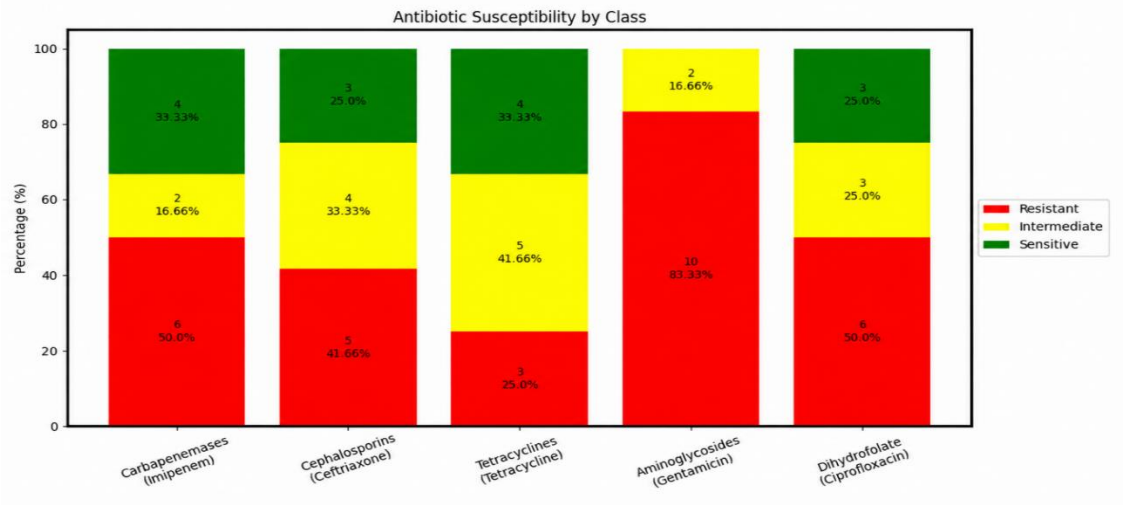
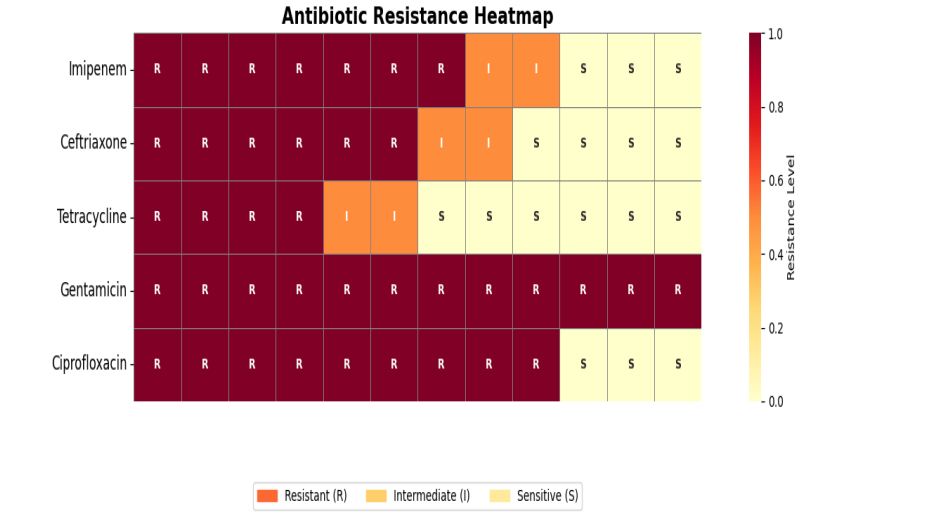


