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Colistin Resistant and Biofilm Formation among Multi-Drug Resistant *Klebsiella pneumoniae* Isolated from Different Clinical Samples

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Abstract: Carbapenem-resistant *Klebsiella pneumoniae* has listed among the priority pathogen group as it poses great threat to human health. Colistin resistance is considered a serious problem. Thus, in this study aimed to investigate the colistin resistance among carbapenem resistant *K. pneumoniae* in a sample from Iraqi patients. A total of 182 clinical samples (including urine, pus, blood, and ear swabs) were gathered from various clinical specimens. The result showed 124 (68.13%) isolates of *Klebsiella pneumoniae* were found out of the total samples, they were identified using morphological and cultural characteristics and the VITEK 2 system. *K. pneumoniae* isolates from Urine samples showed highest percentage (69.4%). Isolates determined in female samples was 79.8% which is higher than isolates in male samples (47.5%). The disc-diffusion method and VITEK 2 system were used to determine *K. pneumonia's* antibiotic resistance to the list of antibiotics in addition to Colistin and Imipenem. The results indicate that all isolates are completely resist to Ampicillin, Amoxicillin-Clavulanic Acid, Meropenem, Norfloxacin, and Cefotaxime, while some isolates displayed high resistance to Cefazolin, Ceftazidim, Ceftriaxone, and Cefepime, moderate resistance to Piperacillin-Tazobactam, Gentamicin, Cefoxitin, and Nitrofurantoin and demonstrate low resistance to Amikacin, Imipenem, Ciprofloxacin, Levofloxacin, Tigecycline, Tirmethoprim Sulfamethoxazole and Ertapenem (7.7%), while all isolates exhibit susceptibility to Fosfomycin, out of them, the Colistin resistance isolates about 31 (25%), and 93 (75%) were sensitive. Due to Colistin's low agar diffusion, 12 isolates that demonstrated Colistin sensitivity by disk diffusion were determined to be resistant by E-test. Additionally, isolates were more common 39 (73.5%) among strong biofilm formers, while 9 (16.9%) isolates were moderate and only 5 (9.4%) were week biofilm formers (*p-value* $\geq$ 0.05). Colistin resistant *K. pneumoniae* showed in strong biofilm isolates 24 (45.28%) and moderate biofilm isolates 2 (3.77%) but the weak biofilm formation isolates were only in 3 (5.66%).

Keywords: Antibiotic Resistance, Biofilm, Colistin, Carbapenem, *K. pneumoniae*

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Introduction

Numerous illnesses, including pneumonia, sepsis, meningitis, soft tissue infections, diarrhea, urinary tract infections, ankylosing spondylitis, and other spondylo-arthropathies are caused by the genus *Klebsiella* (1). *K. pneumoniae* is the principal cause of nosocomial infections. The majority of infections are brought on by hospital environment contamination and colonization, including patient and staff skin, sinks, carpeting, and medical equipment. Through the use of ventilators, intravenous catheters, and other methods, patients are exposed to bacteria through the respiratory tract, blood stream, and urine tract. Direct transmission can occur when patients and healthcare professionals' hands are

contaminated. The fact that *K. pneumoniae* cannot spread through the air is the most significant finding. A significant hospital-acquired infection, particularly in intensive care units, is drug resistant *K. pneumoniae* (2).

The polysaccharide capsule, which is one of numerous variables that contribute to *Klebsiella's* pathogenicity, is the most dangerous one. The second element is the pili, which are filamentous protrusions on the bacterial surface. Another factor is lipopolysaccharides (LPS), which are made up of lipid A, core, and o-polysaccharide antigen and are necessary for the bacterium to withstand complement-mediated death. In *K. pneumoniae*, biofilm development is regarded as a key virulence component that contributes to pathogenicity (3, 4, 5).

The carbapenemase-producing *K. pneumoniae* (CpKp) strain, which is linked to hospital settings and infects vulnerable people, has been widely dispersed in recent years. The hypervirulent *K. pneumoniae* (hvKp) strain, which was first identified in the Asian Pacific Rim and produces metastatic diseases such endophthalmitis, meningitis, and septic arthritis in diabetics and immunocompetent people, does not appear to be related to the CpKp strains (opportunistic infections) (6, 7).

