Colistin Susceptibility in Carbapenem Resistant *Klebsiella Pneumoniae* and their Ability of Biofilm Formation

Sarab Murad Kadum[*]**, Dalal. S. Al Rubaeye**
Department of Biotechnology, College of Science, University of Baghdad**,** Baghdad**,** Iraq

Abstract

A total of 157 clinical samples were collected from different clinical specimens (urine, sputum, blood, swabs, and cannula) from several hospitals in Iraq. Among the samples, 51 isolates (32.48%) of *Klebsiella pneumoniae* were identified according to morphologicaland cultural characteristics as well as the Enterosystem 18R test. Higher numbers of *K. pneumoniae* isolates were observed in urine samples (26, 52%) than the other samples, and in females (70.6%) than males (29.4%) (female: male ratio of about 2.4:1). Antibiotic susceptibility of *K. pneumoniae* against 12 commonly used antibiotics was determined through the disc-diffusion method. The results revealed a higher resistance rate in 51 isolates (100%) against Cephalexin, followed by Ceftazidime (50, 98%), while the lowest resistance rate (24, 47%) was against each of Imipenem and Meropenem. Also, the investigation of the minimum inhibitory concentration (MIC) of Colistin using E-test (strips) demonstrated that 33 isolates were resistance, as compared to 31 using the disk diffusion assay. DNA was extracted from *K. pneumoniae* isolates and molecularly tested using polymerase chain technique (PCR) with a specific primer and 108 bp product to detect the *rpoB* gene that represents this bacteria . Also, all of the 51 isolates of *K. pneumoniae* identified by the *rpoB* gene were detected for the expression of the Colistin drug resistance gene *mgr-B* , which was amplified (347 bp) using a specific primer. Colistin resistance gene *mgr-B* was amplified and sequenced from the twenty isolates. Only 6 isolates appeared with a single nucleotide substitution; G instead A, A instead G, C instead G and G instead C. Also, this study tested biofilm formation from *K. pneumoniae isolates* , using the microtiter plate method, in association with Colistin and Carbapenem resistant. The Colistin and Carbapenem resistance pattern was compared to the ability of biofilm-formation as weak formation versus strong and also, Multi-drug resistant isolates were more common among weak versus strong biofilm formers.

Keywords: Carbapenem Resistant *Klebsiella pneumoniae*; Colistin; Biofilm formation

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الحساسية للكوليستين في عزلات الكليبسيلا الرئوية المقاومة للكاربابينيم وقدرتها على تشكيل الغشاء الحيوي

سراب مراد كاظم[*] **، دلال صالح الربيعي**
قسم التقنيات االحيائية، كلية العلوم، جامعة بغداد

الخلاصه

تم جمع 157 عينة سريرية من عينات سريرية مختلفة (الادرار ، البلغم، الدم المسحات، والكانولة) من عدة مستشفيات في العراق. من بين 157 عينة تح تحديد 51 عزلة (32,48) من الكليبسيلا الرئوية وفقا الصفات

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*Email:haiderm19801@gmail.com

المظهرية، الخصائص الزرعية، واختبار Enterosystem 18R. كانت عزلات الكليبسيلا الرئوية في عينات
البول أكثر من العينات الأخرى بنسبة 26 (52 %) ، وكانت عند الإناث أعلى (70.6 %) من تلك الموجودة
في الذكور (29.4 %) بنسبة حوالي 2.4: 1. تم تحديد الحساسية للمضادات الحيوية لكليبسيلا الرئوية ضد
(12) من المضادات الحيوية شائعة الاستخدام من خلال طريقة نشر القرص. أظهرت النتائج أن نسبة العالية
المقاومة 51 (100%) في العزلات كانت في مضاد Cephalexin، ثم في Ceftazidime 50 (98%) ،
بينما كان أدنى معدل مقاومة لوحظ 24 (47%) في Imipenem و Meropenem لكل منهما. أيضا، تحقق
من التركيز المثبط الادنى (MIC) من Colistin لجميع العزلات (51) باستخدام اختبار E (شرائط)، الذي
اظهر انه كان 33 عزلة مقاومة بينما في نشر القرص كان 31 (فقط 2 من العزلات التي أظهرت حساسية
كوليستين عوضا عن 2 كانت مقاومة في MIC). تم استخلاص DNA من الكليبسيلا الرئوية وشخصت
جزئيئا باستخدام سلسلة تفاعل البلمرة لجين rpoB الذي يمثل الكليبسيلا الرئوية باستخدام البادئ rpoB الخاص
وناتج 108 زوج قاعدة. كذلك جميع الـ 51 عزلة من الكليبسيلا الرئوية، تم تضخيم مقاومة مضاد Colistin
بواسطة جين mgr–B (347 زوج قاعدة) باستخدام بادئ خاص. تم تضخيم الجين mgr–B لمقاومة
Colistin وكذلك عمل التسلسل النيوكليوتيدات من عشرين عزلة. ظهرت 6 عزلات فقط مع استبدال
النيوكليوتيدات المفردة، G بدلاً من A، A بدلاً من G، C بدلاً من G و G بدلاً من C. أيضا ، هذه
الدراسة كشفت عن تشكيل الغشاء الحيوي من الكليبسيلا الرئوية عن طريق استخدام طريقة لوحة microtiter،
والربط مع مقاومة Colistin و Carbapenem. حيث اظهرت المقاومة Colistin و Carbapenem في
الكليبسيلا الرئوية مع قدرة تكوين الأغشية الحيوية في ضعيفة التشكيل مقابل القوية وكذلك، كانت العزلات
المقاومة للمضادات المتعددة أكثر شيوعًا في ضعيفة التشكيل مقابل القوية.

