GENETIC ELEMENTS RESPONSIBLE FOR EXTREME DRUG RESISTANCE (XDR) IN KLEBSIELLA PNEUMONIAE VAR PNEUMONIAE ISOLATED FROM CLINICAL SAMPLES OF IRAQI PATIENTS

Rawa Abdul Redha Aziz1* and Sawsan Sajid Al-Jubori2

1Dept. of Biology, Collage of Science, Kufa University, Najaf, Iraq.
2Dept. of Biology, Collage of Science, Al-Mustansiriyah University, Baghdad, Iraq.

ABSTRACT

Twenty four isolates primarily diagnosed as Klebsiella pneumoniae were isolated from different samples from patients submitted to Baghdad hospitals\Iraq. Bacterial diagnosis was performed using complementary Vitek system. A house keeping gene(primers were designed for the 16s and 23s rRNA region) was used for genotypic diagnosis with amplified size 639bp. The results of antimicrobial susceptibility testing (disc diffusion method) revealed that most isolates displayed extremely high drug resistance (XDR) patterns. The highest resistance percentage was towards Ampicillin (95.83%) followed by Ceftazidime (87.50%) compared with other used antibiotic groups. Results also reveal that 16\24 (66.67%) isolates were resistant to Colistin sulphate, while only 2\24 (8.33%) isolates were resistant to Tigecycline. The MIC test for polymyxin B revealed that 22\24 (91.67%) isolates were resistant to >32µg/ml of this antibiotics. The results of modified Hodge test and rapid ESβLs production test revealed positivity of 100%(24/24) and 58.33% (14/24), respectively. About genotypic screening test for antibiotic resistance genes, the results revealed that the highest percentage were towards aminoglycoside modifying enzyme group type aac(6)-Ib followed by aac(3)-I (100% and 75%, respectively) as compared with other genes in the same group. For efflux pump gene about 88% of the isolates were harbored mexX gene and only 29% harbored mexY. Also 16.67% of the isolates harbor the metalo β-lactamase gene blaIMP-1 while none of the isolates were positive to blaVIM gene. The ESBLs genes bla SHV, bla AMP-C, and bla CMY revealed 58.33%, zero%, and 33.33% among K. pneumonia local isolates tested in this study. The highest percentage was towards rmtF (66.67%) followed by rmtB (54.17%) as compared with...
with other gene in the methylation group. Some of the isolates encode the quinolones resistance genes ParC2 and GyrA (54.17% and 75%, respectively). For oxacillinace genes (OXA58, OXA51, OXA24, and OXA23), only OXA 23 was detected (8.33%) among K. pneumonia isolates. As a conclusion, most isolates harbor more than one resistant mechanisms which might considered a sign for emergency pan drug resistant not only XDR pattern.

**KEYWORDS:** XDR Klebsiella pneumonia, ESBLs, Hodege test, Different resistance mechanism.

**1. INTRODUCTION**

*K. pneumonia* is opportunistic pathogen can be carried asymptptomatically in the intestinal trac, skin, nose and throat of healthy individuals but can also cause a range of infections in hospitalized patients, most commonly pneumonia, wound, soft tissue, or urinary tract infections.\(^1\) Increasing reports of infections due to multidrug resistance (MDR) and extremely drug resistance XDR fermentative Enterobacteriaceae such as *Klebsiella* spp. might become the worst health problems and issues.\(^1,2\) It is pointed out that MDR pathogens could be resistant towards 3-5 antibiotic classes such as antipseudomonal cephalosporins, antipseudomonal carbapenems, β-lactam–β-lactamase inhibitor, fluoroquinolones, and aminoglycosides, while XDR pathogens are susceptibility to only 2 or less antibiotic classes.\(^3\) However, PDR (pan drug resistance) pathogens have no options for treatment as they diminished susceptibility to all classes.\(^3\) MDR *Klebsiella pneumonia* remains susceptible only to more agents such as the polymyxins, and it convert to PDR if they resist this antibiotic.\(^3\) Usually *Klebsiella pneumonia* has the ability to resist wide range of antibiotics via different mechanisms including production of Extended spectrum β-lactamases (ESBLs) and carbapenemases, production of aminoglycoside-modifying enzymes, over expression of efflux pumps, reduction in porin channels, and target-site alteration including changing in penicillin binding proteins and topoisomerases.\(^4\) *K. pneumoniae* is resistant to penicillins extended-spectrum β-lactamase, and expanded-spectrum cephalosporins through the production of β-lactamases that are encoded mainly by the *bla SHV*, *bla TEM*, and *amp C* genes. β-lactams such as imipenem and meropenem, which are highly resistant to hydrolysis by TEM, SHV, and AmpC β-lactamases remain effective antibiotic options.\(^4\) *Klebsiella pneumonia* contain many plasmids that differ in numbers and molecular weight, carrying different types of genes including those encoding extended-
spectrum β-lactamases (ESBLs), AmpC β-lactamases, inhibitor resistant TEM β-lactamases and metallo β-lactamase enzymes. These enzymes confer resistance to various antimicrobial agents including the third and fourth generation cephalosporins, cephemycins, monobactam β-lactamase, β-lactamase/inhibitor combinations and carbapenems.[5] Due to the dearth of antimicrobial effect with novel mechanisms of action, very few options remain to treat the infections caused by XDR pathogens.[6] Klebsiella pneumonia has emerged as a common cause of serious epidemic and nosocomial infections in hospitals, resulting in high morbidity and mortality.[3,6] ESBL-producing K. pneumoniae is resistance to other antibiotics, including fluoroquinolones, aminoglycosides, trimethoprim, and sulfamethoxaxoles.[7] It is also reported that clinical outcome in bacteraemic infections caused by ESBL-producing K. pneumoniae appears to be worse than that of patients with non-ESBL-producing isolates.[8] This prevalence of ESBLs has limited the available therapeutic options and necessitated the increased use of carbapenems against Klebsiella pneumoniae infections. In response to use of carbapenems, carbapenem resistance has been emerged.[9] Modified Hodge test (MHT) has been widely used as primary screening test for carbapenemase activity.[10] In our study, we focus on the detection of Iraqi extensively drug-resistant (XDR) among the isolates and studying their resistance pattern towards different antimicrobial groups by PCR.