Fig (3) Milk sample and multidrug resistance percentage



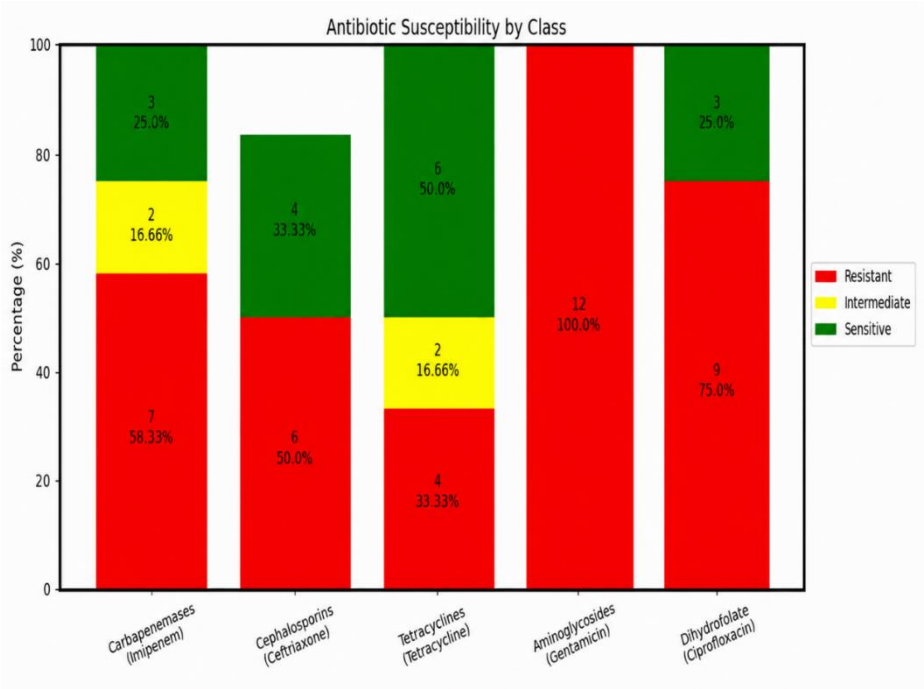


Fig (4) Urine sample and multidrug resistance percentage

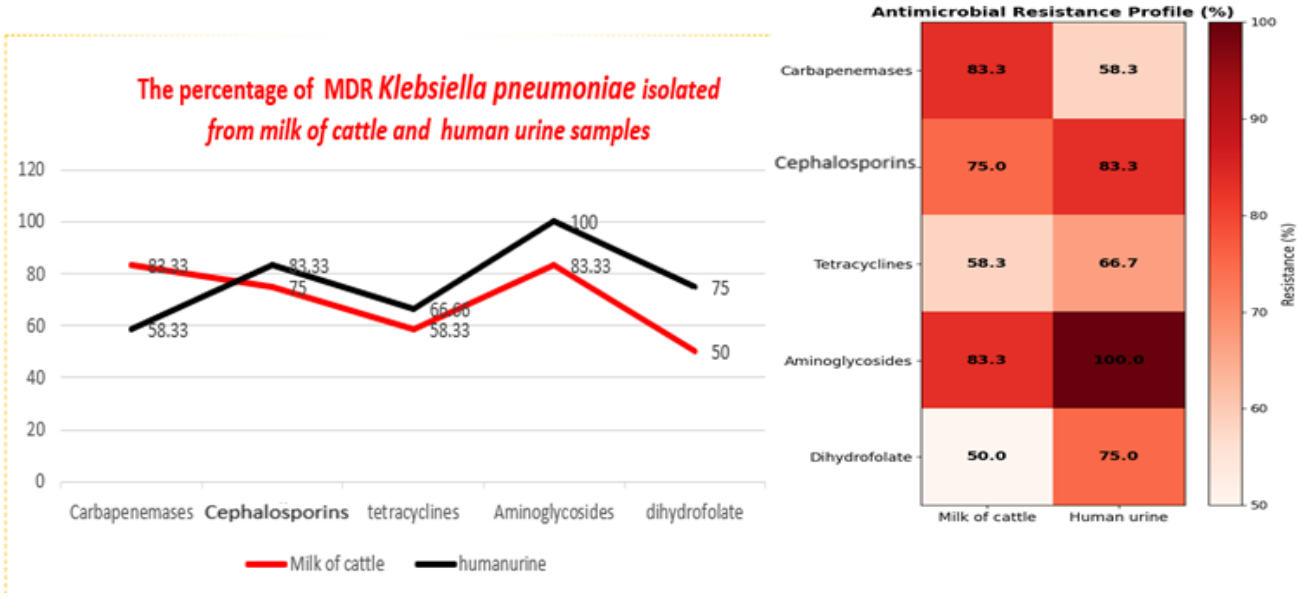


Figure 5: The correlation of MDR *Klebsiella pneumoniae* isolated frommilk of cattle and humans urine samples with 5 antimicrobial agents.