Introduction

The genus Klebsiella is responsible for many diseases, such as pneumoniae, sepsis, meningitis, soft tissue infections, diarrhea, UTI, ankylosing spondylitis and other spondylo-arthropathies [1].

The most common nosocomial infections are caused by *K. pneumoniae*. The majority of the infections are caused by contamination and colonization of hospital environments such as the skin of patients and workers, sinks, carpeting and medical tools. Patients are exposed to bacteria by entering the respiratory tract, blood stream and urinary tract through using ventilators, intravenous catheters and other routes. Transmission can happen directly via contaminated hands of patients and healthcare workers. The most important observation is that *K. pneumoniae* does not spread through air. Drug resistant *K. pneumoniae* is considered as an important hospital-acquired pathogen, especially at intensive care units [2].

The pathogenicity of *Klebsiella* is associated with many factors, the most virulent one being the polysaccharide capsule. The second factor is the filamentous projections on the bacterial surface, which are called as the pili. Another factor is the lipopolysaccharides (LPS), which are essential for the microorganism to resist complement-mediated killing and consist of lipid A, core, and O-polysaccharide antigen. Biofilm formation in *K. pneumoniae* is considered as an important virulence factor which plays a role in pathogenicity [3, 4, 5].

In recent years, there has been a distribution of carbapenemase-producing *K. pneumoniae* (Cp*Kp*) strain, which is associated with hospital environments and causes infections in weakened patients. Cp*Kp* strains appear to be distinct from the hyper-virulent *K. pneumoniae* (hv*Kp*), a strain that was firstly described in the Asian Pacific Rim and causes community-acquired liver abscess invasion and metastatic infections like endophthalmitis, meningitis and septic arthritis in diabetics and immunocompetent individuals (opportunistic infections) [6, 7].

The CP*Kp* isolates are considered as a major public health problem over the past ttwo decades, because they may cause severe infections with high mortality rates due to increasing the enzymatic and nonenzymatic resistance of *K. pneumoniae* against most available antibiotics (Beta-lactams, Carbapenems, Fluoroquinolones, and Aminoglycosides). This strain is rapidly spread across hospitals all over the world, causing a limitation in therapeutic options. Carbapenem is the drug of choice for the treatment of the life-threatening infections [8, 9].

CP*Kp* causes Carbapenem resistance to *K. pneumoniae* strains by plasmid-encoded carbapenemase enzymes. CP*Kp* can produce metallo-β-lactamases which include New Delhi metallo-β-lactamase,

Imipenemase metallo-β-lactamase and Verona integron-encoded metallo-β-lactamase, in addition to Oxacillin-type Carbapenemases (class D) [10].

The increase in Carbapenem resistance among Enterobacteriaceae leads to limitations in the treatment options against these drug-resistant bacteria. Therefore, treatment against Carbapenem-resistant Enterobacteriaceae includes aminoglycosides, tigecycline, fosfomycin and colistin [11]. Infection due to CP*Kp* is commonly treated with Colistin, but a Colistin resistant *K. pneumoniae* (CoR*Kp*) has been reported and is causing an emergency in public health. Especially, Colistin has become the last line of treatment for infections caused by the new multidrug-resistant (MDR) Gram negatives [12]. Colistin is mainly active against gram-negative organisms. Its mode of action involves inducing changes in the permeability of the cell wall by binding anionic lipopolysaccharide molecules and displacing calcium and magnesium, thus causing cell leakage and death [12].

Due to the increasing interest to study *K. pneumoniae* as a result of the global dissemination of MDR clones and the hvKp strains, this study aimed to focusing on the frequency of Colistin resistance *K. pneumoniae* among a sample of Iraqi patients along with its relation to biofilm formation.

Materials and methods
Collection of the sample
The clinical samples were collected during the period from December 2018 until April 2019. The samples consisted of urine, sputum, blood, swabs (burns and wounds) and cannula from five hospitals (Al-Yarmouk hospital, Child Center hospital, Karblaa hospital, Al karkh hospital and Baghdad hospital).

Bacterial isolation
Clinical specimens included different swabs (burns and wounds) taken carefully from the site of infection and placed in tubes containing ready-made media to maintain the swab wet during transferring to laboratory. Urine samples were collected as clean-catch midstream urine at morning, which is highly concentrated and would provide accurate colony counts. Blood samples were collected in a sterile bottle, while feces samples were collected in clean containers and transported to the laboratory for immediate processing. Stool specimens were kept at room temperature and cultured within 1 hr of collection. The specimens that were not sent immediately to the lab were placed in an appropriate enteric transport media. Each specimen was cultured on the surface of MacConky agar by streaking a loopful of culture from brain heart infusion broth for primary selection of *Klebsiella* spp. The plates were incubated at 37°C for 18-24 hrs.

