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DETERMINATION OF *ACRAB, TOLC* EFFLUX PUMPS GENES IN STRONG BIOFILM FORMER *KLEBSIELLA PNEUMONIA*E

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Abstract: *Klebsiella pneumonia*e (*K. pneumoniae*) is a gram-negative bacilli, encapsulated, facultative anaerobic, lactose-fermenting, non-mobile, rod- shaped bacterium, they do have a high tendency to become antibiotic resistant. **Aims of study**: Detection of *acrAB, tolC* efflux pumps genes in strong biofilm of *K. pneumoniae*. **Methods**: Micro-titer plate (MtP) quantitative method used to determine the biofilm production. The phenotypic and qualitative finding of the efflux pump was achieved by Ethidium Bromide-agar Cart Wheel Method, All isolates were screened for their antibiotic resistance against a selected (35) clinically antibiotics by VITEK MIC method. **Results**: *K. pneumoniae* was mainly isolated from urine samples 52% followed by sputum 16%, swap 14%, body fluids 10%, bedsore 5% and folly's catheters were 3%, the distribution of *K. pneumoniae* isolates according to sex showed that 55% occurs in males and 45% of isolates were from females. Most of the *K. pneumoniae* isolates were collected from patients with age group ranged from (1 to 80 years). All of isolates were biofilm producers, with (43%) isolates as strong, (32%) isolate as moderate and (25%) isolates as weak biofilm producers. DNA has been extracted from (51 isolates) that are only strong biofilm and positive efflux pumps. Highly EP founded in the bacteria with different concentration of EtBr 85 (85.00%), 91 (91.00%), 94 (94.00%), and 94 (94.00%) with EtBr (0, 0.5, 1, 1.5, 2 mg/l) respectively. *K.pneumoniae* is found to be resistant to many antibiotics MDR 33% and XDR 67%. The results of efflux pumps genes were *acrAB* (72.5%) and *tolC* was (43%).

Keywords: *K. pneumoniae;* biofilm formation; Phenotypic; genotypic; *acrAB; tolC.*

Introduction: *Klebsiella pneumoniae* (*K. pneumoniae*) is gram-negative bacilli, nosocomial, community acquired and multi-drug resistant (MDR) (1), belong to the Enterobacteriaceae family, a remarkable medical human pathogen in the mucosal surface of mammals and the environment (2 and 3). In humans, *K. pneumoniae* typically colonize the oropharynx and the gastrointestinal tract where it can easily enter the circulation and other tissues causing infections. It contributes to the high frequency of opportunistic infections problem among neonates and infants, elderly individuals within the healthcare setting (4). It occurs among patients with malnutrition, children, immunocompromised conditions such as: bladder neuropathy or diabetes mellitus (5). *K. pneumoniae* is encapsulated (surrounded by a capsule, which increases its virulence by acting as a physical barrier to evade the host's immune response involved in resistance to phagocytosis, and may participate in the resistance of complement system, they do have a high tendency to become antibiotic resistant. These bacteria are harmless in human intestines or stool. But if they spread to another part of the body, such as lungs, they can cause severe infections (6). Biofilm are

community of bacteria that are embedded within an extracellular matrix, adhere to each other and or to a surface. The matrix contents of a biofilm may differ in quantity and nature of its constituents, depending on the environmental factors (7). The biofilm-forming ability by *K. pneumoniae* allows the protection of strains from the host immune response and antibiotics in MDR isolates. Life of a biofilm represents the predominate mode of growth for microbes in most environments. Biofilm microbes are typically surrounded by an extracellular matrix (8). Efflux pumps are known as protein-based units that are capable of transporting of different toxins out of the cells, they are a cell membrane protein channels that are localized and embedded within the plasma membrane of the bacterium, that selectively admits or excludes noxious substances from the cytoplasm, such as drugs, chemicals, dyes, antiseptics detergents and compounds in bacteria, these pumps are found in all species of bacteria (9). *K. pneumoniae* adopt various mechanisms of drug resistance towards different classes of antimicrobial agents including efflux pumps, change in permeability of cell membrane, formation of certain enzymes, modification of targeted site, and adoption of alternative metabolic routes that are inactivated by antibiotics (10). The resistance results in a dramatically growing worldwide problem in regards to the choice of effective antibiotic protocol for hospital-acquired infections, the extensive use of antimicrobial compounds in the human clinical setting has been identified as one of the main causes for the emergence and is considered to be one of the root causes for selection of resistant bacteria (11).