2. MATERIAL AND METHODS

2.1. Collection and diagnosis of Bacterial isolates Twenty four isolates of Klebsiella were obtained from patients admitted to several teaching hospitals in Baghdad during a period between March 2015 to September 2015. They were collected from the midstream urine from patients with urinary tract infections (5 isolates), from bacteraemia (7 isolates), wound swabs from burn unit (4 isolates), 2 isolates were collected from sputum, ear, and pus (for each), finally 1 isolate collected from both of wounds infections and stool. Isolates were re-diagnosed by Vitek 2 compact automated system (Biomeriux, USA), and the probability was 99% Klebsiella pneumonia ssp pneumonia.

2.2 Genotyping detection for isolates Specific primers were designed for the 16s rRNA and 23s rRNA region (housekeeping gene, amplified size was 639 bp) using Genieous Software/primer 3. The sequence for the forward primer was 3’ TGTACACACCGCCCCTC-5’ and for the reverse was R-3’GGTACTTAGATGGTTTACCAGTTC-5’. Total DNA was extracted using Genomic DNA Extraction Kit (Wizards, Promega, USA) following manufacture’s protocol and was used as a template for the PCR process. The mixture of PCR composed from 12.5 of
GoTaq® Green Master Mix (2x), 5 µl template DNA, 1.5 µl primers (for each) final concentration 0.6 pmol/µl and nuclease free water up to 25 µl (4.5 µl). PCR products were stored at -20 ºC, then nucleotides sequence was carried out at NICEM Company, USA. Results of sequencing were analyzed using genious software 7.01R compareid with the NCBI standered strain.

2.3. Antimicrobial sensitivity tests: Resistance tested for the isolates towards thirty types of different antibiotics including β- Lactams, aminoglycoside, tetracyclines and quinolones groups besides other groups. Susceptibility was determined based on the interpretative criteria recommended by the Clinical and Laboratory Standards Institute (CLSI).[11] The antibiotics were: from β- Lactams, Ampicillin (25mcg), Piperacillin (100µg), Cefixime (5µg), Cefazolin (30µg), Cephalothin (30µg), Ceftaxime (30µg), Ceftazidime (30µg), Cefepime (30µg), Amoxicillin/clavulanic acid (20/10µg), Ticarcillin-clavulanic acid (75/10µg), Aztreonam (30µg), Imipenem (10µg), Meropenem (10µg) and Doripenem (10µg). In the Aminoglycoside group: Kanamycin (30 µg), Gentamycin (10µg), Tobraycin (10µg), Netilmicin (30µg) and Amikacin (10µg). The quinolones group include Naldixic acid (30µg), Levofloxacin (5µg), Gatifloxacin (5µg), Ofloxacin (5µg). Other antibiotics include sulpha group: Trimethoprim (5µg) and Trimethoprim-Sulphamethoxazole (1.25/23.75µg), and Tetracyclines: Tetracycline (30µg) and Doxycycline (30µg). Tigecycline (15µg) and colistin sulphate (25µg) were also tested.[12, 13] Polomyxin B was also used to achieve the minimum inhibitory concentration (break point 2µg/ml).[14] E. coli HB101 used as the negative control strain. Phylogenetic analysis for antibiotic susceptibility pattern was done using Tamura –Nei genetic destine model and UPGMA tree build method in order to analyze antibiogram similarity.