Since they have increased *K. pneumonia's* enzymatic and nonenzymatic resistance to the majority of current antibiotics, the CPKp isolates are thought to be a significant public health issue that could lead to severe infections and high mortality rates (Beta-lactams, Carbapenems, Fluoroquinolones, and Aminoglycosides). Hospitals all throughout the world are experiencing a rapid spread of this strain, which restricts treatment options. The preferred medication for the treatment of infections that pose a serious threat to life is carbapenem (8, 9). There are fewchoices for treating these drug-resistant bacteria due to the rise in Carbapenem resistance among *Enterobacteriaceae*. Aminoglycosides, tigecycline, Fosfomycin, and colistin are used in the treatment of Carbapenem-resistant Enterobacteriaceae (10).

Colistin is frequently used to treat infections caused by CPKp. However, a new strain of *K. pneumoniae* that is resistant to Colistin (CoRKp) has been identified and is posing a serious threat to public health. In particular, Colistin has evolved into the final line of defence against infections brought on by the new MDR Gram negative bacteria. Colistin works primarily against gram-negative bacteria. By binding anionic lipopolysaccharide molecules and displacing calcium and magnesium, it alters the permeability of the cell wall, leading to cell leakage and death (11).

Materials and methods

Collection of the samples

The clinical samples were collected during the period from November 2021 until March 2022. The samples consisted of urine, blood, swab (burns and ear) and pus (abdomen mucoid) from Medical city (Baghdad Teaching hospital and The National Center for Teaching laboratories).

Bacterial isolation

Different swabs (burns and ear) were used as clinical specimens. They were carefully removed from the infection site and placed in tubes with ready-made medium to keep the swab moist while being transported to the lab. Clean-catch midstream urine, which is

highly concentrated and would offer precise colony counts, was collected for urine samples in the morning. A sterile vial was used to collect the blood samples. For the primary selection of *Klebsiella spp*., each specimen was cultured on the surface of MacConky agar by streaking a loopful of culture from brain heart infusion broth. The plates were incubated for 18–24 hours at 37 °C.

Identification of *Klebsiella* spp.

Colony morphology was used to identify potential isolates. All isolates were recognized mostly based on the general culture feature of the colony on MaCconky agar and CHROM agar (color, shape, texture, and size).

Antimicrobial susceptibility testing
Agar disk diffusion method

124 *Klebsiella* isolates were tested for antibiotic susceptibility using the single disk diffusion Kirby-Bauer method (12) (Colistin, and Imipenem) and the diameter of the inhibition zone was measured and compared to a chart given by the Clinical and Laboratory Standard Institute. Other antimicrobial agents were evaluated by the VITEK 2 system (13).

Colistin MIC strip testing for *K. pneumoniae* susceptibility (E-test)

The colistin MIC strip was used, and it was interpreted in accordance with the manufacturer's instructions (Liofilchem, Italy). The testing process made use of Mueller Hinton agar after incubation, read MIC at the point where ellipse intersects the scale. If a MIC value between two twofold dilutions is seen, always round up to the highest value. Read the MIC value at complete inhibition of all growth. If the intersect differs on either side of the strip, the MIC as the greater value was read. (14).

Biofilm formation (quantitative)

The microtiter plate assay was used to detect the production of biofilms in all isolates of *Klebsiella* spp. Adherence of Biofilm Formation= Mean of OD630 of bacterial samples (ODs) / Mean OD630 of control (ODc) where the classification of bacterial adherence (15, 16, 17).

Results and discussion
Samples Collection

In five months, a total of 182 clinical samples were gathered. The samples came from Medical City, chosen at random. 135 (74.17%) of the total samples were urine samples, 25 (13.73%) were blood samples, 15 (8.24%) were burns swabs, 4 (2.19%) were ear swabs, and 3 (pus abdomen mucoid) (1.64%). In comparison to the 60 samples collected from males, 122 samples (67.03%) were collected from females. (32.96%), as displayed in table-1. This study found that a significant portion of the isolates were found in urine samples, followed by blood. This is because, in current practice, urinary tract infections are a highly frequent basis for consultation and antibiotic prescription. Due to the fact that more women than men experience UTIs, female urine samples were significantly more numerous than male urine samples. This may be explained by the female urethra's short, broad, and close proximity to the anus(18).

