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

Fatima S. Ali, Sawsan H. Authman, Mohammed F. AL Marjani[*]

Department of Biology, College of Science, Mustansiriyah University, Baghdad, Iraq

Abstract

Klebsiella pneumoniae is a prominent opportunistic pathogen for community-acquired but are much less common than nosocomial 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 (23.08%). 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 then moderate patterns. The *ompA* gene was presence 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 **(1).**

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 lipo-polysaccharides 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 multidrug 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 was 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 antimi-crobial agents as follows: Amikacin (AK), Amoxicillin/clavulanic-acid (AUG), Ampicillin (AM), Ampicillin/sulbactam (SAM), Aztreonam (AZT), Cefazolin (CZ), Cefepime (CPM), Cefotaxime (CTX), Ceftazidime (CAZ), Ceftriaxone (CRO), Ciprofloxacin (CIP), Doxycycline (DXT), Gentamicin (GM), Imipenem (IMI), Levofloxacin (LEV), Meropenem (MEM), Nitrofurantoin (NIT), Piperacillin (PRL), Piperacillin/tazobactam (PTZ), Tetracycline (T) Ticarcillin/clavulanate (TIM), Tobramycin (TM), Trimethoprime/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 35C°. 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 prepersed any remaining biofilm was stained with 200 μL of crystal violet 1 % (w/v) for 30 min after drying at room tempreture for 15 min .Washed with PBS again. After drying at room tempreture, 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 (+++).

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

Primer name	Sequence of primers (5'-----3')	Product size (bp)	Reference
ompA	F: GCTACTATGCTTGTTGCTGCT R: CGCTTCTTGACCAGGTTGAAC	1023	**(9)**
bap	F: ATGCCTGAGATACAAATTAT R: GTCAATCGTAAAGGTAACG	1449	
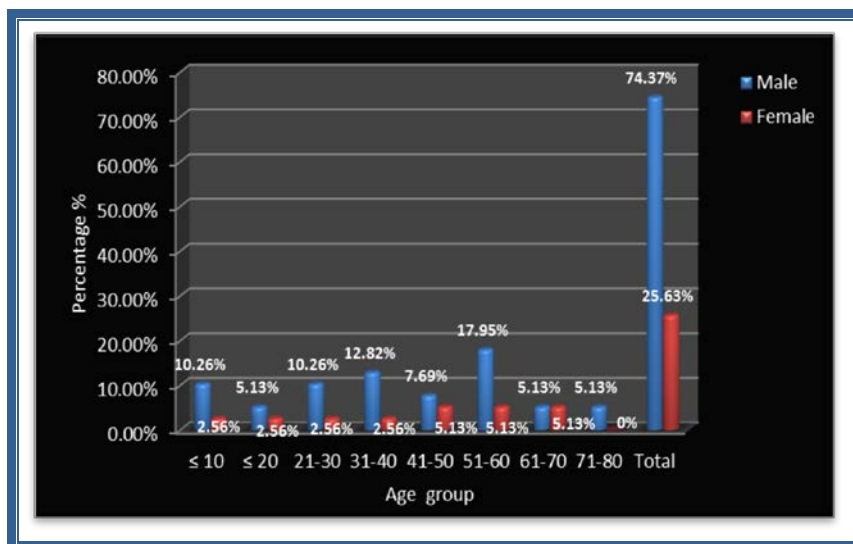
Figure 1: Distribution of *K.pneumoniae* according to age and sex

Molecular detection of *omp*A and *bap* genes

The imipenem-resistant *K. pneumoniae* isolates were screened for the presence of the *omp*A 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 12.5µl 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 (3min), denaturation 94°C (1min), annealing 54°C (1min) and extension72°C(1min 30 sec) in 30 cycles. Final extension 72°C (10min).

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 and ear infection which showed, 4(10.26%); 2(5.13%), respectively.