2.4: Indirect three dimensional agar diffusion method to detect ESBLs The detection of ESBLs production was performed using modified indirect three dimensional method in which Muller Hinton Agar (MHA) plates were seeded with a lawn of a standard strain E.coli HB101 adjusted to MacFarland (0.5) standard. A slit was performed inside the MHA plate using a sharp scalpel emerged with the test isolate. The following discs were placed 2mm away towards the performed line. They were amoxicillin/clavulanic acid (20/10µg), ceftazidime (30 µg), ceftriaxone (30 µg), and cefexime (5µg). The plate was incubated at 37ºC for 16-18 hours. The distortion of inhibition zone around the antibiotic indicates ESBLs production.[15]
2.5: Modified Hodge test (MHT) Modified Hodge test (MHT) was performed according to\textsuperscript{[10]} for detection of carbapenemase production. Briefly, 5 ml of brain heart infusion broth culture for \textit{Escherichia coli} HB101 equal to 0.5 McFarled was prepared then 100μl from 1:10 dilution was streaked as lawn on to a Mueller Hinton agar plate. Imipenem susceptibility disk (10μg/disc) was placed in the center of the test area. The test isolate was streaked in a straight line from the edge of the disk to the edge of the plate. The plate was incubated overnight at 37ºC for 24 hours. After 24 hours, MHT positive test (carbapenemase producer) showed a clover leaf-like indentation of the \textit{Escherichia coli} 25922 growing along the test organism growth streak within the disk diffusion zone. MHT negative test represent by normal growth for the standard strain or without the formation of clover leaf-like shape.

