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ORIGINAL ARTICLE

Efflux Pump and Extended Spectrum Beta-Lactamases as Virulence Determinants in Multi Drug Resistant *Klebsiella Pneumoniae* Isolated from Patients with Respiratory Tract Infection

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MDR K.pneumoniae, CTX-M, AcrAB efflux pump

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ABSTRACT

Background: *Antibiotic resistance is a real life threatening problem. Klebsiella pneumoniae (K.pneumoniae) is an important pathogen which is usually associated with respiratory tract infection. ESBL and efflux pump are two major causes of antibiotic resistance in K.pneumoniae.* ***Objectives:*** *The aims of this study were to determine the prevalence of multidrug resistant (MDR) K.pneumoniae strains among respiratory tract infection patients in Chest Department, Mansoura University Hospital (MUH) and to evaluate the presence of CTX-M ESBL and AcrAB efflux pump in these strains.* ***Methodolgy:*** *The study was conducted on 50 K.pneumoniae isolates from respiratory tract infected patients which were subjected to antimicrobial susceptibility testing to determine the MDR isolates that were phenotypically tested for presence of ESBL production and efflux pump mediated antibiotic resistance by double disk synergy test and microdilution test respectively followed by detection of CTX-M and Acr-A genes by PCR in the phenotypically positive isolates.* ***Results:*** *Antibiotic sensitivity testing of the 50 K.pneumoniae isolates revealed that all of them (100%) were MDR, of which 8 (16.0%) were positive for ESBL production and 33 (86.8%) were positive for efflux pump system by phenotypic tests. PCR revealed that CTX-M gene was founed to be harbored by 4 (8.0%) in those MDR strains and Acr A gene was detected in 33(86.8%) in the phenotypically positive strains.* ***Conclusion:*** *AcrAB efflux pump was more prevalent in MDR K.pneumoniae strains than CTX-M ESBL; this may be helpful in improving the health outcome during designation of the patient treatment regimen.*

INTRODUCTION

Antimicrobial resistance (AMR) is a major health concern worldwide, as well as a major cause of treatment failure in infectious diseases, which is accompanied by increased morbimortality, and financial burden on the healthcare system. It is estimated that about 700000 deaths occur annually owing to AMR. In addition, it is predicted that if appropriate control and prevention measures are not taken, AMR could become a main etiology of mortality in all cases whatever admitted to hospital or not and in all nations.[1] The emergence of MDR *K.pneumoniae* is considered to be a global public health issue. The carriage of *K.pneumoniae* and development of MDR bacteria have increased owing to the frequent use of broad-spectrum antibiotics for hospitalized cases.[2] In a lot of nations, *K.pneumoniae* has developed resistance to the most common antibiotics and even the higher classes of antibiotics, such as third-generation cephalosporins. In addition, *K.pneumoniae* is recorded to be the most common pathogenic bacteria to develop resistance to broad-spectrum β-lactam antibiotics via ESBL.[3]

Klebsiella is a G-ve, non-motile, encapsulated, lactose fermenting, facultative anaerobe belonging to the *Enterobacteriaceae* family. *Klebsiella* spp involve *K.pneumoniae, K. ozaenae, K. oxytoca*, and *K. rhinoscleromatis*.[4]

The most important *Klebsiella* spp is *K.pneumoniae* as it is the most common causative agent of nosocomial and community acquired infections. It causes lot of types of diseases such as pneumonia, bacteremia, and urinary tract infection.[5] *K.pneumoniae* has developed a lot of mechanisms for unresponsiveness to different antibiotics. One of these mechanisms is efflux pump system and extended spectrum β-lactamase enzymes[6]. Efflux pumps are protein-based structures that are capable to extrude the different toxic substances out of the cell. The AcrAB efflux pump system, which belongs to the resistance nodulation division, plays an important role in the development of *K.pneumoniae* MDR. AcrA gene coding the membrane fusion protein Acr A and is a part of AcrAB efflux system.[7] Extended spectrum β-lactamases (ESBLs) are a heterogenous group of β-lactamase enzymes that are found in a lot of members of the family *Enterobacteriaceae.* There are more than 150 different ESBLs that are described. Almost all enzymes belong to one of four ESBL families (TEM, SHV, CTX-

M, OXA)[8] .The CTX-M β lactamase enzymes are the most widespread enzymes which are categorized in five groups which are: CTX-M1, CTX-M2, CTX-M8, CTX-M9, and CTX-M25.[9]

METHODOLOGY

Study design:

This is a cross-sectional study that was conducted over a period of 12 months starting from April2022 to March2023. During that period 210 sputum samples were isolated from respiratory tract infected patients admitted in Chest Department in MUH. 50 samples were *K.pneumoniae* positive and all isolates were MDR strains. Phenotypic and genotypic tests were made to detect presence of ESBL and efflux pump in these strains. The protocol of this study was accepted by the ethical committee in the Faculty of Medicine, Mansoura University, code number "MS.21.12.1779"

Microbiological studies:

The collected samples were processed and examined in Microbiology and Infection Control Unit in the Department of Medical Microbiology and Immunology, Faculty of Medicine, Mansoura University. Sputum samples were cultured on blood agar and macConkey`s agar media.