Table (1): Gender distribution among collected samples

Sample Source	Gender		
	Male	Female	Total
Urine	32	103	135
Burn Swab	9	6	15
Blood	15	10	25
Ear Swab	2	2	4
Pus (abdomen mucoid)	2	1	3
Total	60	122	182

Distribution of *Klebsiella spp*. according to gender and age group

According to table 2, 124 (68.13%) *Klebsiella spp*. were isolated from distinct (182) samples and were recognized by colony morphology and the VITEK 2 system. The percentage of females with *K. pneumoniae* was higher than that of males, at 67.7% compared to 32.2% for males. This finding in agree with study conducted by Anil and Chandrika (2013) who presented nearly the same ratio. The percentage of urine samples that were greater than the other samples was 86 (69.3%), and the percentage in female urine samples was higher than that in male urine samples *(p-value* < 0.05). This was previously explained by the physical variations between male and female bodies (Dielubanza and Schaeffer, 2011). *K. pneumoniae* was discovered in a high percentage of 44 (35.4%) people between (50-69).

Table (2): Distribution of *K. pneumoniae* according to gender and its relationship with age group

Gender	Age group					
	10-29	30-49	50-69	70-89	> 9	Total
Male	8	10	18	3	1	40
Female	18	29	26	11	0	84
Total	26	39	44	14	1	124

Antimicrobial susceptibility of *K. pneumoniae*

Disc diffusion method

According to table-3, the 124 isolates were completely resistant (100%) to Ampicillin, Amoxicillin\Clavulanic Acid, Meropenem, Norfloxacin, and Cefotaxime while some isolates showed high resistance to Cefazolin (93.3%), Ceftazidim (93.3%), Ceftriaxone (90%) and Cefepime (90%) Moderate resistance to Piperacillin\Tazobactam (69.2%), Gentamicin (63.3%), Cefoxitin (63.3%), Nitrofurantoin (61.5%) and show low resistance to Amikacin (52%), Imipenem (51%), Ciprofloxacin (34.5%), Levofloxacin (31%), Tigecycline (3.8%), Tirmethoprim\ Sulfamethoxazole (46.7%), and Ertapenem (7.7%) and all isolates show sensitivity to Fosfomycin. , out of them, the Colistin resistance about 53 (42.7%), and 71 (57.2%) were sensitive, which is in disagreement with a study by Liu and colleagues 2021 who showed that Colistin resistance among Carbapenem-resistant *Klebsiella pneumoniae* was 4.5%.

Table (3): Antibiotic sensitivity of *Klebsiella pnemonaie* towards selected antibiotics

Antibiotic	Sensitive		Resistance	
	No.	%	No	%
Imipenem	61	49	63	50.8%
Ampicillin	0	0.00	124	100%
Piperacillin\Tazobactam	38	30.6%	86	69.3%
Cefazolin	9	7.25%	115	92.7%
Cefoxitin	46	37%	78	62.9%
Ceftazidim	9	7.25%	115	92.7%
Ceftriaxone	12	9.6%	112	90.3%
Cefepime	12	9.6%	112	90.3%
Amikacin	59	47.5%	65	52.4%
Gentamicin	46	37%	78	62.9%
Ciprofloxacin	81	65.3%	43	34.6%
Levofloxacin	86	69.3%	38	30.6%
Tigecycline	119	95.9%	5	4.03%
Nitrofurantoin	48	38.7%	76	61.2%
Tirmethoprim\Sulfamethoxazole	66	53.2%	58	46.7%
Ertapenem	114	91.9%	10	8.06%
Amoxicillin\Clavulanic Acid	0	0.00%	124	100%
Meropenem	0	0.00%	124	100%
Fosfomycin	124	100%	0	0.00%
Norfloxacin	0	0.00%	124	100%
Cefotaxime	0	0.00%	124	100%

MIC of Colistin by E-test

The E-test is used to test the colistin's *in vitro* susceptibility. Unlike some automatic approaches, the E-test is accurate at identifying resistant populations. The European Committee on Antimicrobial Susceptibility Testing (EUCAST) MIC breakpoints for colistin for 2 g/ml susceptible, >2 g/ml resistant serve as the basis for interpretation because the Clinical & Laboratory Standards Institute (CLSI) has not established breakpoints for Enterobacteriaceae to test colistin or polymyxin B (20).