Identification of *Klebsiella* spp.
Identification of suspected isolates was performed by colony morphology. All isolates were identified primarily according to the general culture characteristic (color, shape, texture and size) of the colony on MaCconky agar, whereas other characteristics were also observed, such as lactose fermentation [13].

Identification of *Klebsiella* spp. by Enterosystem 18R.
ENTEROSYSTEM 18R was used for the identification of Gram-negative, oxidase negative bacteria isolated as Enterobacteriaceae. Enterosystem 18R biochemical test's strip contains dehydrated bacterial media/ bio-chemical reagents in 18 separated sections. Bacterial species were identified by the media and bacterial reaction which produces different colors [14].

Antimicrobial susceptibility testing
Agar disk diffusion method
Kirby-Bauer [15] single disk diffusion method was used to test the susceptibility of 51 *Klebsiella* isolates to different antimicrobial agents (Ampicillin, Augmentin, Ceftazidime, Cefepime, Ciprofloxacin, Colistin, Ceftriaxone, Cefotaxime, Cephalexin, Imipenem, Meropenem, and Trovafloxacin). The diameter of the inhibition zone was measured and compared to the chart provided by Clinical and Laboratory Standard Institute [16].

Colistin Susceptibility of *K. pneumoniae* by MIC strip (E-test)
The colistin MIC strip was performed throughout this study and interpreted according to the manufacturer's procedure (Liofilchem, Italy). Mueller Hinton agar was used in the testing procedure [17].

Biofilm formation assay (quantitative)
The detection of biofilm formation for all isolates of *klebsiella spp* was achieved by using the microtiter plate assay [18, 19, 20].

Molecular Detection of *K. pneumoniae* and Colistin resistance gene
DNA Extraction
All bacterial genomic DNA was extracted by a Bioneer DNA extraction Kit.
Estimation of DNA concentration
The concentration and purity of the purified DNA was determined by measuring the UV absorbance at 260 and 280 nm using the nanodrop. DNA concentration was calculated with the OD260nm. The purity was estimated with the OD260nm/OD280nm ratio, with a ratio of approximately 1.8, indicating a low degree of protein contamination [21].
Detection of *K. pneumoniae* and Colistin resistance gene by polymerase chain reaction
This test was performed with species-specific primersforward primer 5'-CAACGGTGTGGTTACTGACG-3' and reverse primer 5'-TCTACGAAGTGGCCGTTTTC-3' were used for the amplification of the *K. pneumoniae* target genes (*rpo B*) because they are the most prominent species among *Klebsiella* spp. While the Colistin resistance (*mgr-B*) gene used the specific forward primer primer 5'-AACCACCTCAAAGAGAAGGCGTT-3' and the reverse primer 5'-GGCGTGATTTTGACACGAACAC-3'. The PCR program included initial denaturation in one cycle for 5min at 95°C, amplification in 35 cycles each of 30 sec. at 94ºC, 30 sec. at 55ºC, and 30 sec. at 72ºC, followed by a final extension cycle for 7min. at 72ºC.
PCR reaction preparation and gel detection
The reaction was performed in a 25 μl volume, 12.5 μl of a ready mix (GO Taq Green Master Mix 2X), one microliter of each primer, and five microliters of DNA, while nuclease free water was used to complete the volume. The compounds were mixed by Exispin system. Positive and negative controls were amplified with each run. After gel electrophoresis, the samples were stained with ethidium bromide 0.5 μg/ml for 15 min. Stained PCR products were visualized under UV light (320nm). The sizes of the bands were compared to the molecular weight marker, Gene Ruler 1 Kb plus DNA ladder.
Sequencing and Alignment
Sequencing was performed to detect resistance related mutations; the conventional PCR products of *mgr*-B of the most resistant and some sensitive 20 isolates were sent to Macrogen Company, USA and sequenced using forward and reverse primers of *mgr*-B on genetic analyser (Applied Biosystems) according to Sanger sequencing method (1977). Homology search was performed using Basic Local Alignment Search Tool (BLAST) online program using blastn algorithms available at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov) [22].
Results and discussion
Sample Collection
A total of 157 clinical samples were collected over five months. The samples were selected randomly from five hospitals. Out of the total samples, urine samples were 84 (53.5%), sputum samples were 30 (19.1%), blood samples were 28 (17.8%), swabs were 14 (8.9%), along with one (0.6%) sample from canula. The collected samples form females were 107 (68.2%), which is markedly higher than those from males 50 (31.8%), as shown in Table-1. This study revealed that urine samples were represented in a high percentage of the identified isolates, followed by sputum. This is due to fact that the urinary tract infection is a very common reason for consultation and antibiotic prescription in current practice. Canula samples were found in a very low number; 1 (0.6%). Female urine samples had markedly higher number than   male urine samples, which is attributed to the observation that more women   suffer from UTIs   than men. This may be explained by urethra anatomy (short, wide and adjacent to the anus) in females [23].

