Imipenem Resistance and Biofilm Formation In *Klebsiella pneumoniae* from some Hospitals in Baghdad City

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**Abstract**

*Klebsiella pneumoniae* is a prominent opportunistic pathogen for community-acquired infections or hospital-acquired infections. A total of 39 (22.94%) *K. pneumoniae* isolates were collected from patients from some hospitals in Baghdad city. The lowest incidence was among the (71-80) years old age group (5.13%), whereas the highest incidence was among the (51-60) years old age group (23.06%). Antimicrobial susceptibility test was performed by Vitek-2 system and MICs of imipenem were determined. Biofilm formation was assayed by micro titer plate, then PCR was performed for detection of *ompA* and *bap* genes. The results show that the imipenem-resistance was seen in 23 (58.97%) of *K. pneumoniae* isolates, and all *K. pneumoniae* isolates were classified into MDR firstly, and to XDR or even PDR depending on their resistance profile. Among these isolates, 22(56.41%) were biofilm producers, the current results recorded that the highly percentage of biofilm production and imipenem resistant for *K. pneumoniae* isolates was located within the weak and moderate patterns. The *ompA* gene was present in 9 (23.08 %) isolates of *K. pneumoniae*, while, all of *K. pneumoniae* isolates didn’t have *bap* gene.

**Keywords:** *Klebsiella pneumoniae*, Imipenem-resistance, Biofilm gene.

**INTRODUCTION**

*Klebsiella pneumoniae* is a part of the normal microbiota of humans where they inhabit mucosal surfaces and they can also be found in the environment in surface water, soil, sewage, and on plants. It is a prominent opportunistic pathogen for community-acquired but are much less common than nosocomial infections or hospital-acquired infections (I). The increasing incidence of extended spectrum β-lactamase (ESβL) and carbapenemase producing of *K. pneumoniae* isolates in health care facilities is a cause of global concern (2). Since the mid-1980s, hyper virulent *K. pneumoniae* isolates, generally associated with the hyper mucoviscosity phenotype, has emerged as a clinically significant pathogen responsible for serious disseminated infections, in a generally younger and healthier population (3). Biofilm formation: Pili along with lipopolysaccharides and the capsule contribute to the formation of biofilms, which increase the bacteria’s pathogenicity (4). Biofilm-forming isolates are associated with the ability to survive in hospital environments, on medical implants, and in patient surgical wounds (5). The burden of multiderrug resistance in Gram-negative bacilli (GNB) now represents a daily issue for the management of antimicrobial therapy in intensive care unit (ICU) patients (6). Moreover, infections due to carbapenem-resistant (CR) Gram-negative pathogens have been reported from many countries with variable prevalence and associated morbidity and mortality (7). Present study was designed to evaluate biofilm formation among imipenem resistant in *Klebsiella pneumoniae* isolates from some hospitals in Baghdad city.

**MATERIALS AND METHODS**

**Bacterial Isolates:** A total of 343 different clinical specimens were collected from some hospitals in Baghdad city between October 2015 and March 2016 for isolation *Klebsiella pneumoniae* isolates, the isolates were identified using conventional biochemical tests and vitek 2 system.

**Antibiotic susceptibility testing, MIC to imipenem and phenotypic detection of ESβLs production:** These tests were done by Vitek-2 system using antibiotic sensitivity test number (AST-GN 69) and (AST-N222) cards according to the manufacturer’s instructions, which included antimicrobial agents as follows: Amikacin (AK), Amoxicillin/clavulanic-acid (AUG), Ampicillin (AM), Ampicillin/sulbactam (SAM), Aztreonam (AZT), Cefazolin (CZ), Cefepime (CPM), Cefotaxime (CTX), Cefazidime (CAZ), Ceftriaxone (CRO), Ciprofloxacin (CIP), Doxycycline (DXT), Gentamicin (GM), Imipenem (IMI), Levofloxacin (LEV), Meropenem (MEM), Nitrofurantoin (NIT), Piperacillin (PRL), Piperacillin/tazobactam (PTZ), Tetracycline (T) Ticaricillin/clavulanate (TIM), Tobramycin (TM), Trimethoprim/sulphamethoxazole (TS).

**Detection for Metallo-β lactamase (MβLs):** Disk synergy test used to test isolates to investigate the production of MβLs, the test was done according to (8) as follows: Tested isolates were inoculated according to Kirby-Bauer method onto plates of Mueller-Hinton agar media. Two discs of Imipenem antibiotic were placed on the plate; 5 µl of EDTA solution (final concentration is 0.5 M) was added to one of them. The inhibition zones of the imipenem and imipenem - EDTA discs were compared after 16-18 hr. of incubation at 35°C. An increase in the zone size of at least 7 mm around the imipenem - EDTA disc more than other was considered as produced isolates of MβLs.