Materials and Methods:

A total number of 100 samples was collected from different patient (bed sore, fluid, folly's catheter, sputum, swap and urine), in Baghdad city from December 2022 to February 2023, collected in brain heart infusion broth, cultured on Blood agar and MacConkey agar tested under microscopic examination, biochemical.

Biofilm used Micro-titer plate (MtP), done by (12, 13). Optical density (OD) readings were determined using micro-plate reader (ELISA reader) in (630nm) (14).

The phenotypic and qualitative measurement of the efflux pump was done by Ethidium Bromide-agar Cart-Wheel Method (15). Muller-Hinton agar plates containing (0, 0.5, 1, 1.5, 2 mg/l) of Ethidium Bromide (EtBr). The isolates that had efflux pumps did not show emission of fluorescence (+ve results), in contrast to the emission of fluorescence (-ve) under UV trans-illuminator (9).

VITEK-2 compact system is consists of 64 biochemical tests and 35 antibiotic tests, so it was used for confirmative identification of *K. pneumoniae* by using Gram negative (GN) card and Antibiotic sensitivity test card (AST-GN327).

Extraction of DNA based on Geneaid/Taiwan kit, then DNA collected and storage at−20°C to avoid degradation, until used for PCR. **The primers** were suspended by dissolving primers as shown in table (1) .PCR amplification mixture has been prepared according to the manufacturer's instructions (Bioneer/ Korea).

Table (1): primers

Target gene	Primer sequence (5′ to 3′)	Annealing temperature	References

		(°C)	
acrAB	F/ATCAGCGGCCGGATTGGTAAA R/CGGGTTCGGGAAAATAGCGG	53	(16)
tolC	F/ ATCAGCAACCCCGATCTGCGT R/ CCGGTGACTTGACGCAGTCCT	57	(16)

PCR conditions were as follow: Initial denaturing, annealing, extension steps and finally one cycle for the final extension (14), show in table (2 A, B).

Table (2 A, B): PCR program of all genes:

A: *AcrAB*

Phase	**Tm (°C)**	**Time**	**No. of cycle**
Initial Denaturation	94°C	5 min.	1 cycle
Denaturation -2	94°C	45 sec.	40 cycle
Annealing	53°C	35 sec.	
Extension-1	72°C	45 sec.	
Extension -2	72°C	7 min.	1 cycle

B: *TolC*

Phase	**Tm (°C)**	**Time**	**No. of cycle**
Initial Denaturation	94°C	5 min.	1 cycle
Denaturation -2	94°C	45sec.	35 cycle
Annealing	57°C	45sec.	
Extension-1	72°C	45sec.	
Extension -2	72°C	7 min.	1 cycle

Statistical analysis:

The Statistical Analysis System- SAS (2018) program was used to detect the effect of difference factors in study parameters. T-test and Least significant difference –LSD test (Analysis of Variation-ANOVA) was used to significant compare between means. Chi-square test was used to significant compare between percentage (0.05 and 0.01 probability in this study (17).

Results and Discussion:

The identification of (100) isolates in table (3) has been mainly from urine samples 52%, sputum

16%, swap 14%, body fluids 10%, bedsore 5% and folly's catheters were 3%, these finding are consistent with (17) who found that *K. pneumoniae* was isolated mainly from urine (95.7%), followed by burns (91.7%) and sputum (85.1%), these findings confirm that *K. pneumonia* plays a critical role in occurrence of UTI. However, these results are different from those obtained by (18) who found that the percentage of *K. pneumoniae* isolated from urine was 4% only. The percentage of *K. pneumoniae* isolated from sputum in the present study is the second highest percentage of samples (16%) this agrees with (19) who emphasized that *K. pneumoniae* typically colonizes human mucosal surfaces and for this reason, *K. pneumoniae* is considered to be the most common cause of hospital-acquired pneumonia.