Identification of *K.pneumoniae* isolates:

K.pneumoniae isolates were identified by their characteristic shape as they appeared large, pink and mucoid on macCkonkey`s agar and gray white, mucoid on blood agar media. Gram- stained smears were done from the suspected bacterial colonies revealing the Gram- negative bacilli. Further identification of the isolates was performed using different biochemical reactions as they were citrate and urease tests positive with appearance of yellow slant and butt in TSI agar, positive indole, motility, with decarboxylation of ornithine and appearance of alkaline slant and butt in LIA agar.

Antimicrobial susceptibility testing:

Antimicrobial susceptibility testing was performed for all selected *K.pneumoniae* isolates according to the recommendations of CLSI.[10] Isolates that were found to be non-susceptible to ceftazidime, cefepime, ceftriaxone or cefotaxime were considered MDR strains and subjected to further testing.

Phenotypic detection of ESBL by double disk synergy test:

Multidrug resistant *K.pneumoniae* were examined for production of ESBL by double disk synergy test that depends on inhibition of ß-lactamase enzyme- produced by the bacteria- by clavulanic acid, which leads to increased diameter of inhibition zone around third generation cephalosporine disk[11]

Phenotypic detection of efflux pump mediated antibiotic resistance:

For phenotypic detection of efflux pump activity, MIC of ciprofloxacin either alone or in the presence of phenylalanine-arginine β-naphthylamide (EPI) was determined. Microdilution assay in accordance with CLSI recommendations was followed with Muller-Hinton Broth[12]

The minimum inhibitory concentration of *K.pneumoniae* isolates ,which were found to be resistant or moderately sensitive to ciprofloxacin were detected by using the microdilution approach , based on CLSI recommendations[12]. The last concentrations in the wells were adjusted between 1024-0.5µg/ml for ciprofloxacin. The lowest antibiotic concentration which inhibited the growth is regarded as the MIC value. 50µL of Muller Hinton broth was added to the wells of a sterile microdilution plate. By the addition of 50µL of suitable concentrations of ciprofloxacin to the 1st line of wells, serial dilutions were conducted. 20µg/ml constant PßNA (200µg/ml) solution to each well. Positive values which revealed a decrease in the MIC values of ciprofloxacin was assessed as the existence of the efflux pump[13]

Detection of CTX-M ESBL and Acr A efflux pump genes by PCR:

DNA extraction:

Only 2 colonies of overnight growth bacteria were used. The colonies were put in a test tube comprising one ml of dH2O and boiled for ten min in a $H_{2\ O}$ bath, and after that were centrifuged for 5 min at 1000 r.p.m. In addition, 5 µL of the supernatant were utilized for the PCR[14]

PCR techniques:

For a total of 25µl reaction volume, the following materials were added in thin walled PCR tube on ice: 12.5 µl of Taq PCR Master MIX following being briefly vortexed to evade localized differences in salt concentration,The primer solutions were thawed on ice and mixed well before use. One µl of each primer was added to the PCR tub,One µl of template DNA was added to each tube,9.5 µl of nuclease-free H2O were added.The samples were gently vortexed and briefly centrifuged to collect all drops to the bottom of the tube.The samples were overloaded with mineral oil (50 µl) and placed in the thermal cycler.PCR products were electrphoresed in 1.5 % agarose gel to measure the detected bands size.

Primers used and cyclic conditions:

Primers used for detection of CTX-M gene were (bla-CTX-M-F 5'-ACCGCCGATAATTCGCAGAT-3') and (bla-CTX- M-R 5'GATATCGTTGGTGGTGCCATA-3') Sigma, band size 588 base pair, cyclic conditions were initial denaturation at 94°C for 5 minutes then three step cycling x 30times: Denaturation at 94°C for 1 minute, annealing at 59.2°C for 30 seconds, elongation

at 72°C for 1 minute And final extension at 72°C for 5 minutes.[15] whereas primers used for detection of Acr A gene were **(**AcrA-F,5'-ATGAACAAAAACAGAGG-3') and (AcrA-R,5'-TTTCAACGGCAGTTTTCG-3') Sigma, band size 495 base pair, cyclic conditions were initial denaturation at 94°C for 5 minutes, three step cycling x 30 times:denaturation at 94°C for 1 minute, annealing at 52°C for 1 minute, extension at 72°C for 1 minute and final extension at 72°C for 5 minutes.[16]

Statistical analysis:

Statistical package of social science (spss), software version 24 for windows was used for entry and statistical analysis of data and the appropriate statistical tests were performed.