**Detection of Biofilm Formation:** In the present study, we screened the isolates for their ability to form biofilm by micro titer plate according to the method described by (9). Briefly, *K. pneumoniae* isolates were grown overnight in Luria-Bertani broth+0.25 % glucose (LBG) prepared at 37 °C. On the next day, the culture was diluted 1:50 in freshly prepared LBG pre-warmed to 37°C. Then, 200 ml of this suspension was used to inoculate sterile 96-well polystyrene microtitre plates, followed by incubation at 37 °C for 72 h. After three washes with PBS prepsred any remaining biofilm was stained with 200 µL of crystal violet 1 % (w/v) for 30 min after drying at room temperature for 15 min. Washed with PBS again. After drying at room temperature, the dye bound to the adherent cells was resolubilized with 200 ml ethanol:acetone (80:20, v/v) and OD570 quantified using an ELISA reader. Each assay was performed in triplicate and repeated three times. The adherence capabilities of the test isolates were classified into four categories; OD above the mean optical density of the negative control (contained broth only) was considered as the cut-off optical density (ODc). Isolates were classified as follows:

- If OD ≤ ODc, the bacteria were non-adherent.
- If ODc < OD ≤ 2×ODc, the bacteria were weakly adherent.
- If 2×ODc < OD ≤ 4×ODc, the bacteria were moderately adherent.
- If 4×ODc < OD, the bacteria were strongly adherent.

Biofilm formation was characterized as: non-adherence tendency (−), weak (+), medium (+++) and strong (++++).

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Biofilm formation was characterized as: non-adherence tendency (−), weak (+), medium (+++) and strong (++++).
The imipenem-resistant *K. pneumoniae* isolates were screened for the presence of the *ompA* and *bap* genes by using primers (table 1). DNA genomes from isolates were extracted by using a commercial purification system (Genomic DNA Purification Kit) and PCR was used to amplify genes. PCR mixture was set up for each gene alone in a total volume of 25 µl included 12.5 µl of Go Taq Green Master Mix, 1.5 µl of each primer (10 picomole/µl) and 4 µl of template DNA. The volume remaining was completed with sterile nuclease free water. Negative control was used with all PCR experiments, which it contained all material except DNA.

PCR reaction tubes were vortexed and finally placed into thermocycler PCR instrument. The PCR conditions including: Initial denaturation 94°C (3 min), denaturation 94°C (1 min ), annealing 54°C (1 min ) and extension72°C(1 min 30 sec) in 30 cycles. Final extension 72°C (10 min).

**RESULTS AND DISCUSSION**

In this study, a total of 343 different clinical specimens were collected from some hospitals in Baghdad city. 39 isolate (22.94 %) *Klebsiella pneumoniae* was isolated from these specimens. It was clear that it could be found as causative agent of RTI (sputum) 11(28.20%), also it is mainly associated with blood samples 9(23.08%); beside to 7(17.95%) from burns and 6(15.38%) from wound swabs; with low percentage from urine samples 9(23.08%); beside to 7(17.95%) from burns and (sputum) 11(28.20%), also it is mainly associated with blood samples was clear that it could be found as causative agent of RTI (%).

For, the age and sex distribution of the infected patients with *K. pneumoniae* was summarized in figure (1). Among the infected patients which their ages ranged from less than 1 month to 80 years, the lowest incidence was among the (71-80) years old age group (5.13%) (male, 5.13% and female, 0%), while, the highest incidence was among the (51-60) years old age group (23.08%) (male,17.95% and female,5.13%). The figure also shows that the incidence was higher among males (74.37%) than that of females (25.63%).

Study done by (14) found that *K.pneumoniae* colonization in females were slightly less than the males. There was a clear indication of the increase in colonization in age group of 26-50, then 51 and above, and 6-25 when compared with 0-5 age group. While, (15) found the maximum number of bacteria 177 (26.4%) and 153 (22.8%) were isolated in the age groups of 46-60 years and more than 60 years respectively. The lowest incidence was among the (16-30) years old age group 63 (9.4%).