Table (3): Distribution of sample study according to Sources

Sources	No	Percentage (%)
Urine	52	52.00
Sputum	16	16.00
Swap	14	14.00
Fluid	10	10.00
Bed sore	5	5.00
Folly Cath	3	3.00
Total	100	100%

Detection of Biofilm production by *K. pneumoniae*:

All of isolates were biofilm producers, with (43%) isolates as strong, (32%) isolate as moderate and (25%) isolates as weak biofilm producers, as shown in table and figure (1).The results of present study agree with results of (20, 21) who found that (85.6%) of *K. pneumoniae* isolates were biofilm producers, also the result has agreed with that of (22, 23) who revealed that (75%) isolates produce biofilm and (25%) were non-biofilm producers. This study has disagreed with another study by (22) who found that non-biofilm producers were more resistant to treatment than biofilm producers indicating that resistance may be related to other mechanisms than biofilm. Moreover, studies by (21 and 23) have reported that biofilm producers were more resistance than non-biofilm producers. Biofilms have major medical significance as they decrease the microbial susceptibility to antimicrobial agents, thus their virulence (26). Hospital and community acquired infections caused by *Klebsiella* are wide spread and are becoming difficult to treat due to antibiotic resistance and biofilm formation ability of this pathogen (27 and 28), until today, it has been shown that there is a significant correlation between MDR (multi-drug resistance) phenotype and the biofilm forming ability of *K. pneumoniae* isolates (25). The extensive use of antimicrobial agents led to a high prevalence of MDR *K. pneumoniae* strains, these bacteria are often associated with outbreaks in hospitals with increased mortality rate and longer stays in the hospitals (24). The increasing rate of *K. pneumoniae* strains that is resistant to multiple antimicrobials is a global public health problem (24). Biofilm is a densely packed community of microbial cells that attach and grow on living and non-living surfaces then surround themselves with a secreted polymer. This pathogen is known to form successful biofilms and these biofilms are often the root of serious medical

complications (25).

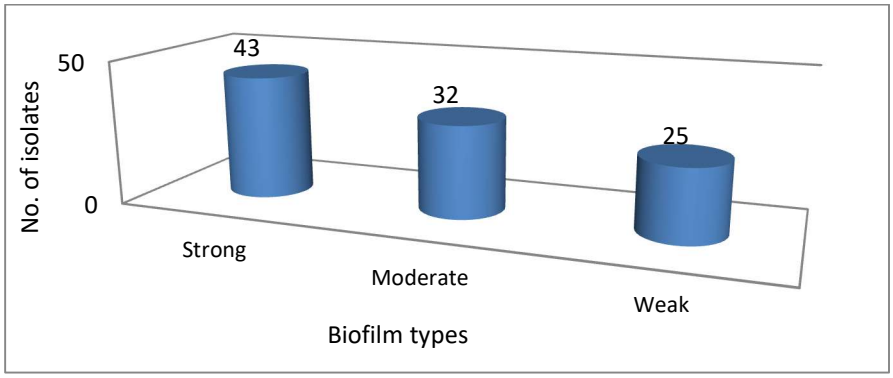


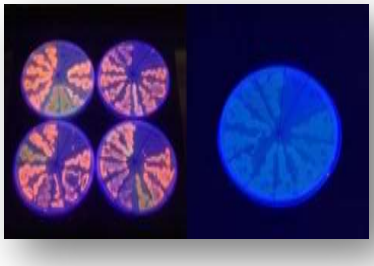
Figure (1): Types of biofilm formation in *K. pneumoniae*

Detection of efflux pumps

The result revealed highly EP founded in the bacteria 85 (85.00%), 91 (91.00%), 93 (93.00%), and 95 (95.00%) with different concentration of EtBr (0, 0.5, 1, 1.5, 2 mg/l) respectively compared with positive results (no EP) with greatly significant (P≤0.01), as in table and figure (4). Subsequently 24 hr. of incubation at 37 degree centigrade, then all plates were examined under UV trans-illuminator, a certain range of fluorescent bacterial aggregates were exposed according to their capability to efflux Ethidium Bromide.

Table and figure (4): Positive and negative no. of isolates in different EtBr concentration.