2.6: Detection of antibiotic resistant genes using PCR amplification technique All the twenty four isolates were subjected to molecular screening study using PCR amplification technique to detect XDR profile. Different primers (table 2) were used. PCR mixture was composed from 5μl template DNA which was extracted using Genomic DNA Extraction Kit (Wizards, Promega, USA), 12.5μl of GoTaq®Green Master Mix, 1.5μl from forward and reverse primers (final concentration 0.6pmol/μl). The volume was completed to 25μl with nuclease free water. PCR was run under the following conditions: primary denaturation step 95°C for 5 min; 30 -40 repeated cycles (according to gene listed in table 2) of 94°C for 30sec , 43-62°C annealing temperature (table-2) for 60 sec and 72°C for 1 min then final extension step at 72°C for 6 min. PCR products were electrophoresed in 1% agarose gel and visualized under UV light.\textsuperscript{[16]}
Table 2: Primers used for detection antibiotic resistant genes.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequences (5' _3') F</th>
<th>Sequences (5' _3') R</th>
<th>Size product</th>
<th>Tm</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>16s RNA &amp; 23s RNA</td>
<td>F-TGTACACACCCGCCGTC</td>
<td>R-GGTACTTAGATGTTTCAGTTC</td>
<td>639</td>
<td>59</td>
<td>This study</td>
</tr>
<tr>
<td>mexX</td>
<td>F- TGA AGG CGG CCC TGG ACA TCA GC</td>
<td>R- GAT CTG CTC GAC GCG GGT CAG CG</td>
<td>326</td>
<td>62</td>
<td>Lianes et.al.,[17]</td>
</tr>
<tr>
<td>MexY</td>
<td>F- CCGCTACAACGGCCTATCCCT</td>
<td>R-AGCGGGATGAGCAGCAGTTTC</td>
<td>250</td>
<td>59</td>
<td>Xavier et.al.,[18]</td>
</tr>
<tr>
<td>aac(3)-I</td>
<td>F- AGGCCGCATGGATTTGA</td>
<td>R- GGCATACCGGAAGAAGTT</td>
<td>227</td>
<td>43</td>
<td>Xavier et.al.,[18]</td>
</tr>
<tr>
<td>aac(6)-Ib</td>
<td>F- TTG CGA TGC TCT ATG AGT GGC TA</td>
<td>R- CTC GAA TGC CTG GCG TGT TT</td>
<td>482</td>
<td>56</td>
<td>Ndegwa et.al.,[19]</td>
</tr>
<tr>
<td>ant(4)’Iib-</td>
<td>FGACGACGACAGCTATGGAAATTG CCAATATATT</td>
<td>RGGAACAGACCGTTCAATTCAA TTCAATTT</td>
<td>364</td>
<td>57</td>
<td>Ndegwa et.al.,[19]</td>
</tr>
<tr>
<td>aph(3’)VI</td>
<td>F-TAT CTC GGC GGC GGT CGA GT</td>
<td>R-CAC GGC GGG AAA CGC GAG AA</td>
<td>800</td>
<td>55</td>
<td>Haldorsen et.al.,[20]</td>
</tr>
<tr>
<td>blaSHV</td>
<td>F-AAGATCCACTATGCAGCAGC</td>
<td>R-TTGCTTCTGCTTTTCCAGCGG</td>
<td>200</td>
<td>59</td>
<td>Vaziri et.al.,[21]</td>
</tr>
<tr>
<td>blaIMPc</td>
<td>F-ATGCAACAACGGACAATCCATC</td>
<td>R-GTTGGGGTAGGTCGAGTTGG</td>
<td>1150</td>
<td>58</td>
<td>Vaziri et.al.,[21]</td>
</tr>
<tr>
<td>blaCMY</td>
<td>F-GACGACGCTTCTTCTCCACA</td>
<td>R-TGGAACAGGAGGCTACGTA</td>
<td>1014</td>
<td>50</td>
<td>Oliveira et.al.,[22]</td>
</tr>
<tr>
<td>IMP-1</td>
<td>F-CTACGCCAGCGAGTCTTGTG</td>
<td>R-AAACAGTTTTGCTTATACCT</td>
<td>500</td>
<td>55</td>
<td>Ramazanzadeh et al.,[23]</td>
</tr>
<tr>
<td>VIM</td>
<td>F-GTTTTGCCTGACATATCCAGC</td>
<td>R-AATGGACAGACCACTGGATAG</td>
<td>382</td>
<td>57</td>
<td>Mendes et al.,[24]</td>
</tr>
<tr>
<td>RmtA</td>
<td>F-CTA GCG TCC ATC CTT TCC TC</td>
<td>R-TTG CTT CCA TGC CTT TGC C</td>
<td>635</td>
<td>58</td>
<td>Haldorsen et al.,[20]</td>
</tr>
<tr>
<td>RmtB</td>
<td>F-CCC AAA CAG ACC GGA GGA GAC GC</td>
<td>R-CTC AAA CTC GGG CAA CAG GC</td>
<td>584</td>
<td>59</td>
<td>Doi et al.,[25]</td>
</tr>
<tr>
<td>RmtC</td>
<td>F-CGA AGA AGT AAC AGC CAA AG</td>
<td>R-ATC CCA ACA TCT CTC CCA CT</td>
<td>711</td>
<td>53</td>
<td>Mendes et al.,[24]</td>
</tr>
<tr>
<td>RmtD</td>
<td>F-TCAAAAAGAAAGGAGAAG</td>
<td>R-CGATCGCAGATCCATTC</td>
<td>500</td>
<td>52</td>
<td>Tijet et al.,[26]</td>
</tr>
<tr>
<td>RmtF</td>
<td>F-GCGATACAGAAACCGAAGG</td>
<td>R-GGCGAGAGCTTCATCAGAA</td>
<td>453</td>
<td>51</td>
<td>Lee et al.,[27]</td>
</tr>
<tr>
<td>OXA 51</td>
<td>F 5’-TGATGGTGATGGCTTGATTG-3'</td>
<td>R5'- GGATTGACTATCATCTTGG-3'</td>
<td>353</td>
<td>53</td>
<td>Morovat et al.,[28]</td>
</tr>
<tr>
<td>OXA58</td>
<td>F 5’-AGATGTTGAGGCCTTGGGTCTT-3'</td>
<td>R 5’-CCCTTGGCTCTACATAC-3</td>
<td>599</td>
<td>53</td>
<td>Morovat et al.,[28]</td>
</tr>
<tr>
<td>OXA23</td>
<td>F 5’-ATTTGCTTGGGCTTATACACGGA-3</td>
<td>R 5’-ATTTGCTTGGCACAGTTCATC-3</td>
<td>501</td>
<td>53</td>
<td>Morovat et al.,[28]</td>
</tr>
<tr>
<td>OXA24</td>
<td>F 5’-GGATTGATGGCCCTTAAA-3'</td>
<td>R 5’-AGTGGAGCGAAAAGGGATT-3</td>
<td>246</td>
<td>53</td>
<td>Morovat et al.,[28]</td>
</tr>
<tr>
<td>gyr A</td>
<td>F-GGCATTTATTCGCTACCGGC</td>
<td>R-TTCGAGAATTACGTTAAAAAGG</td>
<td>886</td>
<td>57</td>
<td>This study</td>
</tr>
<tr>
<td>Par C2</td>
<td>F-GTTACCGTATGCGAGCGGTA</td>
<td>R-TGATTTCACCTGAGGACGCG</td>
<td>314</td>
<td>57.1</td>
<td>This study</td>
</tr>
</tbody>
</table>
3. RESULTS AND DISCUSSION