In the present study culture positivity for *K. pneumoniae* in sputum was good agreement with results of studies by **(10, 11, 12)** were absorved that isolation percentage reached to (28.4%), (30.90%), (25.8%) in sputum, respectively. While, study by **(13)** found the highest isolates of *K. pneumoniae* was from wounds (40), blood (39), urine (33) followed by sputum (27), and low isolated from ear (8) and fluid (2).

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, ceftazdime 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 pepracillin / tazobactam, amikacin and trimethoprim/sulfamethoxazole, then moderate resistance rate reached to (48.72%) for nitrofurantation, (30.77%) for tetracyclin and doxycycline, and recorded lowest resistance to ciprofloxacin and levofloxacin reached to (10.26%) and (7.69%), respectively (table 2).

Table 2: Antimicrobial sensitivity test results of *K.   pneumoniae* isolates.

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

Pattern	No(39)	A	B	C	D	E	F	G	H	I	J	K	L	Classification
Kp1	5													XDR,possible PDR
Kp2	3													MDR,possible XDR
Kp3	3													XDR
Kp4	2													XDR
Kp5	1													MDR
Kp6	1													MDR
Kp7	5													MDR
Kp8	1													MDR,possible XDR
Kp9	1													MDR
Kp10	1													MDR
Kp11	1													MDR
Kp12	2													MDR
Kp13	1													MDR
Kp14	1													XDR
Kp15	1													XDR
Kp16	1													XDR
Kp17	1													MDR
Kp18	1													MDR
Kp19	1													MDR,possible XDR
Kp20	2													MDR
Kp21	1													MDR
Kp22	3													MDR

	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: A) Classification of 39 *K. pneumoniae* isolates into three antibiotic resistance level (MDR, XDR, and PDR) by calculating their antimicrobial susceptibility patterns toward eleven antibiotic categories tested in current study according to criteria revealed by Magiorakos *et al.* (2012). **Antibiotic categories:- A:** Aminoglycosides **B:**Antipseudomonal penicillins+β-lactamase inhibitors, **C:** Carbapenems, **D:** Non-extended spectrum cephalosporins; 1st and2nd generation cephalosporins, **E:** Extended-spectrum cephalosporins; 3rd and 4th generation cephalosporins, **F:** Fluoroquinolones, **G:** Folate pathway inhibitors, **H:** Monobactams, **I:**Penicillins, **J:** Penicillins+β-lactamase inhibitors **K:**Tetracyclines, **L:** Cephamycins **(absence)**.

(12.82%) MDR possible XDR

(20.51%) XDR

(53.85%) MDR

39(100%) K.pneumonia MDR
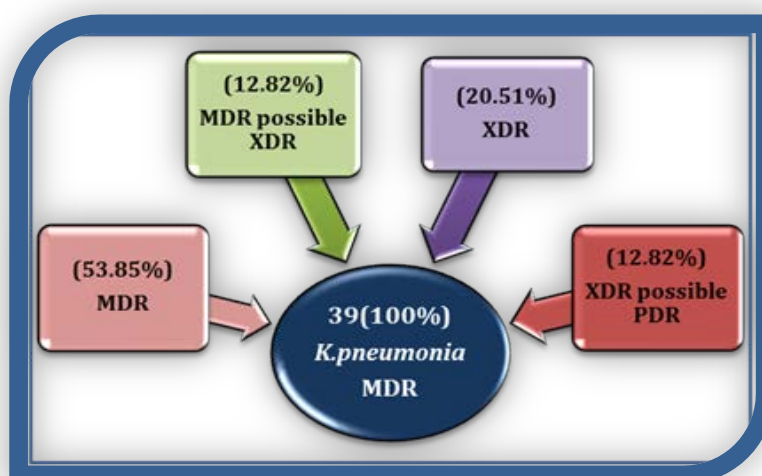
(12.82%) XDR possible PDR