In the current study, for frequency of positive *K. pneumoniae* isolates was higher in Respiratory care unit (RCU); 12(30.77%) followed by Leukemia and Internalization department; 7(17.95%), Burns department; 6(15.38%), Surgical department; 5(12.82%), and finally 2(5.13%) from all other departments of hospitals; while no Frequency of positive isolates in (ICU). While, (16) revealed that *K. pneumoniae* isolates (n=3449) were more commonly isolated from internal medicine departments (1473; 43 %), followed by surgical departments (696;20%), intensive care units (ICU; 611; 18 %), emergency department (405; 12%) and paediatric departments (264; 8 %). On the other hand, *K. pneumoniae* isolates in current study showed a varied levels of resistance rate to antibiotic, with a highest resistance rate reached to (100%) for ampicillin, (89.74%) piperacillin, (84.62%) for cefazolin, cefazidime and cefepime, (82.05%) for ampicillin/sulbactam, ceftriaxone, aztreonam and cefotaxime, (76.92%) for gentamicin, (71.79%) for ticarcillin/clavulanic-acid, (66.67%) for amoxicillin/clavulanic-acid and tobramycin, (61.54%) for pepsicillin / tazobactam, amikacin and trimethoprim/sulfamethoxazole, then moderate resistance rate reached to (48.72%) for nitrofurantain, (30.77%) for tetracyclin and doxycline, and recorded lowest resistance to ciprofloxacin and levofloxcin reached to (10.26%) and (7.69%), respectively (table 2).

### Table 1: Primers used for detection of genes encoding biofilm for *K. pneumoniae* isolates.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence of primers (5'——3')</th>
<th>Product size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ompA</td>
<td>F: GCTACTATGCTTGTTGCTGT</td>
<td>1023</td>
<td>(9)</td>
</tr>
<tr>
<td></td>
<td>R: CGCTTCTTGACCAAGGTGGAAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>bap</td>
<td>F: ATGCCGAGATACAAATATT</td>
<td>1449</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R: GTCAATCGFAAAGGTAAACG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Figure 1:** Distribution of *K.pneumoniae* according to age and sex
Table 2: Antimicrobial sensitivity test results of *K. pneumoniae* isolates.

<table>
<thead>
<tr>
<th>Antimicrobial agents</th>
<th>Resistance (%)</th>
<th>Antimicrobial agents</th>
<th>Resistance (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amikacin</td>
<td>24(61.54)</td>
<td>Gentamicin</td>
<td>30(76.92)</td>
</tr>
<tr>
<td>Amoxicillin/clavulanic-acid</td>
<td>28(66.67)</td>
<td>Imipenem</td>
<td>23(58.974)</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>39(100)</td>
<td>Levofoxacin</td>
<td>3(7.69)</td>
</tr>
<tr>
<td>Aztreonam</td>
<td>32(82.05)</td>
<td>Meropenem</td>
<td>22(56.41)</td>
</tr>
<tr>
<td>Ampicillin/sulbactam</td>
<td>32(82.05)</td>
<td>Nitrofurantoin</td>
<td>19(48.72)</td>
</tr>
<tr>
<td>Cefazolin</td>
<td>33(84.62)</td>
<td>Piperacillin</td>
<td>35(89.74)</td>
</tr>
<tr>
<td>Cefepime</td>
<td>33(84.62)</td>
<td>Piperacillin/tazobactam</td>
<td>24(61.54)</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>32(82.05)</td>
<td>Tetracycline</td>
<td>12(30.77)</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>33(84.62)</td>
<td>Ticarcillin/clavulanate</td>
<td>28(71.8)</td>
</tr>
<tr>
<td>Ceftriaxime</td>
<td>32(82.05)</td>
<td>Tobramycin</td>
<td>26(66.67)</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>4(10.26)</td>
<td>Trimethoprim/sulphamethoxazole</td>
<td>24(61.54)</td>
</tr>
<tr>
<td>Doxycycline</td>
<td></td>
<td></td>
<td>12(30.77)</td>
</tr>
</tbody>
</table>


The isolate were not tested for susceptibility to any agent listed in the category

The isolates were sensitive to all agents listed in the category

The isolates were resistant to some, but not all agents listed in the category

The isolates were resistant to all agents listed in the category

Figure 2: B) The percentages of MDR, XDR, and PDR levels among 39 *K.pneumoniae*
The results of antimicrobial susceptibility test of K. pneumoniae in current study were consistent with other results as the study done by (17) who showed that the resistance rate of K. pneumoniae isolates toward the antimicrobials was (92.2%) to piperacillin, followed by (59.2%) to nitrofurantoin, (50%) to gentamicin and (48.5%) to tobramycin. Other local study by (18) noticed that the highest resistance for K. pneumoniae was (100%) to ampicillin, cefazidime, ceftriaxone and cepfime followed by levofloxacin and tetracycline (66.6%), tobramycin and ciprofloxacin (50%). Recently, the study by (19) obtained the antimicrobial resistance patterns of isolates was (100%) resistance for ampicillin, (87.6%) cefazolin, (85.71%) cefazidine and trimethoprim/sulf., (83.92%) cefepime and ceftriaxone, (78.58%) ampicillin/sulf., (66.07%) gentamicin (57.14%) tobramycin. It was clear that tetracyclines, fluoroquinolones class still effective against isolates rather than other classes, this finding was also supported by others studies as the study done by (20) who obtained the resistance rate of K. pneumoniae to ciprofloxacin and tetracycline by (37.5%, and 31% resp.,