*Klebsiella pneumoniae* is a leading cause of hospital-acquired (HA) infections and serious community-acquired (CA) infections, including pyogenic liver abscess, pneumonia, and meningitis.[3] In the current study, all the 24 isolates were diagnosed depending on a housekeeping gene 16S rRNA gene and 23SrRNA and gave a positive result (figure 1). The amplified DNA segment (figure 2) as compared with the standard strain *Klebsiella pneumoniae subsp. pneumoniae* KPNIH29: CP009863 (NCBI) was exactly 639 bp start from 18,101 bp to 17,467 bp in the genomic map and passing through 3 important housekeeping genes (red area) the 16s rRNA, tRNA-Glu and 23s rRNA. Pairwise identity was 91% which represent the percentage of residues that are identical in the alignment. Some differences were recognized between the local isolate and the recorded NCBI strain as clear with gaps in the upper green identity line. The reason of choosing 16S rRNA gene to identify *Klebsiella pneumonia* isolates in this study is that the widespread use of this gene sequence for bacterial identification and taxonomy of many non-cultured bacteria, and the fact that it is an essential part of the description of a novel organism.[28]

![Figure 1](image1.png)

Figure(1): Agarose gel electrophoresis (1% agarose, 5 V/cm for 90min) for 16SrRNA gene of *Klebsiella pneumonia* isolates. Lane M: 100bp DNA ladder, lanes 1,2,3,4,5,6, and 7 are positive results with 639bp amplicon of *Klebsiella pneumonia* blood 1, 2, 3, 4, 5, 6, 7. Lanes 10, 11 and 12 are positive results with 639bp amplicon of *Klebsiella pneumonia* Urine 1, 2, and 3; lane 8 and 9 show negative results.
Antibiotics are widely used in clinical settings whether for treating simple cases or life-threatening infections caused by microorganisms. Now a days, many antibiotic resistant gram negative bacteria come up and have made global concern such as *Klebsiella pneumonia*. The broad use of antibiotics had created a strong selective pressure, which consistently had resulted in the survival and spread of resistance that has evolved with the increased number, volume and diversity of antimicrobial applications. Among the 24 isolates, rate of resistance (figure3) for penicillin group the rates were 95.83% for ampicillin, 79.17% for piperacillin, 58.33% for ticarcillin-clavulante, 70.83% for amoxicillin/clavulanic acid. While for cephalosporins, the percentage of resistance were 87.50% for ceftazidime, 83.33% for cefotaxime, 79.17% for ceftriaxone, 79.17% for cefepime, 75.00% for cefazolin, 66.60% for cephalothin, 58.33% for cefixime. As for Monobactam the rat was 62.50% for aztreonam. Resistance towards aminoglycoside group were 38%, 20.83%, 4.17% and 29.17% for kanamycin, gentamycin, amikacin, and tobramycin, respectively, but none of the isolates were able to resist netilmicin. Tetracyclines was also tested and the results revealed that 62.50% of the isolates showed resistance for tetracycline and the rate decreased to 58.33% for doxycycline. In addition, flouroquinolones sensitivity test showed that 20.83% of the isolates resisted each of gatifloxacina, florofloxacina, and levofoxacin, while nalidixic acid resistance was 66.67%. Finaly rate of resistance toward thrimethoprim and trimethoprim-sulfamethoxazole were 70.83% and 70.80%, respectively. Similar work was done by Radha *et.al* where antibiotic sensitivity test of 25 *K. pneumoniae* isolates ranged between intermediate and resistant according to the National committee for clinical laboratory standard (NCCLS). Moreover, the results of the current study revealed that there was no
resistant isolates towards netlimicin, meropenem, and doripenem, however, 29.17% of *Klebsiella pneumoniae* isolates were resistant to imipenem. It has been revealed that, ESBL producers showed resistance pattern towards imipenem due to the production of carbapenemase, so these bacteria cannot be eliminated by carbapenems that are the usual drug of choice against them.\[^{30}\] It is reported that 62 (50%) *K. pneumonia* isolates resistant to imipenem.\[^{31}\] It is important to reveal that 16 (66.67%) *K. pneumonia* isolates (K. pne. b4, b5, b6, b7, U2, U3, U4, U5, S2, W1, ES2, Bu1, Bu2, Bu3, Bu4, and P1) were resistant to colistin sulphate, while only two (8.33%) isolates (K. pneumonia b5 and W1) were resistant to a tigecycline. It was pointed out that tigecycline, a broad-spectrum glycylcycline, is tigecycline is considered as therapeutic option for MDR gram positive and gram negative bacteria, and it was found to be effective against *Klebsiella* infections.\[^{31}\] However, Rhee found that their local *Klebsiella pneumoniae* collected from patients with diabetes mellitus and chronic renal failure on He modialysis in South Korea displayed intermediate pattern to tigecycline, so he concluded that the isolates could be defined as XDR.\[^{32}\] Galani et al. showed that colistin sulphate was less active against *Klebsiella pneumonia* tested in their study\[^{13}\] although colistin sulphate (polymyxin E) has been considered an effective therapeutic option for such XDR pathogen.\[^{33}\] Finally, minimum inhibitory concentration test (MIC) of Polymyxin revealed that 91.67% (22/24) isolates were resistant at 32µg/ml (break point ≥2µg/ml), leading to conclude that *K. pneumoniae* isolates are PDR pathogens. This conclusion is supported in literature, for example Falagas and his team found that the mortality rate was 41.7% among hospitalized patients at a tertiary-care center when tested 23 *Klebsiella pneumoniae* isolates. They conclude that PDR gram-negative bacterial infections are associated with considerable mortality, although not as high as expected given the fact that the isolates were resistant to all tested antibiotics, including polymyxins.\[^{14}\] It was clear that each isolate showed its ability to resist more than one antibiotic group. As illustrated in figure(4) each isolates of *K. pne.* B5, ES2, and Bu2 were able to resist 23-24 antibiotics related to different groups followed by *K. pne.* W1 and P1 that were resistant to 21 antibiotics as compared with other local isolates tested.
Figure 3: the percentage of different antibiotic groups against *Klebsiella pneumonia*.