Table 1- Gender distribution percentages among the collected samples

Sample source	Female	Male	Total	(%)
Urine	65	19	84	53.51
Sputum	15	15	30	17.81
Blood	17	11	28	8.92
Swab	10	4	14	19.12
Cannula	0	1	1	0.64
Total	107	50	157	100

Distribution of *Klebsiella* spp
As shown in Table- 2, the results following identifiication by colonies morphology and

Enterosystem 18R revealed that 56 (35.6%) *Klebsiella* species were isolated from the total samples, among which 51 (91%) were identified as *K. pneumoniae* and 5 (8.9%) as *K. oxytoca* by Enterosystem 18R test. The Enterosystem 18R is a reliable method for bacterial identification and uses a small medium that may not need automated systems [24]. The number of *Klebsiella* isolates in females was 36 (70.6%), which was higher than 15 (29.4%) in males, in a ratio of about 2.4:1. Almostsimilar ratio was presented by Anil and Chandrika (2013). Urine samples were higher in number than the other samples (26, 52%), with a higher percentage in females than that in males. This was explained previously by the anatomical differences between males and females [23].

Table 2- Percentage of *Klebsiella* isolates according to gender and samples source.

Samples	Female (% from total)	Male (%from total)	Total (%from total)
Urine	20 (39.2)	6 (11.8)	26 (51)
Blood	8 (15.7)	5 (9.8)	13 (25.6)
Swab	4 (7.8)	0 (0)	4 (7.8)
Sputum	4 (7.8)	3 (5.9)	7(13.7)
Canula	0 (0)	1(1.9)	1 (1.9)
Total	36 (70.6)	15 (29.4)	51 (100)

The overall mean age of the 51 patients at time of sampling was 34.04 ±3.8 with a range from below one year to more than 70 years. Gender/age group distribution of donors identified with *Klebsiella* isolates shown in Figure-1 revealed that the age groups of 11-2 and 21-30 in females represented the highest percentage (7%), followed by that of less than 1 year old children and more than 70 years old adults , (5% and (6%, respectively). Age distribution in males showed that the highest percentage was represented in adults with more than 61 years age, which agrees with previously published studies [25, 26]. Finally, patients at the age group of 41-50 years showed no distribution in *Klebsiella* isolates, which may be due to the notion that this young group has a higher resistance to infections than the more susceptibile ages of children and elderly [27].
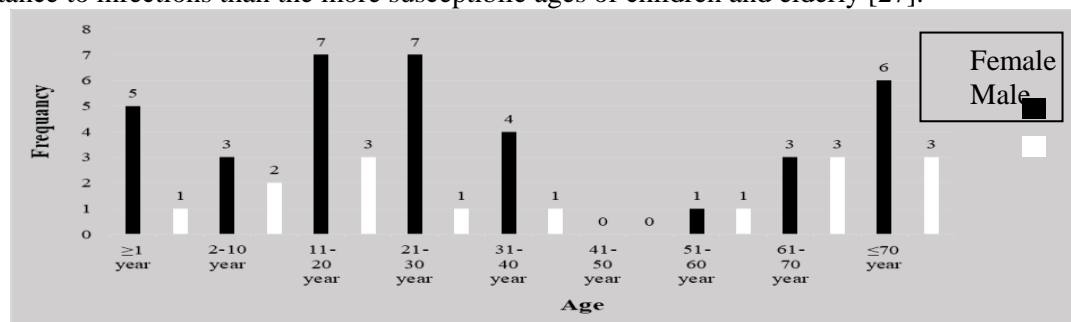

Figure 1- Distribution of K. pneumoniae according to patient gender and age group.

Antimicrobial Susceptibility of *K. pneumoniae*
Different Antibiotic groups

Antibiotic susceptibility results in Figure- 2 showed that the highest resistance rate was agaiinst Cephalexin (51, 100%), followed by Ceftazidime (50, 98%), and each of Ampicillin and Ceftriaxone (47, 92%). The lowest resistance rate observed was 24 (47%) against each of Imipenem and Meropenem. A highest resistance in 31 isolates (60.7%) was recorded to the most conventional antibiotics, whereas Imipenem and Meropenem showed susceptibility rates in 10 (32.2%) and 9 (29%) samples, respectively. Similarr results were observed by Rossi *et al*., (2017) who found that the susceptibility to Carbapenem among all colistin-resistant Gram-negative isolates was 21.5%. This should be highlighted because most clinical laboratories release the Colistin test only when the isolates are resistant to Carbapenems, which silently contribute to the spread of Colistin resistance by the carbapenem-susceptible and *mcr-1*-positive phenotypes. Hence, it is important to keep in mind the presence of Colistin resistant isolates in patients treated with colistin and those who are not undergoing Colistin therapy, because resistant bacteria could later be selected in case of Colistin treatment. Colistin has been used as a first-line drug, especially in intensive care units, due to high rates of carbapenem-resistance among *KP*C- producing *K. pneumoniae*. The colistin resistance is a serious problem in controlling infections, especially that it is one of the last options for treatment of MDR in Gram-negative bacteria [28].
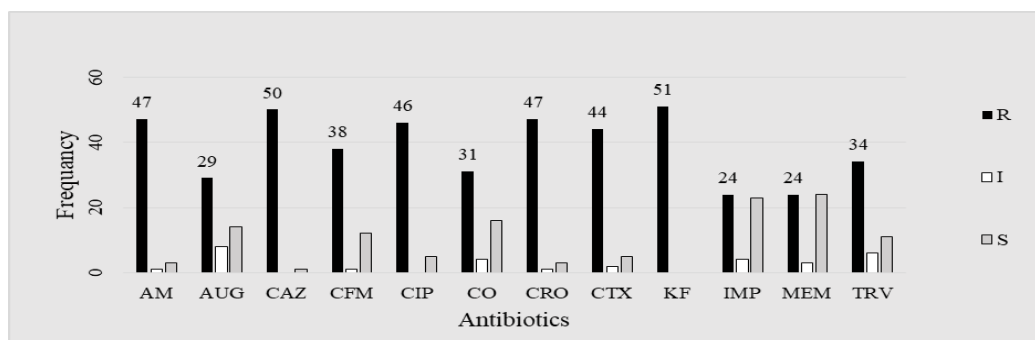
Figure 2- Frequancy of antibiotic susceptibility among 51 *K. peumonia*. isolates. AM: Ampicillin / AUG: Augmenten/ CFM: Cefepime / CTX: Cefotaxime/ CAZ: Ceftazidime /CRO: Ceftriaxone / IPM: Imipenem / MEM: Meropenem/ KF: Keflex (Cephalexin)/ TRV: Trovafloxacin/ Co: Colistin/ CIP: Ciprofaxacin/. S: sensitive/ R: resist/ I: intermediate.

