MOLECULAR STUDY OF BLAOXA1, BLACTX-M AND THEIR RELATED WITH 16SRRNA METHYLTRANSFERASE GENES IN MULTIDRUG RESISTANT KLEBSIELLA PNEUMONIA ISOLATED FROM DIFFERENT CLINICAL SAMPLES IN KIRKUK CITY

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ABSTRACT

Background: Extended Spectrum beta-lactamase producing Klebsiella.pneumoniae which also carry 16S rRNA methyltransferase have been reported to be an important cause of many infections in hospitals. K.pneumoniae have developed resistance by intrinsic & acquired mechanisms to many antimicrobial agents imposes a serious therapeutic problem. Materials and Methods: A total of 51 Klebsiella.pneumoniae isolates were isolated from 120 different clinical samples in some public & private hospitals in Kirkuk city during the period from October 2020 to January 2021. Bacterial identification was done using conventional, cultural & biochemical methods, API 20E and VITEk 2 cards for identification (GN), while the antibiotic susceptibility tests was done using disk diffusion test (DDT) and minimum inhibitory concentration (MIC) testing was performed using VITEK 2 automated system (bioMérieux, France). The highly resistance isolates were subjected to conventional PCR technique to detect the presence of blaoXA1, blactx-M and related 16S rRNA methyltransferase. Results: The results of antibiotic sentivity by (DDT) revealed that highest resistance was against oxacillin(100%), ampicillin and tetracycline (94%), while the rest of antibiotics had resistance percentage of (60.7%) for cefotaxime and ceftriaxone, (54.95%) for cefepime, (62.7%) for ceftazidime and (50%) for cefoxitin and ciprofloxacin. AMK and imipenem by (43%), gentamicin and piperacillin by (49%) and (68.8%) respectively. The MIC of different antibiotics was performed on highest resistance isolates using VITEK2 AST-GN327 showed the isolates were resistant to the antibiotics used except few isolates exhibit sentivity to carbapenems, amikacin, cefoxitin and ciprofloxacin. PCR assay revealed that all resistant K.pneumoniae isolates were harbored blaoXA1 and blactx-M were detected in 18(78.2%) of isolates. Regarding to 16S rRNA methyltransferase genes, armA gene was detected only in 4(17.3%) isolates but all resistance isolates were harbored rmtC gene. Conclusion: The prevalence of multi-drug resistant K.pneumoniae isolates was increased in Kirkuk province. Molecular characterization of ESBLs and 16S rRNA methyltransferase genes provide information about the prevalence of these genes in K.pneumoniae in Kirkuk city. The blaoXA1 was the predominant among the ESBLs genes while the rmtC genes were predominant among 16S rRNA methyltransferase genes in K.pneumoniae in this study.

Key words: Klebsiella.pneumoniae, ESBL, blaoXA1 genes, blactx-M genes, 16S rRNA methyltransferase genes, armA, rmtC.

I. INTRODUCTION

Klebsiella pneumoniae is Gram-negative bacilli, facultative anaerobic and generally measures 0.3 to 1.5 µm wide by 0.5 to 5.0 µm long, non-motile, and lactose fermented, non- spore forming, shorter and thicker when compared to others in Enterobacteriaceae. They can be found singly, in pairs, in chains or linked end to end. K. pneumoniae can grow on ordinary lab medium and do not have special growth requirements, the ideal growth temperature is 35° to 37 °C, while the ideal pH level is about 7.2 (1, 2).
Extended spectrum beta lactamase (ESBLs) are plasmid mediated enzymes capable of hydrolyzing and inactivating a wide variety of beta-lactams, including third generation cephalosporins, penicillins, and aztreonam, but are susceptible to beta-lactamase inhibitors such as clavulanate, sulbactam and tazobactam. They are mainly found in Escherichia coli, Klebsiella species, and other Enterobacteriaceae (3,4). More than 350 different natural ESBL variants are known that have been classified into nine distinct structural and evolutionary families based upon their amino acid sequence comparisons such as TEM, SHV, CTX-M, PER, VEB,GES, BES and OXA (5, 6, 7). CTX-M beta-lactamases (class A) were named for their greater activity against cefotaxime than other oxyimino-beta-lactam substrates (e.g., ceftazidime, ceftriaxone, or cefepime), rather than arising by mutation, they represent examples of plasmid acquisition of beta-lactamase genes normally found on the chromosome of Kluvyvera species, a group of rarely pathogenic commensal organisms.