Tetracycline (T), Meropenem (MEM), Cefepime (FEP), piperacillin (PRL), Aztreonam (ATM), Amikacin (AK), Ticarcillin +Clavulanic acid (TIM), Trimethoprim I, Levofloxacin (LEV), Naldixic acid (NA), Imipenem (IPM), Gentamicin (CN), Cephalothin(KF), Ceftazidime (CAZ), Ampicillin (AP), Kanamycin (K), Gatifloxacin (GAT), Cefazolin (CZ), Cefixime(CFM), Tobramycin(TOB), Ofloxaclin (OFX), Trimethoprim +Slumphemethoxazole (SXT), Doripenem (DOR), Doxycycline (DXT), Cefotaxime (CTX), Ceftriaxone (CRO), Amoxicillin+Clavulanic acid (AMC), netilmicin (NET).

Figure 4: illustrate number of resisted antibiotics related to different by *K. pneumonia*.
Figure 5, dendrogram illustrating phylogenic analysis of Antibiotic sensitivity test toward 30 different antibiotics using Global alignment \(\text{Tamura-Nei (UPGMA)}\) in Geneious software. The tree was with 47 nodes and 24 Tips. The data is separated into 2 groups (A and B). The phylogenetic tree of the current study reflects a high diversity between isolates. It is clear that cluster A2 shows \textit{K.pne U4} having different antibiogram pattern compared with other isolates in the same group. Similarities in antibiogram pattern on the other hand were appeared in cluster B1b2, precisely \textit{K. pne. B5} and \textit{K. pne. Bu2} since they did not have differences and the coefficient was (0). Also, similar phenotypic pattern was noticed between \textit{K.pne} b1 and b3 (B1a cluster) making them segregated within the same clone (red color). The isolate \textit{K.pne .p2} at (0.259) bosses the most differ pattern hence it segregate alone as with the stander strain.

![Phylogenetic tree](image)

Figure 5: The phylogenetic tree for antibiogram phenotype(47 nodes and 24 Tips) for local \textit{K. pneumonia} isolates tested.

Modified Hodge test were performed since this test has been widely used for screening of carbapenemase activity.\cite{34} As it is known that carbapenemases (KPC) are β-lactamase enzymes that inactivate the carbapenems (ertapenem, imipenem and meropenem), and these are responsible for many epidemics in Greece, India, and USA, especially when carbapenemase producers spread in patients with identical risk factors (patients receiving broad-spectrum anti-biotherapy, patients in intensive care units, immune compromised patients, transplant patients, surgical patients).\cite{35} Therefore, early identification of carbapenemase producers in clinical infections is mandatory to prevent development of those
hospital-based outbreaks. All the isolates gave a positive result (100%). Figure 6 shows some of the positive tested isolates presented in leaf–shape.

Figure 6: positive results of Modified Hodage test for *Klebsiella pneumonia*. Isolates no. 1, 3, 5, 13, and 20 showed positive results (leaf shape), while no 16 showed negative results.