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, ceftazidime, ceftriaxone and cefepime 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%) ceftazdime and trimethoprim/sulf., (83.92%) cefepime and ceftriaxone, (78.58%) ampicillin/sulb., (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 cephamycins category including (MDR, MDR possible XDR, XDR, and XDR possible PDR}. This classification was applied in twenty two different antibiotype patterns designated arbitrarily from (**Kp1** to **Kp22**) antibiotypes (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 cephamycins category, so there was a highly probability to be XDR since they resisted cephamycins 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 categoryes. 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 carbapenems 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 aminoglycosides and carbapenems 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 cephamycins category, so there was a highly probability to be PDR since they resisted cephamycins. 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 (8μg/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 (1μg/ml) one isolate and MIC (2μg/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 ESβL production. While, **(18)** revealed that percentage of ESβLs producing isolates was11(36.7%).

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 MβLs 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 MβL 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 MβL 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

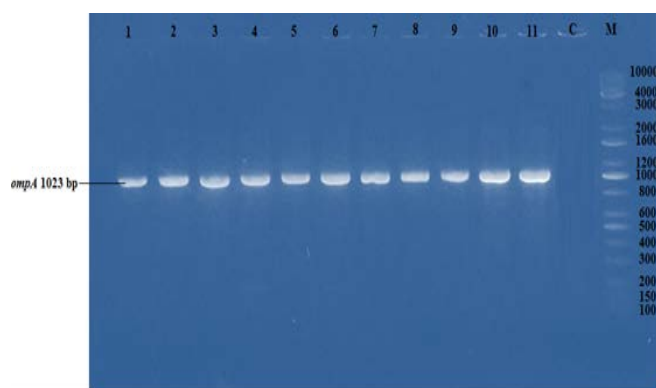| Total Number of Isolates	Biofilm Formation Isolates		(R) from 23 Imipenem Resistant
	Patterns	N(%)	N(%)
39 *K.pneumoniae*	Moderate	1(2.56)	1(4.35)
	Weak	21(53.85)	16(69.56)
	Non-producer	17(43.59)	6(26.09)

N: Number

Figure 3: Detection of *ompA* gene by monoplex PCR of *K. pneumoniae*; Lanes 1-11,*K.pneumoniae* isolates; Lane C, Negative control; Lane M, 100 bp DNA ladder, on agarose gel (1%) at 5 V/cm for 1 h, stained with RedSafe dye and visualized on a UV transiluminator documentation system.

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* (Fiq 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.