The OXA-type beta-lactamases confer resistance to ampicillin and cephalothin and are characterized by their high hydrolytic activity against oxacillin and cloxacinil and the fact that they are poorly inhibited by clavulanic acid (8). Most ESBLs have been found in E. coli, K. pneumoniae, and other Enterobacteriaceae, the OXA-type ESBLs have been found mainly in P. aeruginosa.

*Klebsiella pneumoniae* resistance to Aminoglycosides (amikacin and gentamicin) related to 16S rRNA methylases which are encoded on the plasmids (9), seven 16S rRNA methylase genes have been identified (armA, rmtA, rmtB, rmtC, rmtD, rmtE and npmA) (10). The armA gene was initially identified on plasmid pCTX-M3 in Citrobacter freundii and plasmid pIP1204 in K pneumoniae, both of which also carried an ESBL gene blaCTX-M3. In these plasmids, armA is located downstream of insertion sequence ISCR1. The armA (aminoglycoside resistance methyltransferase) was the first 16S rRNA methylase reported in a clinical isolate of *K. pneumoniae* (11), which confers resistance to kanamycin, amikacin, isepamicin, gentamicin, netilimicin, sisomicin, and tobramycin and fortimicin. The armA gene encoding the methylase was detected in a composite transposon, Tn1548, located in a conjugative plasmid of approximately 90 kb (13).

The rmtC was first reported in a Proteus mirabilis clinical strain that was isolated from a hospitalized patient in Japan in 2003. Located on a non-conjugative plasmid, rmtC is adjacent to an ISEcp1-like element, which also provides the promoter sequence for the expression rmtC. It has been found among *K.pneumoniae* clinical isolates (14), rmtC is being incorporated by MDR/XDR K.pneumoniae, in particular those producing NDM-type carbapenemase. The *rmtC* gene encode resistance to gentamicin, tobramycin, and amikacin but susceptibility to neomycin and apramycin (11).

### II. MATERIALS AND METHODS

#### Bacterial Isolates

A total of 51 *Klebsiella pneumoniae* isolates were collected from different clinical specimens in Kirkuk/Iraq during the period from October 2020 to January 2021. The *K. pneumoniae* isolates (51 isolates) were as follows: urine (25), sputum (19), blood (3) and burns (4). Clinical samples were collected from Kirkuk general hospital, Azadi teaching hospital, Alnaser hospital, Tuberculosis center, Pediatric hospital, General health laboratory, in addition to some private laboratories in Kirkuk city. Bacteria were cultured on MacConkey agar in aerobic condition at 37 °C for 24-48 h. Then they identified by conventional biochemical tests and by using of VITEK 2 Automated system using (GN) cards as recommended by the manufacturer.

#### Antibiotic susceptibility testing

All 51 *K. pneumoniae* isolates were screened for their antibiotic resistance against selected 13 different clinically important antibiotics using disk diffusion test method (DDT). VITEK 2 system using (AST-GN327) was used & the MIC for these isolates was obtained.

#### Molecular detection of ESBLs and 16S rRNA methyltransferase genes:

### Isolation of Genomic DNA from Gram negative Bacteria

This method was achieved according to the genomic DNA purification Kit supplemented by the manufacturing company (Geneaid/Taiwan). All of the DNA extraction steps were achieved according to the genomic DNA purification Kit supplemented by the manufacturing company (Geneaid/Taiwan).