Carbapenem and Colistin detection
Single diffusion method

The 51 *K. pneumoniae* isolates were subjected to a single disc diffusion test. Out of them, the Colistin resistant constituted 31 (60.7%), whereas 4 (7.8%) were intermediate and 16 (31.3%) were susceptible, while each of the Meropenem and Imipenem resistant isolates were 24 (47%). Among the total isolates, Carbapenem resistance to both antibiotics (Meropenem and Imipenem) was found in 19 (37%) isolates, among which a large percentage was resistant to Colistin 17 (89%) (Figure- 3). This indicates that colistin resistance increases in isolates that are also resistant to Carbapenem, which is in agreement with a study by Bhaskar and colleagues who showed that Colistin resistance among Carbapenem-resistant *Klebsiella* was 91%.
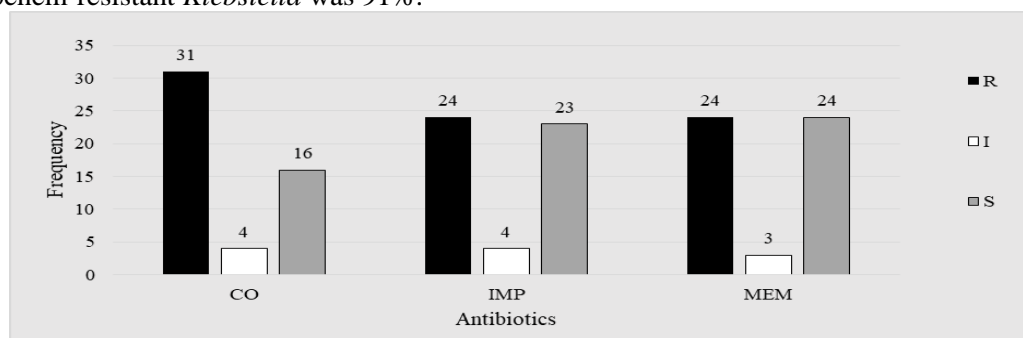

Figure 3- Carbapenem resistance rate among Colistin resistance *K. pneumoniae* isolates. Colistin (CO); Meropenem (MEM) and Imipenem (IMP) antibiotic.

MIC of Colistin by E- test

In vitro susceptibility of colistin was also tested using E-test which is, in contrast to some automatic methods, is reliable for detecting resistant populations [29]. There are no breakpoints for Enterobacteriaceae according to CLSI to test colistin or polymyxin B, so the interpretation depending on the European Committee on Antimicrobial Susceptibility Testing (EUCAST) depicts that MIC breakpoints for colistin as ≤2 for susceptibleand >2 for resistant.