Detection of class1.class2 integron- gene for multidrug resistance *Klebsiella pneumoniae* by conventional PCR.

The results of detecting integrons in *Klebsiella pneumoniae* isolates from cow milk and human urine samples showed variation in the presence of integron genes. Class 1 integron was detected in 100% of all isolates, whereas Class 2 integron showed variability among the isolates, as illustrated in Fig(6, 7).

Discussion

Klebsiella pneumoniae is considered one of the most important opportunistic pathogens associated with animals and is commonly linked to mastitis and respiratory tract infections. Urinary tract infections (UTIs) are also among the most common and severe bacterial infections worldwide. Although UTIs are generally manageable, increasing antimicrobial resistance among uropathogens, particularly members of the Enterobacteriaceae family, has complicated treatment strategies (19). Several risk factors predispose individuals to *K. pneumoniae* infections, including prolonged hospitalization and invasive medical procedures. *K. pneumoniae* is a non-motile, Gram-negative, rod-shaped bacterium characterized by a prominent polysaccharide capsule (20).

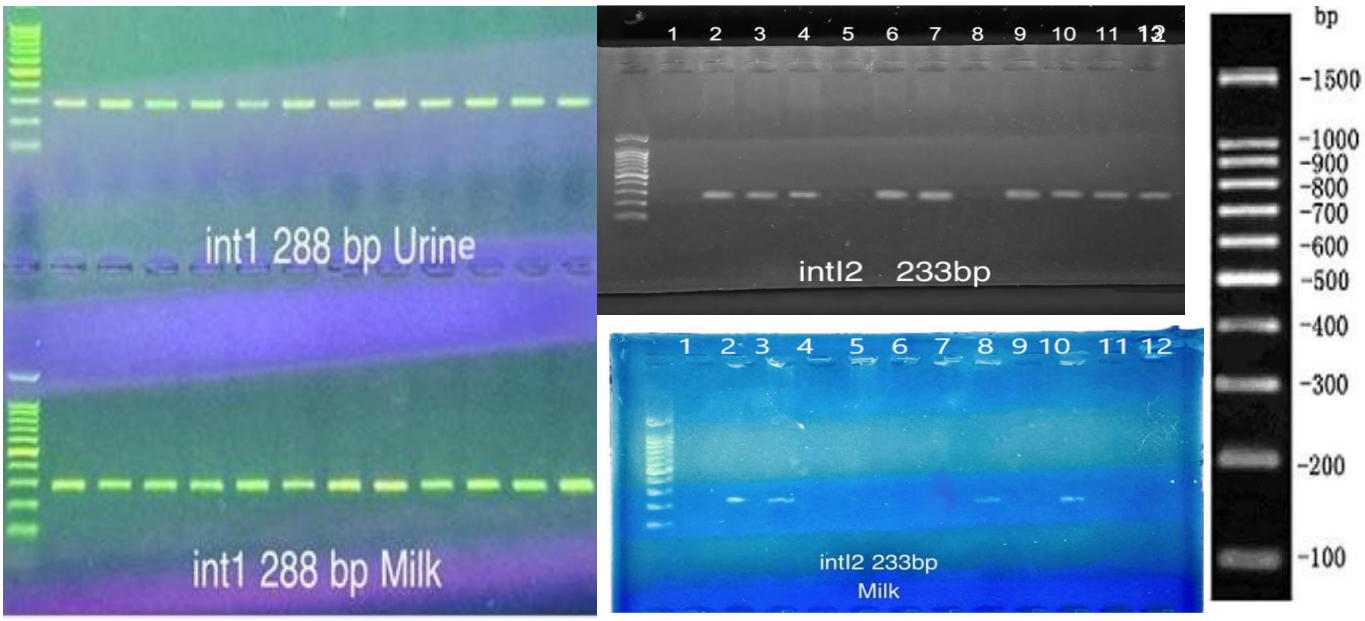


Fig (6) .PCR amplification of int1 (288 bp) and int2 (233 bp) genes in *Klebsiella pneumoniae* isolates. M: DNA ladder (100–1500 bp).