EtBr. Con.	0.5	1	1.5	2
Positive	15	9	7	5
Negative	85	91	93	95



Fluorescence of *K. pneumoniae* was: +ve emission means no efflux pumps in sample, but -ve emission means that the bacteria have efflux pumps (9).The fluorescence of bacteria growth seen as a confluent mass along a radial line of the plates containing four different concentrations of EtBr, clearly, efflux pumps bacteria have no fluorescence at a concentration of 2 mg/l of EtBr. Efflux pumps are of high importance in the resistance to various antibiotics (29). Efflux pumps on the outer membrane of the bacteria serve to extrude the antibiotic to the outside and prevent the permeability of it; the efflux pumps could decrease the intracellular concentration of antibiotics that is an important reason of bacterial survival (30).

Antibiotic sensitivity test:

All of isolates were screened for their antibiotic resistance against a selected (35) clinically antibiotics, as in figure (2), based on the type of specimens it was noted that out of all isolates (80%) of swap and bed sore are resistant to (35) antibiotics used, this high percentage may be

related to overuse or abuse of antibiotics by the patients and/or contamination of the health workers (19). *K.pneumoniae* is found to be resistant to many antibiotics such as Amikacin (57%), Ampicillin (28%), Cefepime (61%), Cefotaxime (22%), Doxycycline (59%), Ceftazidime (83%), Ceftriaxone (54%), Ciprofloxacin (87%), Gentamicin (54%), Imipenem (73%), Tobramycin (83%), Piperacillin (57%) and Tetracycline (59%), Ticarcillin (72%), Cefpodoxime (57%). Ceftazidime (83%) and ceftriaxone was (54%).

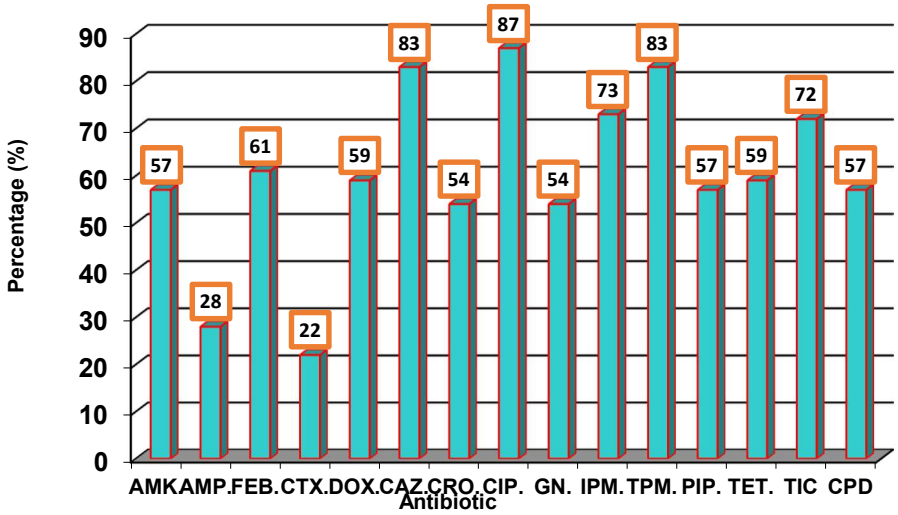


Figure (2): Percentage of *K. pneumoniae* resistant against different antibiotics (AMK: Amikacin; AMP: Ampicillin; FEB: Cefepime; CTX: Cefotaxime; DOX: Doxycycline; CAZ: Ceftazidime; CRO: Ceftriaxone; CIP: Ciprofloxacin; GN: Gentamicin; IPM: Imipenem; TPM:Tobramycin; PIP: Piperacillin; TET: Tetracycline; TIC: Ticarcillin; CPD: Cefpodoxime).

Multi-drug resistant (MDR) among *K.pneumoniae* isolates mean isolate resist to at least one antimicrobial drug in 3 or more antimicrobial categories, while extensive drug resistance (XDR) mean non-susceptibility of one bacteria species to all antimicrobial agents except in two or less antimicrobial categories. Within XDR, pandrug resistant (PDR) is resistant bacteria to all classes of antibiotics (31). About 33 (33%) of *K.pneumoniae* isolates were found to be resistant to at least 3 or more antibiotic classes tested and considered as MDR isolates. And 67 (67%) of *K.pneumoniae* isolates were resistant to all but one or two antibiotic classes and were an XDR isolates, as in table (5).