All isolates (51) that were tested by single disc diffusion were subjected to E-test to find the MIC for each isolate. The E-test strip (ranged between 1.016 to 256 µg/ml) showed that the MIC of Colistin resistant isolates was >3 while that of the susceptible isolates had a range of 1-2 ug/ml (Figure-4). The disc diffusion test showed a lower error in comparison with the E-test, which disagreed with a previous study [30] who showed a high error when using the disk diffusion and microdilution methods. Only 2 isolates that showed Colistin sensitivity by disk diffusion were confirmed as resistant by MIC, which may be due to Colistin poor agar diffusion, thickness of culture, incubation time, and molecular weight of the antibiotic, with inhibition values of 12-13 mm should be confirmed with MIC by E-test.
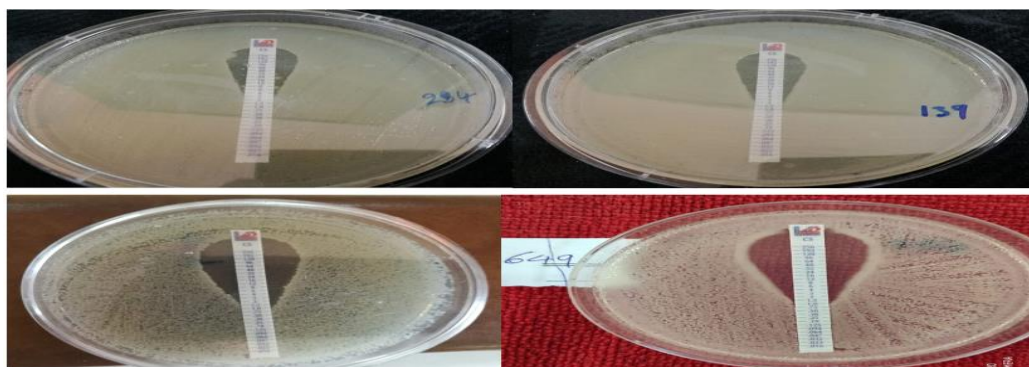
Figure 4- Colistin E test strip to determine MIC for *K. pneumoniae*. Upper: Resistant isolates. Lower: Sensitive isolates.

Moleculer identification of *Klebsiella spp*
DNA Extraction
The genomic DNA was extracted from 56 *klebsiella* spp isolates using a Bioneer kit. Purity and concentration of all DNA samples were tested by nanodrop, where the purity range was from 1.8 to 2 and the concentration range was from 60 to 130 ng /ul.
Identification of *K. pneumoniaee* by PCR
  Most of the 56 isolated *Klebsiella* spp. samples that were diagnosed by morphological and Enterosystem 18R test were confirmed as *K. pneumoniae* by PCR through (51, 91.1%) using a specific *rpo*B primer that represented *K. pneumoniae* with a 108 bp product (Figure-5). Whereas, 5 (8.9%) isolates showed no PCR product, which were already diagnosed as *K. oxytoca* by Enterosystem 18R test. Similar results were obtained by a previous report [31] which found that the percentage of *K. pneumoniae* reached to 87.9%. Another study [32] showed that *K. pneumoniae* is the most important species that caused nosocomial diseases and that it was more prevalent than *K. oxytoca*.
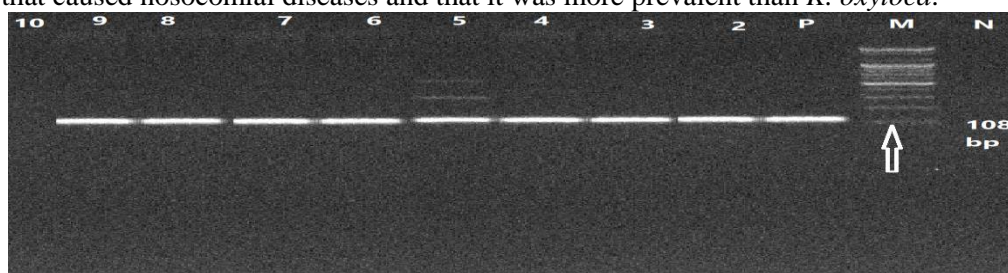

Figure 5- Electrophoretic banding patterns of the amplified products of the *rpo*B specific gene (108 bp) for *K. pneumoniae* by PCR in 1% agarose gel, 90 V/cm for 80 min, stained with ethidium bromide dye and visualized on a UV transilluminator. Lane M-100bp DNA ladder. Lane N- negative control (PCR product without the DNA template). Lane P- positive control (*K. pneumoniae* from Central Health Laboratory). Lanes 1-10 Clinical isolates. The amplified products and their sizes are indicated in the right.

Polymerase chain reaction for the detection of Colistin resistance *mgr-B* gene.
To detect any genetic alterations associated with colistin resistance, *mgr-B* gene was amplified using a specific primer.
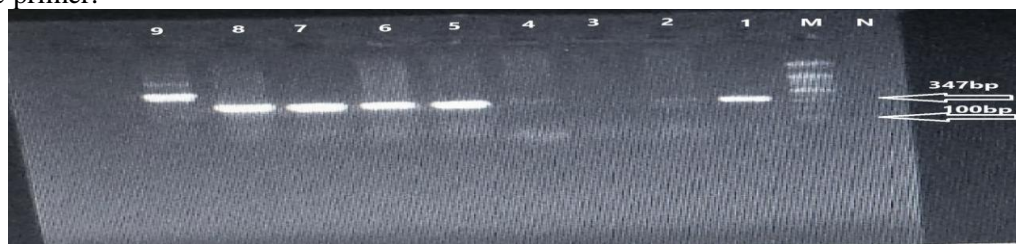

Figure 6- Electrophoretic banding patterns of the amplified products of the *mgr-B* gene (347 bp) for Colistin resistance in *K. pneumoniae* by PCR in 1% agarose gel, 90 V/cm for 80 min, stained with ethidium bromide dye and visualized on a UV transilluminator. Lane M-100bp DNA ladder. Lane N- negative control (PCR product without the DNA template). Lanes 1-9. Clinical isolates.