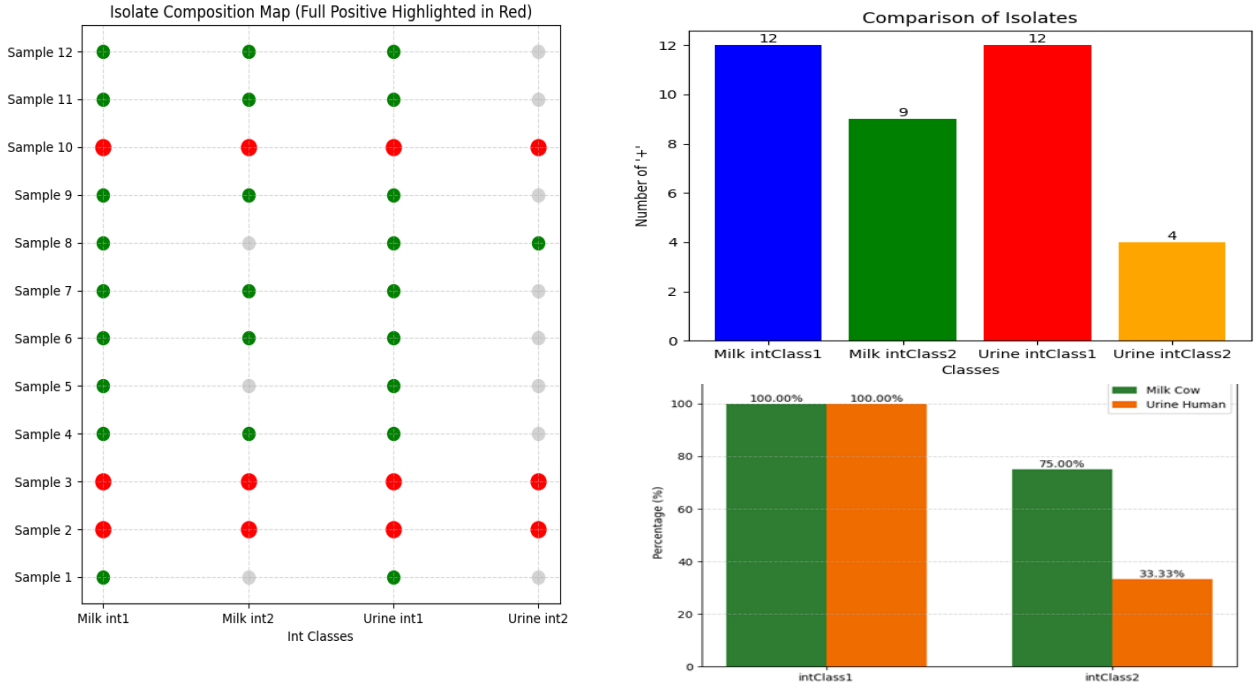


Fig (7) comparison het maps of the distribution of int1 and int2 gene among milk cow and urine human samples

In the present study, a total of 200 samples were examined, including 100 milk samples collected from cows with mastitis and 100 human urine samples. Following culturing on selective and differential media, bacterial growth consistent with *Klebsiella* species was observed. The findings demonstrated that 25% of isolates from both milk and urine samples were suspected *Klebsiella* spp. on MacConkey and EMB agar. On Klebsiella Chrome agar, characteristic blue to metallic blue mucoid colonies were observed in 48% and 46.66% of isolates from milk and urine samples, respectively.