REFERENCES

1. Shon, A. S.; Bajwa, R. P. S. and Russo, T. A. Hypervirulent (hypermucoviscous) *Klebsiella pneumoniae*: A new and dangerous breed. Virulence, 2013, 4(2), 107–118.
2. Díaz, M. A.; Hernández, J. R.; Martínez-Martínez, L.; Rodríguez-Baño, J. and Pascual, A. Extended-spectrum beta-lactamase-producing *Escherichia coli* and *Klebsiella pneumoniae* in Spanish hospitals: 2nd multicenter study (GEIH-BLEE project, 2006). Enferm. Infect. Microbiol. Clin. 2009, 27:503–510.
3. Lee, C.-R.; Lee, J. H.; Park, K. S.; Jeon, J. H.; Kim, Y. B.; Cha, C.-J; Jeong, B. C.; Lee, S.H. "Antimicrobial resistance of hypervirulent *Klebsiella pneumoniae*: epidemiology, hypervirulence-associated determinants, and resistance mechanisms". Front. Cell. Infect. Microbiol. 2017, 7: 483.
4. Vuotto, C.; Longo, F.; Balice, M. P.; Donelli, G., and Varaldo, P. E. Antibiotic Resistance Related to Biofilm Formation in *Klebsiella pneumoniae*. Pathogens. 2014, 3(3), 743–758.
5. Hall-Stoodley, L.; Costerton, J.W. and Stoodley, P. Bacterial biofilms: from the natural environment to infectious diseases. Nat. Rev. Microbiol. 2004, 2: 95-108.
6. Ruppé, É.; Woerther,P-L. and Barbier,F. Mechanisms of antimicrobial resistance in Gram‑negative bacilli. Ann. Intensive Care. 2015, 5:21.
7. Xu, A.; Zheng, B.; Xu, Y.C.; Huang, Z.G.; Zhong, N.S. and Zhuo, C. National epidemiology of carbapenem-resistant and extensively drug-resistant Gram-negative bacteria isolated from blood samples in China in 2013. Clin Microbiol. Infect. 2016, 22(Suppl 1):S1–8.
8. Bashir, D.; Thokar, M. A.; Fomda, B. A.; Bashir, G.; Zahoor, D.; Ahmad, S. and Toboli, A. S. Detection of metallo - beta-lactamase (MBL) producing *Pseudomonas aeruginosa* at a tertiary care hospital in Kashmir. Afr. J. Microbiol. Res. 2011, 5(2), 164-172.
9. Badmasti,F.; Siadat,S.D.; Bouzari,S.; Ajdary,S. and Shahcheraghi,F. Molecular detection of genes related to biofilm formation in multidrug-resistant *Acinetobacter baumannii* isolated from clinical settings. J. Med. Microbiol. 2015, 64:559-564.
10. Manikandan,C. and Amsath,A. Antibiotic susceptibility of bacterial strains isolated from patients with respiratory tract infections. Int. J. Pure Appl. Zool. 2013, 1(1): 61-69.
11. Ravichitra, K.N.; Prakash, P.H.; Subbarayudu, S. and Rao,U.S. Isolation and antibiotic sensitivity of *Klebsiella pneumoniae* from pus, sputum and urine samples. Int.J.Curr. Microbiol. App.Sci. 2014, 3(3): 115-119.
12. Mansoor, S.; Qadir, S.; Javed, S.; Jehan,F.S. and Kundi,M. Antimicrobial susceptibility of organisms isolated from sputum culture of Karachi, Pakistan. Pak. J. Pharm. Sci. 2017, 30 (6): 2247-2252.
13. Acheampong,D.O.; Boamponsem,L.K. and Feglo, P.K. Occurrence and species distribution of *Klebsiella* Isolates: A case study at Komfo Anokye teaching hospital (Kath) in Ghana. Pelagia Research Library. Adv. Appl. Sci. Res. 2011, 2 (4):187-193.
14. Rampure,R.; Gangane,R.; Oli,A.K. and Chandrakanth. R.k. Prevalence of MDR-ESBL pro-ducing *Klebsiella pneumoniae* isolated from clinical Samples. J. Microbiol. Biotech. Res. 2013, 3(1):32-39.
15. Das, P.K. and Debnath, J. Prevalence and Antibiotic susceptibility pattern of *Klebsiella pneu-moniae* isolated from various clinical specimen in a tertiary care hospital of Tripura. Sch. Acad. J. Biosci. 2015, 3(11):931-935.
16. Spyropoulou,A.; Papadimitriou-Olivgeris,M.; Bartzavali,C.; Vamvakopoulou,S.; Marangos, M.; Spiliopoulou,T.; Anastassiou,E.D. and Christofidou,M. A ten-year surveillance study of carbapenemase producing *Klebsiella pneumoniae* in a tertiary care Greek university hospital: predominance of KPC- over VIM- or NDM-producing isolates. J. Med. Microbiol. 2016, 65: 240–246.
17. Al-Sehlawi, Z. S. R. Occurrence and Characterization of AmpC β-Lactamases in *Klebsiella pneumoniae*I solated from Some Medical Centers in Najaf. Ph.D. thesis. Biology department. College of Science, University of Babylon. 2012.
18. Al-Azawi,I.H.S. Detection of Extended Spectrum Beta Lactamase (ESBL) in *Klebseilla pneumoniae* Isolated from Urinary Tract Infections. AL-Qadisiya Med. J. 2014, 10(18):168-173.
19. Hussein,J.D. and Hamed,S.L. Comparison of Three Phenotypic Methods for Detection of Extended-Spectrum β-Lactamase (ESBL) Producing *Klebsiella Pneumoniae*. J. Glob. Phar. Tech. 2017, 09 (9): 157-165.
20. Chakraborty,S.; Mohsina,K.; Sarker,P.K.; Alam,MD.Z.; Karim,M.I.A. and Sayem,S. M.A. Prevalence, antibiotic susceptibility profiles and ESBL production in *Klebsiella pneumoniae* and *Klebsiella oxytoca* among hospitalized patients. Period. Biol. 2016, 118(1):53-58.