Inactivation of the *mgrB* gene, encoding a negative-feedback regulator of the PhoQ-PhoP (two-component regulatory) system, causes colistin resistance in carbapenemase-producing *K. pneumoniae* (KPC-KP) by upregulation of the Pmr lipopolysaccharide modification system [33]. Colistin resistant *K. pneumoniae* (CoR*Kp*) were subjected to PCR to amplify the *mgr-B* gene and to subsequently sequence the product to find any mutation responsible for the Colistin resistance. Figure- 6 shows a 347 bp product by PCR, a result that is consistent with what was reported by a previous study using the same primer [33].

Colistin Detection by PCR and Sequencing

Colistin resistance gene *mgrB* was amplified and sequenced from the twenty isolates (12 Colistin resistant and 8 Colistin sensitive *K. pneumoniae* by E-test). Only 6 isolates (S6, S8, S9, S12, S13, S15) appeared with a single nucleotide substitution. The substitutions of the resistant isolates (S8, S9, S12, S13, S15) were as follows: G instead A at position 49, A instead G at position 221, C instead G at position 279, G instead Cat position 281, and A instead G at position 294, while one sensitive isolate (S6) showed a substitution of C instead G at position 159 (Figure- 7). Similar *MgrB* mutations were reported *in vitro* by a previous investigation [34].
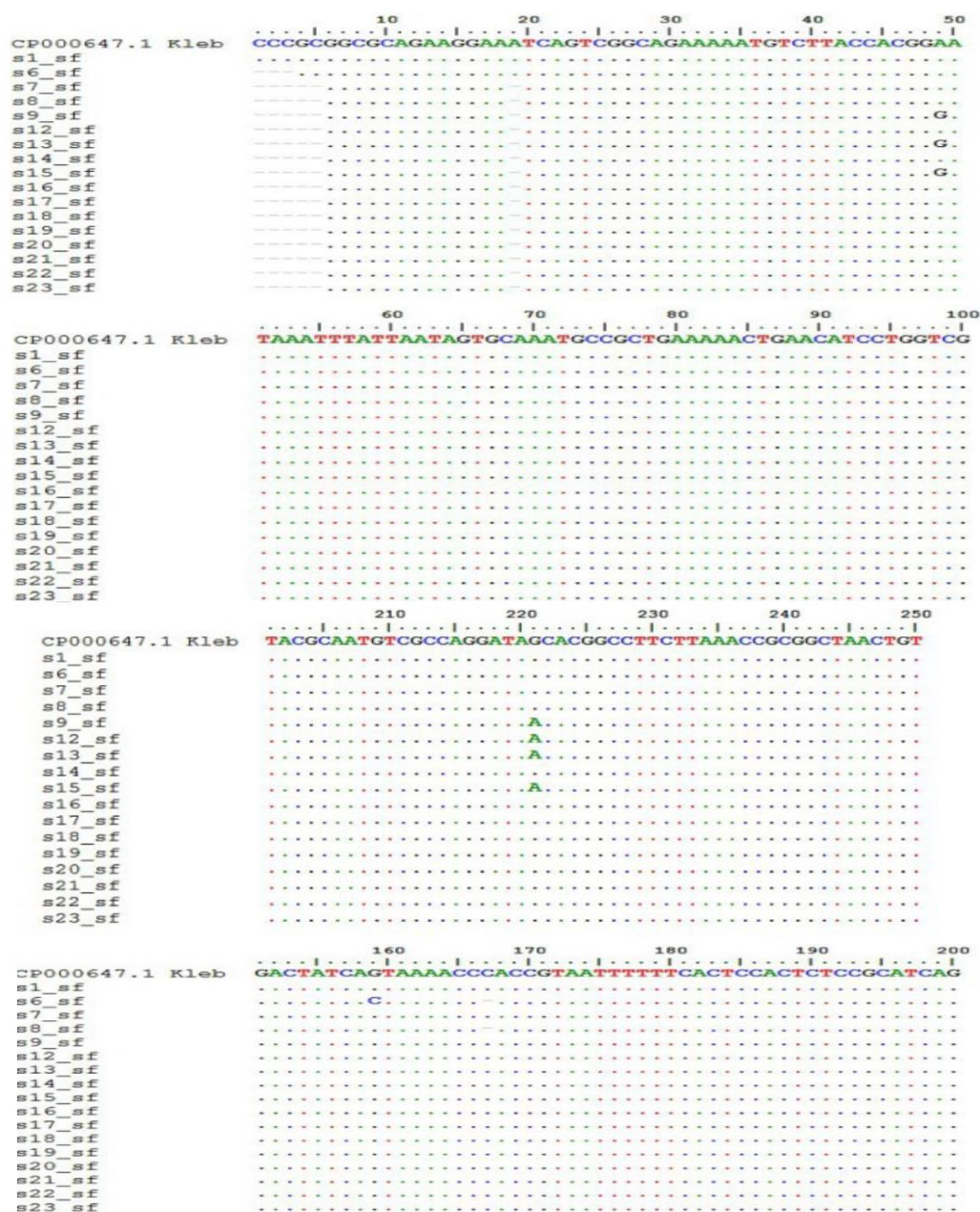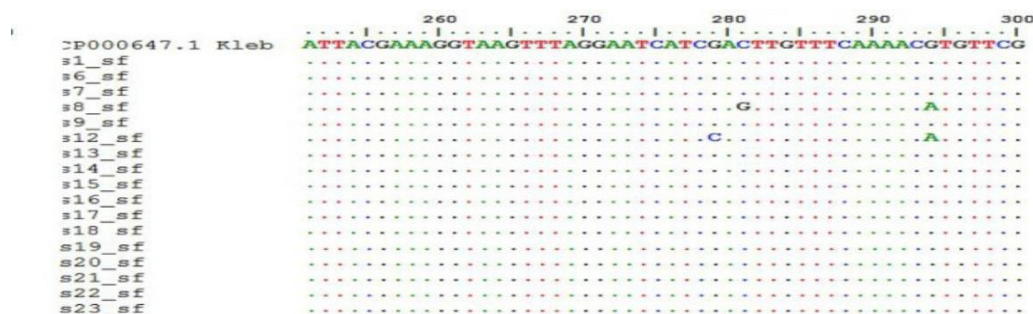
Figure 7- DNA sequence alignments of the *mgrB* gene from 20 isolates, with wild *mgrB* gene of Colistin susceptible *K. pneumoniae* which have been deposited at GenBank nucleotide sequence database under the accession numbers of MF431845.