All suspected isolates were further confirmed using conventional PCR targeting the *rpoB* gene. A total of 24 isolates (12 from milk samples and 12 from urine samples) were selected for molecular analysis, and all isolates (100%) yielded positive results. Similar findings have been reported in several studies investigating the molecular epidemiology of *K. pneumoniae*. For example, 104 strains were isolated from 1,063 mastitis samples (9.78%) collected during 2017–2018 (21). Similarly, in Hubei Province, China, 239 isolates (26.94%) were recovered during 2019–2020 (22). Another study conducted on two large dairy farms in China reported 365 isolates from 1,354 samples (26.96%) in 2020 (23). In addition, a study investigating bloodstream infections caused by *K. pneumoniae* in Saudi Arabia reported that adults (66.4%) were more susceptible to infection than pediatric patients (33.6%), suggesting that age may represent an important risk factor (24).

A total of 24 *K. pneumoniae* isolates (12 from milk samples and 12 from human urine samples) were screened for antimicrobial susceptibility against five antibiotics representing five different antibiotic classes using the Kirby–Bauer disk diffusion method. Variable levels of resistance were observed among all isolates. Milk and urine isolates demonstrated relatively similar resistance profiles against penicillins, tetracyclines, and aminoglycosides. However, urine isolates appeared to exhibit higher resistance rates to carbapenems and folate pathway inhibitors compared with milk isolates. The results demonstrated that all *K. pneumoniae* isolates from both sources were multidrug-resistant (MDR), exhibiting resistance to more than three antibiotic classes. Similar findings were reported by (25), where *K. pneumoniae* isolates recovered from mastitis exhibited variable resistance to cefotaxime, ceftiofur, cephalexin, sulfamethoxazole, trimethoprim/sulfamethoxazole, amikacin, gentamicin, streptomycin, ceftazidime, meropenem, florfenicol, and tetracycline.

Integrons may contribute to the dissemination of antimicrobial resistance genes in *K. pneumoniae*. Among these genetic elements, class 1 integrons (*intI1*) are recognized as the most prevalent and are frequently associated with MDR strains. Integrons are capable of capturing and expressing gene cassettes encoding resistance to several antibiotic classes, including β-lactams, aminoglycosides, and sulfonamides. Class 2 integrons (*intI2*), although detected less frequently, also play a role in the dissemination of resistance determinants. These integrons are commonly associated with transposon Tn7 and generally contain a conserved set of gene cassettes, which may

limit their diversity compared with class 1 integrons. Nevertheless, their presence remains important in horizontal gene transfer among bacterial populations (26). The detection of integron genes in *K. pneumoniae* isolates obtained from cow milk and human urine samples revealed variation in integron prevalence. Class 1 integrons were detected in 100% of all isolates, whereas class 2 integrons showed variable distribution among isolates.

Based on Figure 7, similarities and differences were observed between milk and urine isolates across different samples. Some samples demonstrated consistent results between milk and urine isolates, where all tested markers were positive or negative, suggesting the possibility of shared bacterial origins or closely related strains. Samples 2, 3, and 10 exhibited fully positive profiles across all tested markers, indicating that similar or potentially identical bacterial strains may have been present in both milk and urine samples. This finding may reflect possible transmission pathways or common sources of infection. In contrast, samples 1 and 5 demonstrated variable profiles, with certain markers detected in one source but absent in the other. Such variation may reflect differences in microbial composition or genetic characteristics between isolates. Additionally, urine isolates appeared to exhibit more consistent profiles in certain cases, whereas milk isolates showed greater variability. These differences may be related to variations in the biological environments of the udder and urinary tract, which could influence bacterial behavior and adaptation. Overall, the findings suggest a partial relationship between milk and urine isolates, while also highlighting microbial diversity and environmental influences on bacterial characteristics. A previous study (27) reported that most MDR *K. pneumoniae* isolates recovered from different samples were positive for class 1 integrons, whereas the presence of class 2 integrons was significantly associated with resistance to multiple antibiotics ($P < 0.01$). Class 2 integrons are generally considered less common among Gram-negative bacteria. Several studies have also demonstrated a strong association between the presence of class 1 integrons and MDR phenotypes in Gram-negative organisms. Similarly, another study (28) found that antimicrobial resistance rates were significantly higher among integron-positive isolates compared with integron-negative isolates. Other reports have also documented a high prevalence of integron-positive *K. pneumoniae*. The widespread occurrence of integrons among MDR strains may be attributed to the selective advantage conferred by these genetic elements, particularly in environments with extensive antibiotic exposure, such as hospitals (29, 30).