Table (5): Multi-drug resistant in *K.pneumoniae* isolates

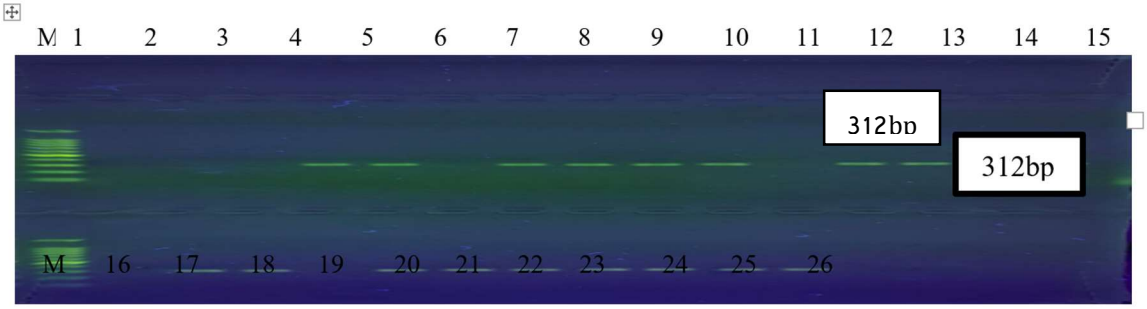
Feature	NO. of Antibiotic Groups	Resistant Isolates
MDR.	Resistant to 3 or more	33 (33%)
XDR.	Resistant to all but one or two	67 (67%)
PDR.	Resistant to all classes	0

Detection of some genes:

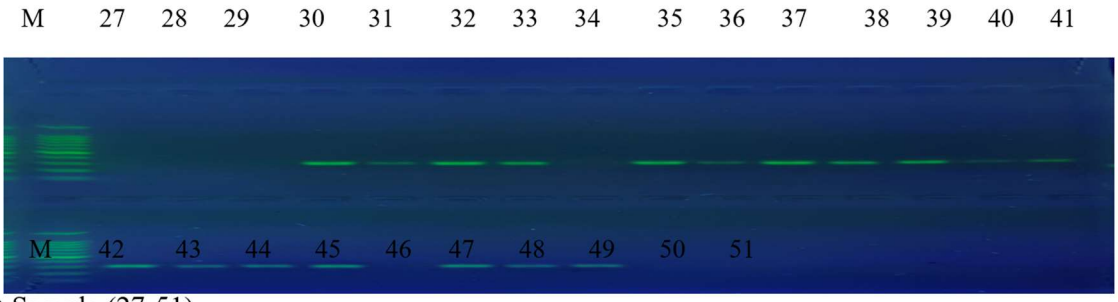
AcrAB has appeared in 37 (72.5%) of the isolates, the *acrAB* was more prevalent in isolates comparing to other efflux pump genes, as shown in table (6). On gel electrophoresis, most of the samples DNA has given the virulence coding gene *acrAB* for efflux pump, except for 14 (27.4%)

samples where DNA has not given the virulence coding gene *acrAB* as demonstrated in figure (3 a, b).

AcrAB-tolC belongs to the Resistance Nodulation Division Family (RND) which is one of five efflux pumps families that are mainly identified in Gram-negative bacteria. *AcrAB-tolC* efflux pump is an intrinsic mechanism of multidrug resistance in Gram-negative bacteria (32). The RND efflux pump *acrAB-tolC* has been identified as an important factor in antibiotic resistance in this bacterium, these efflux pumps play a regulatory role besides its antibiotic resistance role (33), the *acrAB* works with *tolC* to extrude a wide variety of antimicrobial compounds from the cell, including antibiotics, dyes, detergents, and organic solvents (34). Research have shown that *acrAB* and *tolC* were found in Some members of Enterobacteriaceae consistent to what Ali and Al-Dahmoshi have recently reported in Iraq. As *acrAB-tolC* were found in (95/ 86%), these pumps are associated with antibiotic resistance in uro-pathogenic (35).