21. Fouzia, B. and Damle, A.S. Prevalence and Characterization of Extended Spectrum Beta-Lactamase Production in Clinical Isolates of *Klebsiella pneumonia*. J. Med. Microb. Diagn. 2015, 4(2):1-6.
22. CLSI, (Clinical and Laboratory Standards Institute). Performance Standards for Antimicro-bial Susceptibility Testing; Twenty-Fifth Informational Supplement (M100-S25). CLSI Document. Pennsylvania: Wayne. 2015.
23. Magiorakos, A. P.; Srinivasan, A. and Monnet, D. L. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. Clin. Micobiol. 2012, 18:268-281.
24. Li,B.; Yi,Y.; Wang,Q.; Woo,P.C.Y.; Tan,L.; Jing,H.; Gao,G.F. and Liu,C.H. Analysis of Drug Resistance Determinants in *Klebsiella pneumoniae* Isolates from a Tertiary-Care Hospital in Beijing, China. PLoS ONE. 2012, 7(7): e42280.
25. Wang,Q.; Li,B.; Tsang,A.K.L.; Yi,Y.; Woo,P.C.Y. and Liu,C.H. Genotypic Analysis of *Klebsiella pneumoniae* Isolates in a Beijing Hospital Reveals High Genetic Diversity and Clonal Population Structure of Drug-Resistant Isolates. PLoS ONE. 2013, 8(2): e57091.
26. Aljanaby,A.A.J. and Alhasani,A.H.A. Virulence factors and antibiotic susceptibility patterns of multidrug resistance *Klebsiella pneumoniae* isolated from different clinical infections. Afr. J. Microbiol. Res. 2016, 10(22):829-843.
27. Al-Mussawi,A.A. Detection of Extended Spectrum Beta Lactamase (ESBL) Producing *Klebsiella pneumoniae* Associated with Tuberculosis Suspected Patients in Basra Governorate, South of Iraq. Amer. J. Microbiol. Res. 2015, 3(2): 59-61
28. Anoar,K.A.; Ali,F.A. and Omer,S.A. Phenotypic Detection of Metallo -lactamase Enzyme among Gram Negative Bacteria Isolated From Burn Patients In Sulaimani, Iraq. Int. J. Curr. Microbiol. App. Sci. 2014, 3(3): 315-325.
29. Kumar,M.; Dutta,R.; Saxena,S. and Singhal,S. Evaluation of Different Phenotypic Methods to Detect ESBL and MBL Production in *Escherichia coli* and *Klebsiella* Species with Detection of NDM-1 by PCR. Int. J. Microbiol. Res. 2015, 6 (1): 20-26.
30. Swarna, S.R.; Swedha, and Gomathi, S. Biofilm Production in Carbapenem Resistant Isolates from Chronic Wound Infections. Int. J. Med. Res. Health Sci. 2017, 6(2): 61-67.
31. Hsieh,P-F.; Liu,J-Y.; Pan,Y-J.; Wu,M-C.; Lin,T-L.; Huang,Y-T. and Wang,J-T. *Klebsiella pneumoniae* Peptidoglycan-Associated Lipoprotein and Murein Lipoprotein Contribute to Serum Resistance,Antiphagocytosis,and Proinflammatory Cytokine Stimulation.J.Infect.Dis. 2013, 208:1580-1589