RESULTS

Examination of sputum samples that were collected from cases admitted in Chest Department of MUH over 12 months from (April 2022 to March 2023) revealed that about 50 out of total 210 sputum samples were positive for *K.pneumoniae* which represent (23.8%) of total samples while the number of MDR *K.pneumoniae* was 50 samples which represent (100%) of positive *K.pneumoniae* samples and (23.8%) of total samples. (table 1)

Table 1: Prevalence of multidrug resistant *K.pneumoniae* among total sputum samples

Prevalence of multidrug resistant *K.pneumoniae*	Total number of sputum samples (n=210) N	%
K.pneumoniae strains	50	23.8
Others	160	76.19
Multidrug resistant strains	50/50	100
	50/210	23.8

In the 50 MDR *K.pneumoniae* group, the mean age was 54.64 y ranged from 34y to 83 y Most of cases aged between 40-50 y (30%), some cases age was between 50-60 y, while others were between 60-70 y, and the least group were above 70 yrs.The number of male cases was 31 (62%), while the number of female cases was 19 (38%).

The fifty *K.pneumoniae* isolates were screened for their sensitivity toward nine antibiotics. The majority of the isolates revealed resistance to antibiotics, all of the isolates revealed resistance to both Cefotaxime and Ceftazidime, 92% of isolates were resistant to Aztreonam and Cotrimoxazole., while 86% of isolated samples were resistant to Gentamicin. On the other hand, 84% of isolates showed resistance to Amikacin and 70% of isolates were resistant to Imipenem antibiotic as in (table 2)

Table2 shows: Antibiotic sensitivity among positive *K.pneumoniae* samples to various antimicrobial agents.

Antibiotic	*K.pneumoniae* samples (n=50)			
	Sensitive		Resistant	
	N	%	N	%
Aztreonam	4	8.0	46	92.0
Ciprofloxacin	5	10.0	45	90.0
Amikacin	8	6.0	42	84.0
Imipenem	15	30.0	35	70.0
Gentamicin	7	14.0	43	86.0
Cefotaxime	0	0	50	100
Ceftazidime	0	0	50	100
Cotrimoxazole	4	8.0	46	92.0
Cefipime	3	6.0	47	94.0

Positive MDR *K.pneumoniae* isolates were tested for presence of ESBL enzyme by double disk synergy method. From the 50 isolates, 8 isolates (16.0%) were positive ESBL producing *K.pneumoniae*, while 42 samples were non ESBL producing mdr *K.pneumoniae* as in (figure 1).

Fig. 1: The double disk synergy test used for detection of ESBL-producing *K.pneumoniae*. There is an increase in inhibition zones around Cefotaxime disk (CTX), and Aztreonam disk (AT) towards Amoxclavulinic acid disk (AMC) as aresult of inhibition of ESBL enzyme by clavulinic acid.

On the other hand,there were 45 (90%) *K.pneumoniae* isolates out of 50 total positive *K.pneumoniae* isolates resistant to ciprofloxacin. In these isolates, the effect of the 20 µg/ml constant concentration of PAßN on the MIC values of ciprofloxacin was observed. Reduction of ciprofloxacin MIC values of 38 (84.4%) out of 45 *K.pneumoniae* isolates was observed in the presence of PAßN. Of the 45 *K.pneumoniae* isolated in the study, 17 (37.8%) isolates showed decrease in ciprofloxacin MIC by 2 folds while 11 (24.4%) isolates showed decrease in ciprofloxacin MIC by 4 folds, and 10 (22.2%) isolates showed decrease in ciprofloxacin MIC by more than 4 folds. On the otherhand, there were 7 (15.6 %) isolates showed no change in MIC of ciprofloxacin after addition of PAßN. (figure 2)