Biofilm formation in association with Colistin resistant *K. pneumoniae*

   K. pneumoniae have the ability to form biofilms, enhancing the persistence of infection by escaping form host immune reactions. This study focused on biofilm formation in association with the CR phenotype [35].

   Biofilm formation of MDR resistant *K. pneumoniae* isolates is poorly understood and, therefore, studying the relationship between the biofilm formation and drug resistance is very critical to improve therapy. Colistin and Carbapenem resistance patterns in *K. pneumoniae* were compared to the ability of biofilm-formation as weak versus strong. Multi-drug resistant isolates (44, 86.2%) were more common among weak (18, 40.9%) versus strong biofilm formers (6.8%; p ≤ 0.05). Among the total isolates (51), the number of Carbapenem-resistant *K. pneumoniae* was 19 (37.3%), among which the number of the Colistin resistant *K. pneumoniae* was 17 (89.5%). The number of Colistin resistant *K. pneumoniae* was lower in the less strong biofilm formers (3, 10.7%) compared to the weak biofilm formers (10, 35.7%) (Figure-8). This method utilizes the OD cut-off (ODc) value, defined as 3 standard deviations above the average OD of the negative control, to determine biofilm formation. Isolates are either non-adherent (OD ≤ ODc), weakly adherent (ODc b OD ≤ 2× ODc), moderately adherent (2× ODc b OD ≤ 4× ODc), or strongly adherent (4× ODc b OD) [20]. The statistically significant inverse relationship between biofilm formation and antibiotic resistance suggests that virulence may be a trade-off for bacterial survival. Similar results were reported 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).
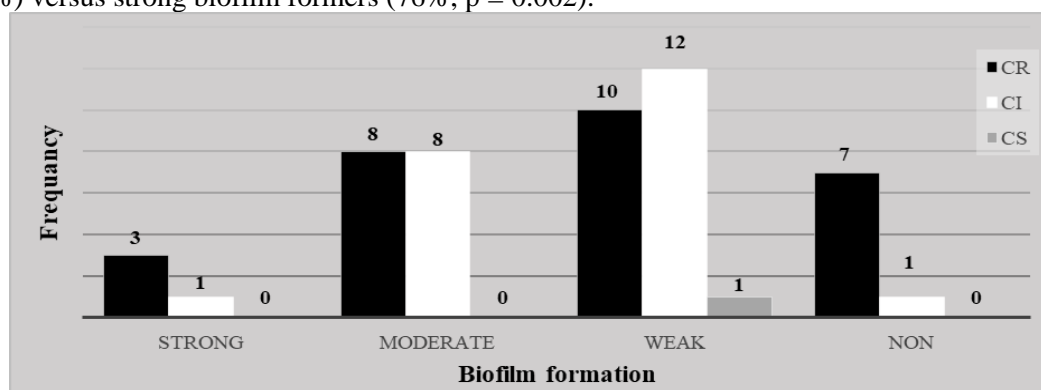

Figure 8- Biofilm formation in association with Colistin susceptibility among 51 *K. pneumoniae* isolates. CR: Colistin resistance, CI: Colistin intermediate, CS: Colistin sansative.

Conclusion

   The *K. pneumonia*e isolates showed more distribution than the other *Klebsiella* species and had higher number in females than males, especially in the young age group. The antibiotic susceptibility for *K. pneumoniae* demonstrated variable resistance for different antibiotics used in this study, with a Carbapenem resistance to Colistin resistant *K. pneumoniae* at 89%. In addition, the disc diffusion test showed a lower error when compared with the E-test (MIC strip). Colistin resistance gene *mgrB* was amplified and sequenced from the twenty isolates, showing five isolates as resistant (S8, S9, S12, S13,

and S15) and only one sensitive isolate (S6) that appeared with a single nucleotide substitution. According to the ability of biofilm-formation and antibiotic susceptibility appearance, *K. pneumonia*e showed less resistant isolates at strong biofilm formation and more resistant isolates at weak biofilm formation isolates.

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