#### Preparation of primers suspension

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The primers were resuspended by dissolving the lyophilized primers after spinning down with nuclease free water depending on manufacturer instruction as stock suspension. Working primer tube was prepared by diluted with nuclease free water. The final picomoles depended on the procedure of each primer.

Detection of ESBLs and 16S rRNA methyltransferase genes by PCR

The ESBLs and 16s rRNA methyltransferase genes were determined for the betalactam and aminoglycosides resistant isolates by using targeting four genes, \textit{bla}OXA1, \textit{bla}CTX-M1, \textit{arm}A and \textit{rmt}C. The PCR amplification mixture has been prepared according to the manufacturer's instructions (BIONEER, Korea).

A. Primers:

The primers and PCR conditions used to amplify the genes encoding the betalactamase and 16s rRNA methyltransferase enzymes are listed in Table -1 below:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5'-3')</th>
<th>Size of product bp</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{bla}OXA1 (F)</td>
<td>GGCACCCAGATTCAACTTTCAAG</td>
<td>564</td>
<td>Perez \textit{et al.}(15)</td>
</tr>
<tr>
<td></td>
<td>GACCCCCAGTTTTCGTGTAAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(R)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>\textit{bla}CTXM1 (F)</td>
<td>TTAGGAAAGTGTGCCCCTGTA</td>
<td>655</td>
<td>Ogutu \textit{et al.}(16)</td>
</tr>
<tr>
<td>(R)</td>
<td>CGGTTTTCTCCCCACAA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>\textit{arm}A (F)</td>
<td>ATCTGTGCTATCCTAATTGG</td>
<td>315</td>
<td>Yamane \textit{et al.}(17)</td>
</tr>
<tr>
<td>(R)</td>
<td>ACCTATATTTATCGTCGTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>\textit{rmt}C (F)</td>
<td>CGAAGAAGTACAGCCAAAG</td>
<td>711</td>
<td>Doi \textit{et al.}(18)</td>
</tr>
<tr>
<td>(R)</td>
<td>ATCCCCACATCTCTCCACT</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B. The reaction mixture:

Amplification of DNA was carried out in a final volume of 25 μl containing the following:

<table>
<thead>
<tr>
<th>No.</th>
<th>Contents of reaction mixture</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>PCR master mix</td>
<td>12.5 μl</td>
</tr>
<tr>
<td>2.</td>
<td>Forward primer</td>
<td>2 μl</td>
</tr>
<tr>
<td>3.</td>
<td>Reverse primer</td>
<td>2 μl</td>
</tr>
<tr>
<td>4.</td>
<td>DNA template</td>
<td>5 μl</td>
</tr>
<tr>
<td>5.</td>
<td>Nuclease free water</td>
<td>3.5 μl</td>
</tr>
</tbody>
</table>

Total volume 25 μl

III. RESULTS

Out of 51 \textit{Klebsiella pneumoniae} isolates, 23 isolates were highly resistant and the MIC of 9 antibiotics listed in Table -3 was done using VITEK2-Compact by using AST-GN327 for testing the antibiotic susceptibility of these isolates and the MIC values were interpreted according to the CLSI 2020 (19).

The results showed that all 23(100%) \textit{K.pneumoniae} isolates were resistant to ampicillin MIC ≥ 32 μg/ml, nearly all 22(95.6%) of isolates were resistant to Piperacillin/Tazobactam MIC ≥128 μg/ml except 1(4.3%) was susceptible MIC ≥ 16 μg/ml. The MIC for the 2\textsubscript{nd} generation cephalosporin cefoxitin indicate that 17(73.9%) isolates were resistant with MIC ≥ 64 μg/ml, the remaining 6(26%) isolates were susceptible MIC (≤4.8
Regarding to quinolone three isolates (MIC ≤ 4 µg/ml) were resistant (K.p-11S,13S,16U) show intermediate resistance to ciprofloxacin with MIC 2 µg/ml, while the remaining 20 (86.9%) K. pneumoniae isolates were resistant MIC ≥ 4 µg/ml.