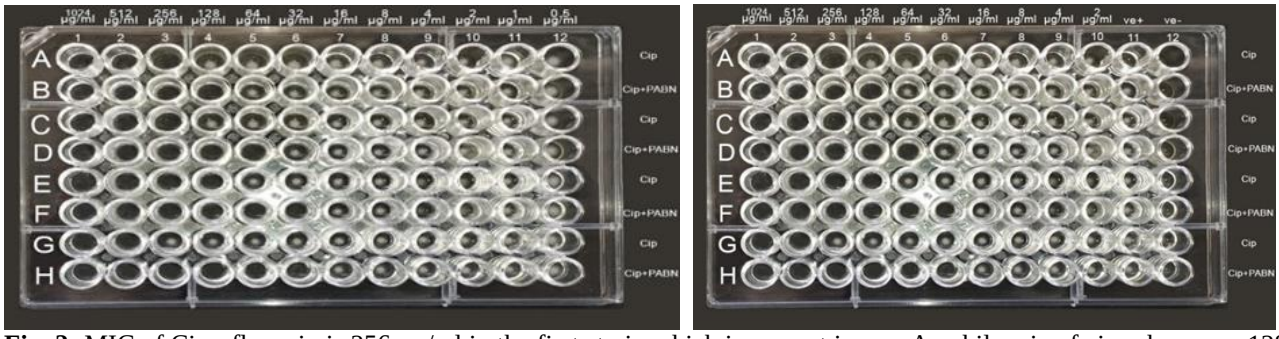

Fig. 2: MIC of Ciprofloxacin is 256 µg/ml in the first strain which is present in row A, while mic of cipro becomes 128 µg/ml after addition of PAßN as an efflux pump inhibitor to the same strain which is present in the row B. (Decrease in mic by 2 folds) In row G mic of cipro is 512 µg/ml, while in row H mic is 32 µg/ml on the same strain after addition of PAßN. (decrease in mic by >4 folds)

Polymerase chain reaction detection of bla CTX-M gene among phenotypically positive mdr *K.pneumoniae* showed that 4 isolates (50%) out of 8 isolates were positive for bla CTX-M gene and 16% of MDR *K.pneumoniae*, giving 588bp band as in (figure3) while detection of AcrA gene by PCR using specific primers demonstrated that 33 strains (84.4%) of phenotypic positive strains harbored acrA gene giving 495 bp band as in (figure 4) and consequently contain AcrAB efflux system.

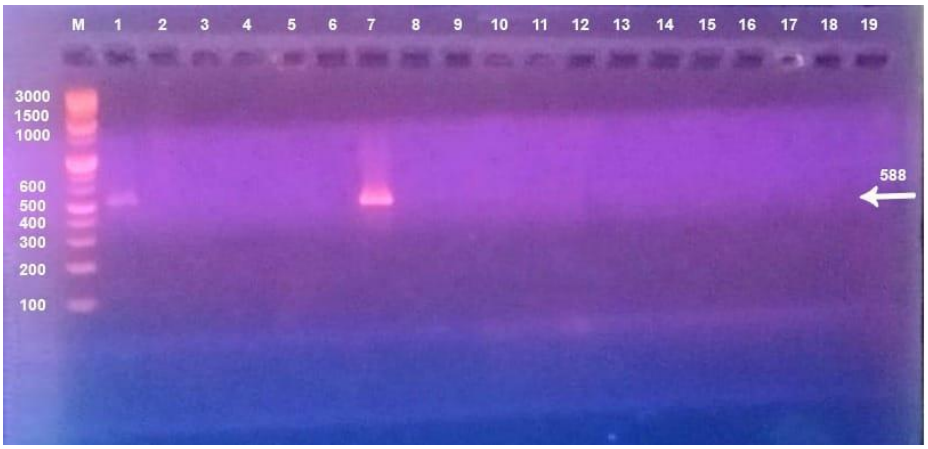

Fig. 3: PCR result of bla $_{ctx-m}$ gene in ESBL-producing *K.pneumoniae* isolates. The figure shows PCR bands of bla$_{ctx-m}$ gene in lanes 1,7. The size of each band was (588 bp)