Moreover, it is revealed that *K. pneumoniae* is identified to be the most common entero bacterial species for spreading ESBL genes in health care facilities during the past 30 years. Resistance to fluoroquinolones, co-trimoxazole, and trimethoprim is frequently observed among ESBLs producers. Therefore, results of screening for extended spectrum β-lactamases via the modified indirect method in this study showed 14 isolates (58.33%) were positive in that 11 isolates (45.83%) were resistant to Amoxicillin/clavulanic acid, 9 (37.5%) isolates were resistant to Ceftriaxone, 8 (33.33%) to Ceftazidime, 4 (16.67%) were resistant to Cefixime. Similar work has been done by Dhara et al., however, their results demonstrated 81.48% ESBLs prevalence among (54 isolates) and the susceptibility pattern of ESBL positive isolates showed all 2nd generation cephalosporins, 3rd generation cephalosporins and aztreonam were resistant. Also it has been reported that 148 (61%) of *K. pneumoniae* isolates from different areas in Iran were ESBLs producing.

It has been reported that the increasing clinical incidence of antibiotic-resistant bacteria is a major global health care issue. It has been grounded on data that characterization of antibiotic resistance determinants at the genomic level plays a critical role in understanding, and potentially controlling, the spread of MDR or XDR pathogens. Of particular concern is the spread of resistant *Klebsiella pneumoniae* which can harbor both extended-spectrum β-
lactamases (ESBL) and carbapenemases capable of hydrolyzing newer carbapenem drugs. Frequently associated with ESBL-producing *K. pneumonia* is resistance to other antibiotics, including fluoroquinolones, aminoglycosides, trimethoprim, and sulfamethoxazoles.\[40,30\]

Therefore, many genes related to different antibiotic resistance mechanisms were tested. Figure 7 represents 1: *aac(6)-Ib*, 2: *aac(3)-I*, 3: *ant(4')IIb*, and 4: *aph(3')VI*, genes related to aminoglycoside modifying enzymes. 5: *mexX* and 6: *mexY*, the efflux pump genes. 7: *blaIMP-1* represent MBL gene 8: multiplex oxacillinase genes (OXA 58, OXA 51, OXA 24, and OXA23). 9: 16s ribosomal methylation enzymes such as *rmtA*, 10: *rmtD*, 11: *rmtF*, 12: multiplex of *rmtB* and the ESBLs encoding gene *bla* SHV. 13: *bla* CMY. Finally, 14: *ParC2* and 15: *GyrA1*, quinolone resistance genes.
Results revealed that all the isolates harbored more than one resistant gene. The most dominant gene among aminoglycoside modifying enzymes tested was 1aac(6)-Ib(100%), followed by aac(3)-I(75%), ant(4’)Ib-(45.83%) and the amikacin resistance gene aph(3)-VI also called (aphA6) (8.33%) (figure 8). It has been known that aminoglycoside are important options for the treatment of infection caused by carbapenem-resistant K. pneumonia strains. The inactivation by enzymatic modification is the most prevalent mechanisms of resistance specially class aph(3)-VI and ant(4’)Ib- confer high-level resistance towards amikacin and are infrequently encountered. Althoughaac (6)Ib is common among Enterobacteriaceae, the clinical relevance is largely unknown. [41] Aac(6)-Ib, the most prevalent aminoglycoside modifying enzyme that confers resistance to tobramycin, kanamycin, and amikacin was first identified in K.15arboring isolates in 1986. [42] Bremmer reported that 20(70%), aac(6)Ib positive K. pneumonia isolates were resistant to amikacin, however, majority showed regrowth supporting the idea that aac(6)-Ib confer intermediated amikacin resistant. [41]

The percentage of Efflux pump genes were 88% and 29.17% for mexX and mexY, respectively (figure 8). mexX and mexY (RND; resistance-nodulation-division) are located on operon within genomic chromosome of Klebsiella pneumonia. [43] There are increasing evidence that the role of efflux pumps in antibiotic resistance in bacteria is significant. [43] Despite high-level resistance may not occur as a result of MDR efflux pumps alone, the
association of over-expression of these genes amongst highly resistant clinical isolates cannot be ignored.[44]

The percentages of resistance for 16s ribosomal methylation enzymes were rmtA (20.83%), rmtB (54.17%), rmtC (zero%), rmtD (33.3%), and rmtF (66.67%) (figure 8). Those enzymes confer high level resistance to most clinically useful aminoglycosides by inhibiting their access to the site of action.[45] As can be seen here, the most dominant gene among local isolates was rmtF that confers high-level resistance to most clinically relevant aminoglycosides. This resistance determinant appears to be spreading quickly among enterobacteria associated with other emerging resistance mechanisms, including NDM-1 carbapenemase.[27] Moreover, rmtB has been detected in various species belonging to the family Enterobacteriaceae, including K. pneumonia, however, only 0.3% presence of rmtB among K. pneumonia isolates in Taiwan screened by amikacin and confirmed by PCR. Detection of this resistance mechanism may pose a challenge in clinical laboratories.[24]