About 15 (65.2%) of K. pneumoniae isolates were resistant to amikacin MIC ≥ 64 µg/ml and 8 (34.7%) were susceptible with MIC ≤ 64 µg/ml. Most 16 (69.5%) of K. pneumoniae isolates were susceptible with MIC ≥ 16 µg/ml, for gentamicin most 21 (91.3%) of K. pneumoniae isolates were resistant MIC ≥16 µg/ml and only 3 (13%) were susceptible with MIC 4 µg/ml. Most 16 (69.5%) of K. pneumoniae isolates were resistant to imipenem, MIC ≥16 µg/ml the remaining 7 (30.4%) isolates were susceptible. The results showed that most of resistant K. pneumoniae were isolated from sputum, burn and blood.

Table 3: MIC values (µg/ml) for K. pneumoniae isolates to different antibiotics using VITEK2

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Specimen</th>
<th>MIC (µg/ml) of selected antibiotics determined by VITEK 2 system</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>AM</td>
</tr>
<tr>
<td>Kp 3S</td>
<td>Sputum</td>
<td>≥32 (R)</td>
</tr>
<tr>
<td>Kp 4S</td>
<td>Sputum</td>
<td>≥32 (R)</td>
</tr>
<tr>
<td>Kp 5S</td>
<td>Sputum</td>
<td>≥32 (R)</td>
</tr>
<tr>
<td>Kp 1B</td>
<td>Blood</td>
<td>≥32 (R)</td>
</tr>
<tr>
<td>Kp 9S</td>
<td>Sputum</td>
<td>≥32 (R)</td>
</tr>
<tr>
<td>Kp 11S</td>
<td>Sputum</td>
<td>≥32 (R)</td>
</tr>
<tr>
<td>Kp 2B</td>
<td>Blood</td>
<td>≥32 (R)</td>
</tr>
<tr>
<td>Kp 2U</td>
<td>Urine</td>
<td>≥32 (R)</td>
</tr>
<tr>
<td>Kp 12S</td>
<td>Sputum</td>
<td>≥32 (R)</td>
</tr>
<tr>
<td>Kp 13S</td>
<td>Sputum</td>
<td>≥32 (R)</td>
</tr>
<tr>
<td>Kp 16S</td>
<td>Sputum</td>
<td>≥32 (R)</td>
</tr>
<tr>
<td>Kp 17S</td>
<td>Sputum</td>
<td>≥32 (R)</td>
</tr>
<tr>
<td>Kp 18S</td>
<td>Sputum</td>
<td>≥32 (R)</td>
</tr>
<tr>
<td>Kp 3B</td>
<td>Blood</td>
<td>≥32 (R)</td>
</tr>
<tr>
<td>Kp 1Bu</td>
<td>Burn</td>
<td>≥32 (R)</td>
</tr>
</tbody>
</table>
All resistant *K. pneumoniae* isolates were further investigated for the presence of ESBLs (*bla*OXA1 and *bla*CTX-M) and 16S rRNA methyltransferase genes (*armA* and *rmtC*) using 4 families of genes and those genes was detected by conventional PCR technique.

PCR analysis showed that *bla*OXA1 was detected in 23(100%) isolates as in Figure -1, while 18(78.2%) of *K. pneumoniae* isolates were carry *bla*CTX-M gene and this gene was not detected in the remaining 5(21.7%) isolates, these are *Kp* 5S, *Kp* 11S, *Kp* 17S, *Kp* 21U, *Kp* 17U respectively as in Figure -2.

**Figure -1:** Agarose gel showing PCR amplification products with *bla*OXA1 gene (564bp) primers for *K. pneumoniae* extracted DNA.