Bacterial populations may also be influenced by overlapping antibiotic usage patterns or transmission through the food chain. These findings support the concept of the “One Health” approach, which emphasizes the interconnected relationship between human, animal, and environmental health in the spread of antimicrobial resistance.

Conclusion

This study confirmed the presence of multidrug-resistant (MDR) *Klebsiella pneumoniae* in both bovine mastitis milk and human urine samples. All isolates were successfully identified by PCR and showed high levels of antimicrobial resistance. Class 1 integrons were detected in all isolates, indicating their major role in the dissemination of resistance genes. These findings highlight the importance of the One Health approach in controlling the spread of antimicrobial resistance between animals and humans.

Conflicts of interest

The authors declare that there is no conflict of interest**.**

Ethical Clearance

This work is approved by The Research Ethical Committee

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أنماط مقاومة مضادات الميكروبات والتوصيف الجزيئي للإنتجرونات من الصنف الأول والصنف الثاني، وجينات الإنتيغريز في بكتيريا *Klebsiella pneumoniae* المعزولة من عينات سريرية بشرية وبيطرية.

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الخلاصة

هدفت هذه الدراسة إلى عزل وتشخيص ومقارنة بكتيريا *Klebsiella pneumoniae* هدفت هذه الدراسة إلى عزل وتشخيص ومقارنة بكتيريا *Klebsiella pneumoniae* من عينات حليب الأبقار وعينات الادرار في الإنسان، وتقييم أنماط مقاومتها للمضادات الحيوية والكشف عن جينات الإنتيغرون المرتبطة بتعدد المقاومة الدوائية. تم فحص ما مجموعه 200 عينة، تضمنت 100 عينة حليب و100 عينة ادرار بشري، وبعد الزرع على الأوساط الانتقائية والتفريقية تم عزل أنواع *Klebsiella*. أظهرت النتائج أن نسبة 25% و30% من العزلات المشتبه بها تم الحصول عليها من عينات الحليب والادرار على التوالي باستخدام أوساط MacConkey وEMB، بينما تم تأكيد 48% من عزلات الحليب و46.66% من عزلات الادرار على وسط Chrome agar *Klebsiella* اعتمادًا على المستعمرات الزرقاء المخاطية المميزة. تم تأكيد التشخيص الجزيئي باستخدام تقنية PCR التقليدية باستهداف جين **rpoB**، حيث أظهرت جميع العزلات المختارة (100%) نتائج إيجابية بحجم 108 زوج قاعدي. أظهرت نتائج اختبار الحساسية للمضادات الحيوية بطريقة Kirby-Bauer أن جميع العزلات تمتلك مستويات متفاوتة من المقاومة لخمس فئات من المضادات الحيوية، حيث أظهرت عزلات الحليب والادرار أنماط مقاومة متشابهة نسبيًا تجاه السيفالوسبورينات والتتراسايكلينات والأمينوغلايكوسيدات، في حين لوحظت مستويات مقاومة أعلى في عزلات الادرار خاصة تجاه الكاربابينيمات والسيبروفلوكساسين، كما تبين أن جميع العزلات متعددة المقاومة (MDR) حيث أظهرت مقاومة لأكثر من ثلاث فئات من المضادات الحيوية. كما أظهرت نتائج الكشف عن جينات الإنتيغرون أن الإنتيغرون من الصنف الأول (int1) كان موجودًا في 100% من جميع العزلات، في حين أظهر الإنتيغرون من الصنف الثاني (int2) تباينًا في نسبة الانتشار حيث تم الكشف عنه في 75% من عزلات الحليب و33.33% من عزلات الادرار . وتُبرز هذه النتائج الانتشار الواسع لبكتيريا *Klebsiella pneumoniae* متعددة المقاومة في المصادر الحيوانية والبشرية، مع الدور المهم للإنتيغرونات في انتشار مقاومة المضادات الحيوية.

الكلمات المفتاحية: *Klebsiella* pneumoniae ,مقاومة مضادات الميكروبات, integrons.