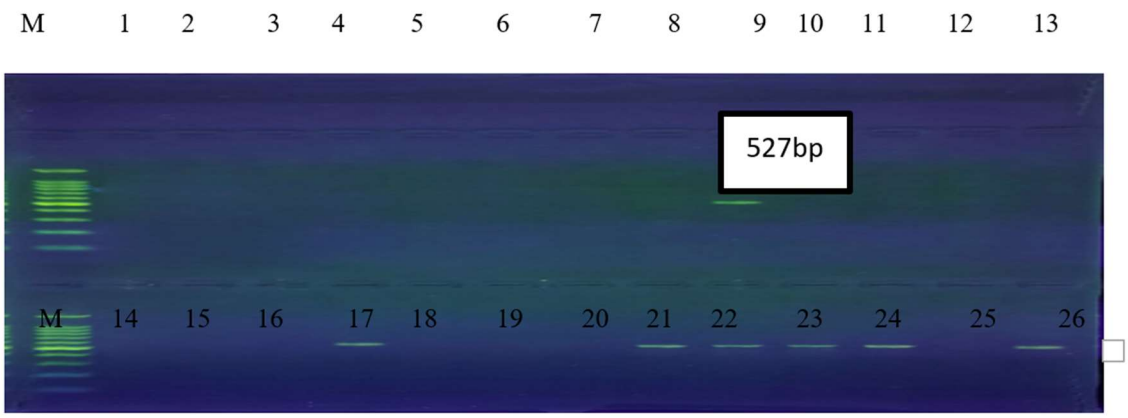
a) Sample (1-26)



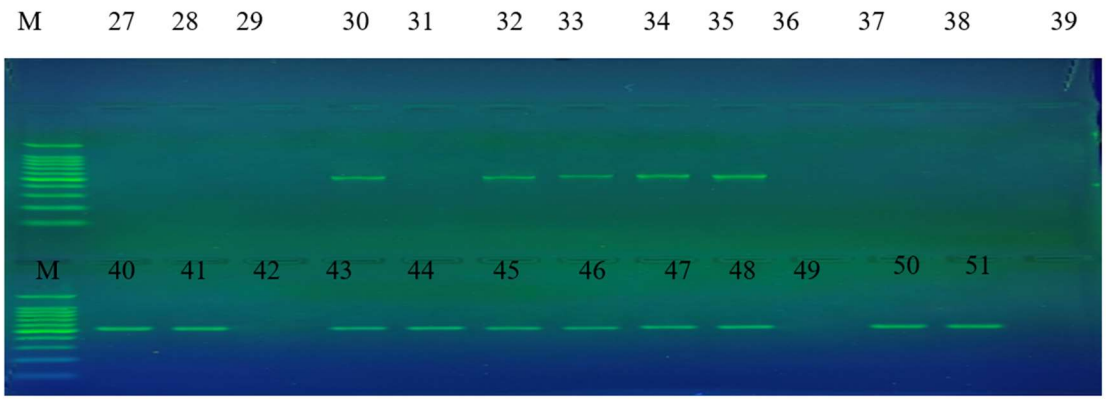
b) Sample (27-51)

Figure (3 a, b): PCR product *acrAB* gene size (312bp) on agarose gel electrophoresis (2%) at (5 volt/cm^2), (1x) TBE buffer for 1hr stained with red safe. (M) Marker DNA ladder (100bp).

***TolC*:** The results of *tolC* genes in table (6) have appeared in 22 (43%) of the strains that mean this strains have *tolC* gene that code for efflux pumps, but other samples DNA have not given the gene *to1C* 29 (56.8%), as in figure (4 a, b).



a) Sample (1-26)



b) Sample (27-51)

Figure (4): PCR product *tolC* gene size (527bp) on agarose gel electrophoresis (2%) at (5 volt/cm^2), (1x) TBE buffer for 1hr stained with red safe. (M) Marker DNA ladder (100bp).

Table (6): Summarizes the results of molecular detection of virulence genes percentage in all samples

genes	Total number of Isolates	No.of positive	%positive	No. of negative
acrAB	51	37	72.5%	14
tolC	51	22	43%	29

Conclusion:

K. pneumoniae was mainly isolated from urine, bedsore, sputum, swap, follows catheter and fluid and it was producing strong biofilm and efflux pumps. *K.pneumoniae* is found to be resistant to many antibiotics MDR 33% and XDR 67%. The results of efflux pumps genes were *acrAB* (72.5%) and *tolC* was (43%).

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