Interestingly, results in present study showed approximately that all 39 (100%) K. pneumoniae isolates were multidrug resistant isolates (MDR). Also, study done by (21) observed that ≥80% of K. pneumoniae isolates were resistant to third generation cephalosporin’s and other antibiotics, making them multidrug resistant (MDR) strains. Consequently, it was necessary to classify these isolates into four categories for the absence of cephemcyclins category including (MDR, MDR possible XDR, XDR, and XDR possible PDR). This classification was applied in twenty two different antibiotic patterns designated arbitrarily from (Kp1 to Kp22) antibiotype (isolates showing intermediate levels of susceptibility were classified as resistant) (22), to define each isolate according to its resistance ability. Classification was done according to the criteria revealed by (23). Consequently, all of 39 K. pneumoniae clinical isolates were classified into MDR in first place, but some of them were converted to XDR or even PDR depending on their resistance profile as presented in figure (2-A and B).

Twenty one isolates (53.85%) were classified as MDR since they were resistant to ≥3 out of all antimicrobial categories that match the criteria used. Represented by pattern, including: Kp5, Kp6, Kp7, Kp9, Kp10,Kp11, Kp12, Kp13, Kp17,Kp18, Kp20, Kp21 and Kp22. Five isolates (12.82%) were considered as MDR possible XDR since they were resistant to 9/11 antimicrobial categories with the absence of cephemcyclin’s category, so there was a highly probability to be XDR since they resisted cephemcyclins category. Pattern Kp2, Kp8 and Kp19 covered this type of resistance. Eight isolates (20.51%) of K. pneumoniae isolates were considered as XDR since they were susceptible to only 2 out of all antimicrobial categories that match the criteria used. Therefore, XDR defined as non-susceptibility to at least one agent in all but remain susceptible to only one or two antimicrobial categories. Represented by patterns Kp3, Kp4, Kp14, Kp15 and Kp16.

Three isolates of K. pneumoniae showed Pattern Kp3 being resistant to all antimicrobial categories but sensitive to tetracyclines category and resistant to some, not all agents listed in the fluoro-quinolones and carbapenemes categories. Pattern Kp4 covered 2 isolates that they were resistant to all antimicrobial categories but resistant to some, not all agents listed in the Fluoroquinolones category. One isolate of K. pneumoniae showed Pattern Kp14 being resistant to all antimicrobial categories but sensitive to tetracyclines category. Pattern Kp15 covered one isolate that there was resistant to all antimicrobial categories but resistant to some, not all agents listed in the fluoroquinolones and aminoglycosides categories. Pattern Kp16 covered one isolate that there was resistant to all antimicrobial categories but resistant to some, not all agents listed in the fluoroquinolones and carbapenemes categories.

Finally, five isolates (12.82%) K. pneumoniae isolates showed Pattern Kp1, being resistant to all antimicrobial categories that match the criteria used were considered as XDR possible PDR with the absence of cephemcyclins category, so there was a highly probability to be PDR since they resisted cephemcyclins. Thus, PDR was defined as non-susceptibility to all agents in all antimicrobial categories for Enterobacteriaceae.

Study done by (24) revealed that from the total of 223 non-repetitive patients who had K. pneumoniae isolates, among which, 137 (61.4%) were MDR isolates, 49 (22.0%) were XDR isolates, 4 (1.8%) were PDR isolates, and 33 (14.8%) were other types of isolates. Other study by (25) obtained a total of 163 K. pneumoniae isolates. The percentage of MDR, XDR and PDR and other types of K. pneumoniae isolates were 104(63.8%), 34(20.9%), 3(1.8%) and 22(13.5%), respectively. While, local study by Aljanaby and Alhasani (26) found from the total of 32 K. pneumoniae isolates 27(84.38%) were MDR, 4(12.5%) were XDR and 1(3.12%) were PDR.