E-test was used to determine the MIC for each of the 31 isolates that were subjected to single strip diffusion testing. The MIC of Colistin-resistant isolates was >2 µg/ml, according to the E-test strip, whereas the MIC of susceptible isolates was between 1-2 µg/ml (the range of the E-test strip was 0.016 to 256 µg/ml) figure-1. According to table (4-6), the disc diffusion test demonstrated high error when compared to the E-test, which was agree to a study by Galani *et al.*, (2008) that demonstrated significant error in the disk diffusion. Due to Colistin's low agar diffusion, 12 isolates that demonstrated Colistin sensitivity by disk diffusion were determined to be resistant by E-test. The inhibition values (12–13 mm) should be validated with MIC by E-test.

Table (4): Comparison between disk diffusion method and E-test strip to determine the resistance to colistin.

Method	N	Mean	Std. Deviation	Std. Error Mean
E- Test MIC	31	2.52	1.14	0.21
Colistin Disk Diameter	31	13.06	2.64	0.48
The t-value is -20.38119. The p-value is < 0.00001. The result is significant at p < 0.05.				

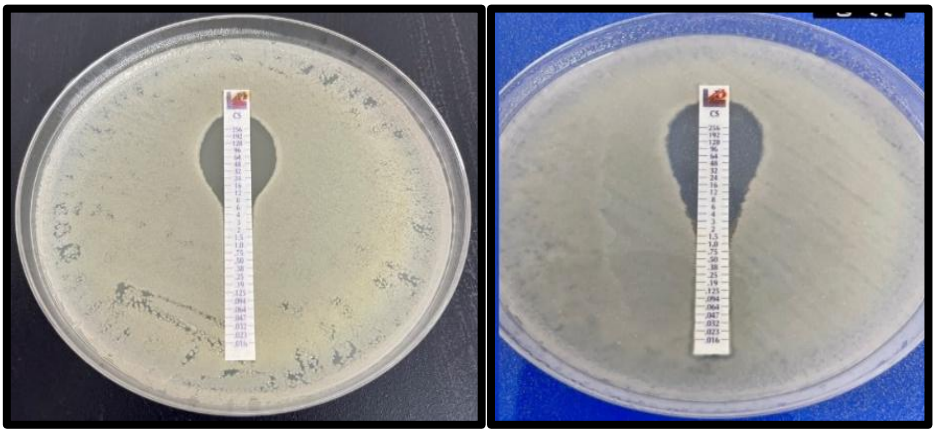

Figure (1): Colistin E test strip to determine MIC for *K. pneumoniae*. Left: Resist isolates. Right: Sensitive isolates.

Biofilm formation among colistin resistant *K. pneumoniae* isolates

The ability of *K. pneumoniae* to produce biofilms enhances the duration of infection by preventing host immune response. This study shows the relationship between biofilm development in connection with Colistin resistance table-5. Since it is unclear how MDR-resistant *K. pneumoniae* isolates produce biofilms, research into the connections between biofilm development and drug resistance is crucial for enhancing treatment. Multi-drug resistant isolates 39 (73.5%) were more common among strong while 9 (16.9%) were moderate and 5 (9.4%) week biofilm formers (*p-value* ≥ 0.05). Colistin resistant *K. pneumoniae* showed more common in strong biofilm isolates 24 (45.28%) moderate biofilm isolates in 2 (3.77%) but the Colistin resistant *K. pneumoniae* showed in weak biofilm formation isolates 3 (5.66%). This results in a *p-value* of 0.099 which is above the defined significance level of 5%. The Chi^2 test is therefore not significant and the null hypothesis is confirmed. These results were disagreement by Jaclyn and colleagues (2019) who showed that the multi-drug resistant isolates were more common among weak (97.9%) versus strong biofilm formers (76%; p = 0.002).

Table (5): The relationship between Biofilm formation and colistin susceptibility

Biofilm Formation	Colistin Susceptibility		
	S	R	Total
Strong	15	24	39
Moderate	7	2	9
Weak	2	3	5
Total	24	29	53

Conclusion

The isolates of *K. pneumoniae* were more prevalent in particular among younger age group (50-69), the number of *K. pneumoniae* isolates was higher in females than in males. Variable resistance was seen in *K. pneumonia's* susceptibility to the several antibiotics utilized in this study. Colistin resistance were shown in 31(25%) among 124 *K. pneumoniae* isolates. Additionally, compared to the E-test, the disc

diffusion test displayed a lower error (MIC strip). *K. pneumoniae* demonstrated more resistant isolates at strong biofilm formation and less resistant isolates at weak biofilm formation, respectively, in accordance with the ability of biofilm-formation and the emergence of antibiotic susceptibility.

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