M: 100 bp standard size reference marker, Lane 1: *Kp* 4S show positive *bla*OXA1, Lane 2: *Kp* 5S show positive *bla*OXA1, Lane 3: *Kp* 1B show positive *bla*OXA1, Lane 4: *Kp* 9S show positive *bla*OXA1, Lane 5: *Kp* 11S show positive *bla*OXA1, Lane 6: *Kp* 2B show positive *bla*OXA1, Lane 7: *Kp* 2U show positive *bla*OXA1, Lane 8: *Kp* 12S show positive *bla*OXA1, Lane 9: *Kp* 13S show positive *bla*OXA1, Lane 10: *Kp* 16S show positive *bla*OXA1, Lane 11: *Kp* 17S show positive *bla*OXA1, Lane 12: *Kp* 18S show positive *bla*OXA1, Lane 13: *Kp* 3B show positive *bla*OXA1, Lane 14: *Kp* 1Bu show positive *bla*OXA1, Lane 15: *Kp* 2Bu show positive *bla*OXA1, Lane 16: *Kp* 3Bu show positive *bla*OXA1, Lane 17: *Kp* 4Bu show positive *bla*OXA1, Lane 18: *Kp* 3S show positive *bla*OXA1, Lane 19: *Kp* 9U show positive *bla*OXA1, Lane 20: *Kp* 16U show positive *bla*OXA1, Lane 21: *Kp* 21U show positive *bla*OXA1, Lane 22: *Kp* 17U show positive *bla*OXA1, Lane 23: *Kp* 14U show positive *bla*OXA1.
Figure -2: Agarose gel showing PCR amplification products with *blaCTX-M* gene (655bp) primers for *K.pneumoniae* extracted DNA.


Regarding 16S rRNA methyltransferase genes, *armA* gene was detected only in 4(17.3%) isolates which are *Kp*16U, *Kp*17U, *Kp*14U and *Kp*21U as in Figure -3, while this gene was not detected in the remaining 19(82.6%)isolates, all 23 (100%) *K.pneumoniae* isolates were carried *rmtC* genes Figure-4.

Figure -3: Agarose gel showing PCR amplification products with *armA* gene (315 bp) primers for *K.pneumoniae* extracted DNA.

M: 100 bp standard size reference marker,Lane 1: *Kp* 4S show negative *armA*, Lane 2: *Kp* 5S show negative *armA*, Lane 3: *Kp* 1B show negative *armA*, Lane 4: *Kp* 9S show negative *armA*, Lane 5: *Kp* 11S show negative *armA*, Lane 6: *Kp* 2B show negative *armA*, Lane 7: *Kp* 2U show negative *armA*, Lane 8: *Kp* 12S show negative *armA*, Lane 9: *Kp* 13S show negative *armA*, Lane 10: *Kp* 16S show negative *armA*, Lane 11: *Kp* 17S show negative *armA*, Lane 12: *Kp* 18S show negative *armA*, Lane 13: *Kp* 3B show negative *armA*, Lane 14: *Kp* 1Bu show negative *armA*, Lane 15: *Kp* 2Bu show negative *armA*, Lane 16: *Kp* 3Bu show negative *armA*, Lane 17: *Kp* 4Bu show negative *armA*, Lane 18: *Kp* 3S show negative *armA*, Lane 19: *Kp* 9U show negative *armA*, Lane 20: *Kp* 16U show negative *armA*, Lane 21: *Kp* 21U show positive *armA*, Lane 22: *Kp* 17U show positive *armA*, Lane 23: *Kp* 14U show positive *armA*. 
Figure 4: Agarose gel showing PCR amplification products with rmtC gene (711 bp) primers for K. pneumoniae extracted DNA.

IV. DISCUSSION

The prevalence of antibiotics resistance among pathogenic bacteria has become a serious matter worldwide. K. pneumoniae is an opportunistic pathogen that causes community and hospital acquired infections (20).

Reasons behind resistance of K. pneumoniae to ampicillin may be due to of production of a chromosomal penicillinase (21). Higher resistant to pencillin antibiotics may be due to mutation of gene-coding penicillin binding proteins (PBPs) (22) or due to production of β-lactamase such as ESBL mainly SHV-1 beta-lactamase besides OXA and TEM β-lactamase as all of isolates in the current study was harbored blaOXA1 genes. On the other hand resistance to piperacillin/Tazobactam was also mediated by blaOXA1 gene.