The determination of the MIC for imipenem antibiotic was done for isolates, the numbers of K. pneumoniae isolates that gave MIC values more than break point for imipenem were 23 (58.97%) and these values assure resistance of studied K. pneumoniae isolates for this antibiotic; while 16(41.03%) isolates that gave MIC values less than break point including: 15(38.462%) isolates that gave MIC values equal to or less than (1μg/ml) and these values assure susceptibility of studied K. pneumoniae isolates for this antibiotic and 1(2.564%) isolates that gave MIC values equal to (2μg/ml). The present study revealed that the MIC values for imipenem resistant isolates ranged from (8ug/ml to ≥16μg/ml) and the lowest MIC (0.25 μg/ml). The highest MIC (≥16 μg/ml) was achieved (22) isolates and MIC(8 μg/ml) one isolate, while, the lowest MIC (0.25µg/ml) was developed by the isolates(14), MIC (1ug/ml) one isolate and MIC (2ug/ml) one isolate.

On other hand, results of phenotypic detection of ES/βLs producing for K. pneumoniae isolates obtained that out of 39 K. pneumoniae isolates, 9(23.08%) isolates were gave positive results for ES/βLs producing. Furthermore, out of (9) positive ES/βLs producing K. pneumoniae isolates, (4) isolates from blood, (2) from urine and one isolate from burn, Sputum and wound (for each).

Results observed in current study for the ES/βLs producing isolates of K. pneumoniae agreed with other local study as (27) who found that out of 28 isolates of K. pneumoniae, six (21.4%) were positive for ES/βL production. While, (18) revealed that percentage of ES/βLs producing isolates was I(136.7%).

Eleven (47.83%) K. pneumoniae isolates in current study were gave positive results for M/βLs production, 4 isolates from blood, 2 from burn, Sputum, wound (for each) and one isolate from ear swab. Study by Anoar (28) observed lowest positive results for M/βLs producing K. pneumoniae isolates reached to 7 (3.95%), 3(1.69%) and 18 (10.17%) by using DDST, CDT and MHT for detection this test. While, (29) observed from a total of 97 isolates were tested for M/βL production, the percentage of production was in 34 isolates (35.1%) by MHT. Of these 34, 21 isolates (21.6%) were also positive by both the combined disk test and M/βL Etest. In addition, results obtained that out of 39 K. pneumoniae, 22(56.41%) were biofilm producers isolates by using micro titer plate (MTP). The current results recorded that the highly percentage of biofilm production and imipenem resistant for K. pneumoniae isolates was located within the weak and then moderate patterns. Therefore, these isolates may be need this resistance but in this type of patterns of biofilm formation more than other patterns, as shown in table (3). From weak pattern of
biofilm producers for *K. pneumoniae*, the results showed that the highly number of biofilm producers isolates and biofilm producers imipenem resistance isolates of *K. pneumoniae* was also isolated from sputum specimens. Others studies such as (30) observed that 6(100%) *K. pneumoniae* isolates formed biofilm including: 2(33.33) strong, 1(16.66) moderate, 3(50) weak by MTP and these results showed that the association of biofilm production among carbapenem resistant isolates obtained from chronic wound infections.

**Table 3:** Number and percentage of biofilm formation and imipenem resistance for all clinical isolates for patterns of MTP method

<table>
<thead>
<tr>
<th>Total Number of Isolates</th>
<th>Biofilm Formation Isolates</th>
<th>(R) from 23 Imipenem Resistant Isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Patterns</td>
<td>N(%)</td>
</tr>
<tr>
<td>39 <em>K. pneumoniae</em></td>
<td>Moderate</td>
<td>1(2.56)</td>
</tr>
<tr>
<td></td>
<td>Weak</td>
<td>21(53.85)</td>
</tr>
<tr>
<td></td>
<td>Non-producer</td>
<td>17(43.59)</td>
</tr>
</tbody>
</table>

One of the present study objectives is to detect the existence and prevalence of common biofilm encoding genes among all clinical isolates by monoplex PCR technique. The results showed that the *ompA* gene was presence in 9(23.08 %) isolates of *K. pneumoniae* (Fig 3). While, all of *K. pneumoniae* isolates didn’t have bap gene. Study by (31) obtained that OMPs are implicated in the maintenance of cell envelope integrity and demonstr-ated that the *K. pneumoniae* OMPs confer protection not only against serum killing, but also against neutrophil phagocytosis and killing.

**CONCLUSION**

In conclusion, the results show that the highly number of this weak biofilm producers isolates and biofilm producers imipenem resistance isolates was isolated from sputum specimens.

**Acknowledgments**

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