The multiplex oxacillinase genes (OXA 58, OXA 51, OXA 24, and OXA23) showed positive results for only OXA 23 (8.33%) (figure 7). It is reported that OXA-23 confers high-level carbapenem resistance.[45] The emergence of carbapenem-resistant K. pneumoniae strains will have a serious impact on remaining therapeutic options. β-Lactamase producing strains are clinically significant, as they are difficult to treat.[46]

Moreover, different types of ESBLs and MBLs are the major enzymes that are increasing in pathogenic microorganisms worldwide.[30] The rate of MBL genes in this study was 16.67% for blaIMP-1 and zero % for blaVIM (figure 7). Also the results showed ESBLs encoding genes bla AMP-C for which no positive results (0.00%), however, bla SHV and bla CMY showed positivity (58.33% and 33.33%, respectively). Ulyashova et. al., revealed in their work that K. pneumonia isolates did not expressed blaVIM.[47] It has been revealed by Hammond et al., that 13/21 clinical isolates tested in their study expressed bla SHV which is prevalent in gram-negative bacteria, and it can hydrolyze penicillin and cephalosporines but not extended spectrum antibiotics oxyimino cephalosporine and monobactams.[47] Reports of broad spectrum β-lactamase types such as SHV, mediated by plasmids, have proliferated with the increased identification of K. pneumonia strains and with the use of cephalosporin during recent years.[48] Acquired MBL genes are located on integron that reside on mobile genetic elements such as plasmids or transposons, thus, enabling widespread dissemination.[45] According to research done in Mexican hospital, 31 strains of Klebsiella
pneumonia isolated from septicemic pediatric patients could harbor bla SHV gene and considered as MDR.\textsuperscript{[48]} Some of these multidrug-resistant isolates produce extended-spectrum $\beta$-lactamases (ESBLs) that are able to hydrolyze expanded-spectrum cephalosporins (e.g., ceftriaxone, cefotaxime, and ceftazidime), aztreonam, and related oxyimino-$\beta$-lactams. The substitution of one or more amino acids in bla SHV gene has altered the configuration of the active site.\textsuperscript{[48]}

ParC2 and GyrA1 (quinolone resistance genes) exhibited 54.17\% and 75\%, respectively, and this is the first time in Iraq to document the existence of quinolons resistant genes among Iraqi Klebsiella isolates. Fluoroquinolones have been frequently prescribed as empirical therapy against most hospital and community infections due to increased appearance of multiple drug resistant gram-negative pathogens and to the disease severity. In countries with extensive clinical use of quinolones, fluoroquinolone resistance has been a problem in clinical medicine for its limiting of available agents in the treatment of many types of infection.\textsuperscript{[49]} Klebsiella pneumonia isolates showed mutation in either gyrA or ParC or both genes in different codons resulting in resistance towards quinolones, and these multiple alterations of gyrA and parC have been associated with ciprofloxacin resistance.\textsuperscript{[50]} It has been shown that 77 of 120 test strain K. pneumonia harbor gyrA and parC genes and could then resist quinolones.\textsuperscript{[51]} Twenty-two K. pneumonia isolates in other study were resistant to gatifloxacin, trovafloxacin, moxifloxacin, levofloxacin, and ciprofloxacin, whereas only 7 were clinafloxacin resistant. This resistant mechanism related to alteration in gyrA and parC genes, too.\textsuperscript{[52]}

Results obtained in this study support data in literature which revealed that K. pneumonia clinical isolates have been shown to manifest all three broad mechanisms of drug resistance in gram-negative bacteria, which are the acquisition of novel antibiotic catalytic genes, mutations of antibiotic targets and membrane proteins, and differential expression of specific genes such as those for efflux pumps which mediate drug effects.\textsuperscript{[40]} In this study, K. pneumonia Sputum 2 and Stool 1 isolates noticed to have 13 genes each out of the 22 antibiotic resistant genes tested compared with other isolates that have range of 3-12 genes (Figure 9).

In conclusion, antibiotic resistance among various species of bacteria is a global public health problem. Molecular analysis has been performed to quantify the hypothesis of these isolates being extremely drug resistant (XDR); therefore, it could be concluded from the results
explained above that most of the 24 *K. pneumonia* local Iraqi isolates covered not only extremely drug resistance (XDR) pattern but also being pan drug resistant (PDR) pathogens. A sign of emergency of un treatable isolates.

**Figure 8:** Prevalence and percentage of Antibiotics resistance genes within *K. pneumonia* local Iraqi isolates.

**Figure 9:** The number of resisted genes within twenty four *Klebsiella pneumonia* isolates.

**REFERENCES**


baumannii Clinical Isolates at a Tertiary Medical Center in Pennsylvania. IMICROBIAL AGENTS AND CHEMOTHERAPY., 2008; 52(11): 3837–3843.