The resistance to cephalosporines may be due to extreme and bad use of these antibiotics in Kirkuk hospitals. The K. pneumoniae has different strategies to resist cephalosporines mainly by production of ESBLs include CTX-M, SHV, TEM and OXA beta-lactamase, loss of outer membrane porins and antimicrobial efflux (23). CTX-M and SHV – enzymes are known to have the highest substrate affinity for cefotaxime and ceftazidime (24). The resistance of K. pneumoniae to imipenem may be due to inappropriate duration of antibiotic therapy and subtherapeutic concentrations of the drug. Resistance to imipenem significantly associated production of KPC-type carbapenemases mediate resistance to extended-spectrum cephalosporins in addition to carbapenems. Although resistance is not limited to this mechanism solely, another methods of resistance includes ESBLs like OXA enzymes that have measurable activity against imipenem and or / AmpC production coupled with outer membrane porin (OMP) alterations delaying the diffusion of antibiotics into the bacterial cell and up-regulation of efflux pump (25).

Klebsiella pneumoniae resistance to aminoglycosides (amikacin and gentamicin) may be as a result of enzymatic modification of this drug, modification of the ribosomal target and decreased intracellular antibiotic accumulation by alterations of the outer membrane permeability, decreased inner membrane transport or active efflux. Also all isolates were harbored rmtC which play important role in resistance to aminoglycosides.

The K. pneumoniae have several mechanisms of resistance to ciprofloxacin. These mechanisms involve the modulation of the target site that bind with the antibiotics by triggering chromosomal genetic mutations in the genes encoding for DNA grease or Topoisomerase resulting in production of different enzyme that is resistant to
these antibiotics, also efflux pumps on the outer membrane of the bacteria that serve to expel the antibiotic to the outside and prevent the permeability of it.

Our results revealed that blaOXA1 carrying strains of *K. pneumoniae* exhibited high level of resistance to cephalosporines due to the fact that blaOXA1 showed strong binding affinity with ceftazidime, resulting in cleavage of the antibiotic which suggests the high efficiency of blaOXA1 in cleaving other β-lactam antibiotics like carbapenem and penciillins.

In our present findings all of blaOXA1 producing strains were resistant to β-lactam inhibitor (piperacillin/tazobactam). Resistance to penicillin/inhibitor combinations among ESBL producers are due to several reasons among these is poorly inhibited penicillinases such as OXA1(28).

The present study found that blaOXA genes were more predominant than blaCTX-M gene in K. pneumoniae which was in accordance with Silago et al (2021) who found that blaCTX-M gene was predominant (98.6%) in K. pneumoniae more than other ESBLs genes and are commonly encoded by conjugative epidemic plasmids, i.e., IncFII which plays an important role in their successful dissemination and, therefore, their predominance.

The result of the present study showed that only one (6.6%) from 15 resistance isolates to amikacin was carried armA gene and 3 (14.2%) from 21 resistance isolates to gentamicin was carried armA gene. Alasht et al (2020) found that (86%) of *K. pneumoniae* isolates were carried rmtC genes which nearly agreed with the result of present study indicating the important role of rmtC in conferring resistance to amikacin and gentamicin among *K. pneumoniae* isolates.

V. CONCLUSION

The prevalence of multi-drug resistant *K. pneumoniae* isolates was increased in Kirkuk province. Molecular characterization of ESBLs and 16S rRNA methyltransferase genes provide information about the prevalence of these genes in *K. pneumoniae* in Kirkuk city. The blaOXA1 was the predominant among the ESBLs genes while the rmtC genes were predominant among 16S rRNA methyltransferase genes in *K. pneumoniae* in this study.

The results revealed importance of accurate identifications of ESBLs and 16S rRNA methyltransferase genes in *K. pneumoniae* to prevent the emergence of complete resistance to the most potent drugs against *K. pneumoniae* in Kirkuk.

REFERENCES