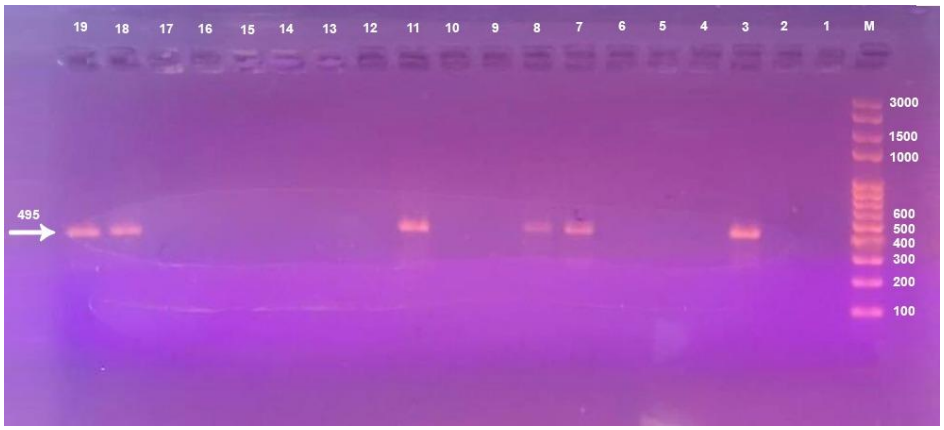

Fig. 4: PCR result of acr-A gene in MDR *K.pneumoniae* isolates. The figure shows PCR bands of acr-A gene in lanes 2,7,8,11,18,19. The size of each band was (495 bp)

DISCUSSION

MDR (MDR) *K.pneumoniae* become common all over the world. *K.pneumoniae* has emerged as a major health problem as a result of increasing prevalence of infections caused by MDR strains. The over-usage of antibiotics led to an increasing prevalence of MDR *K.pneumoniae* strains.[17] There is a meta-analysis study made by Asri et al.[18] showed the difference between nations and regions in the prevalence of MDR *K.pneumoniae*. The results from North America revealed the lowermost value of (12.9%), whereas very high percentage of MDR *K.pneumoniae* (77.7%) was demonstarted in Egyptian hospitals.[19] In our study we found that the prevalence of *K.pneumoniae* spp was 50 strains, which represented 23.8% of the 210 total number of studied sputum samples, and we found that the prevalence of MDR *K.pneumoniae* among total samples was 50 strains (all positive *K.pneumoniae* strains) which representd (100%) of positive *K.pneumoniae* strains. Our study result is close to the study made by Asri et al[18] who found that prevalence of MDR *K.pneumoniae* was (32.8%) from total samples . In addition, another study was made in Egypt and conducted by Kamel et al[20] recorded that MDR *K.pneumoniae* represented (95.77%) of *K.pneumoniae* isolates. While Marwa et al[21] at Al-azhar University Hospital recorded (83.9%) MDR *K.pneumoniae* of *K.pneumoniae* samples and (6.9%) of total samples. In another study, conducted at El-Mansoura University Hospital, El-Kady and Gouda[22] recorded that (49.2%) of isolated *K.pneumoniae* isolate were MDR. The difference in prevalence of *MDR K.pneumoniae* between researches may refer to different clinical samples, different patient condition and different time as prevalence of MDR *K.pnumoniae* is increasing continuously.[23]

As per patient gender, *K.pneumoniae* strains were found in both gender but frequency of isolation is high in male (62.0%) when compared with female (38%).Similarly, the study conducted by Sureka et al[24] recorded close results where frequency of isolated *K.pneumoniae* strains in male was (54 per cent) and in female was(46%).

As per antibiotic sensitivity manner, (100%) of isolated *K.pneumoniae* strains were resistant to both Cefotaxime and Ceftazidime antibiotics and (92.0 %) were resistant to Cotrimoxazole and Aztreonam antibiotics. This result is close to the result conducted by Dalia et al[25] which recorded that (93.3%) of isolated *K.pneumoniae* were resistant to Cefotaxime antibiotic and (90.0%) were resistant to Cotrimoxazole, while (90.0%) of positive *K.pneumoniae* strains were resistant to Aztreonam antibiotic.On the other side, our result revealed that frequency of resistant strains to Imipenem antibiotic was (70.0%) and frequency of strains resistant to Cifipime antibiotic was (94.0%). This result shows some difference to the study made by Rhanda et al [26]that recorded that (31.79%) of *K.pneumoniae* strains were resistant to Imipenem antibiotic and (76.82%) of positive isolated strains were resistant to Cifipeme antibiotic. This disparity might be owing to difference in level of hygiene, sample size, type of specimens and restriction on antibiotic usage.[27]

The mechanisms that are usually involved in AMR in *K.pneumoniae* strains are production of β-lactamase enzymes and multidrug efflux pump system.[28]All MDR strains of isolated *K.pneumoniae* were subjected to double disk synergy test for detection of ESBL enzyme production, (16%) of those isolates were found to be positive for ESBL. Similar result was recorded by Parama et al [29] when he performed double-disk synergy test for detection of ESBL in *K.pneumoniae* isolates and he found that (16.6%) strains were positive.[29] While Lal et al [30] in India recorded that (97.1%) of MDR *K.pneumoniae* strains were established phenotypically to be positive for ESBL production. This variation of prevalence of ESBL-producing bacteria refers to various geographic areas. Less than 10% of isolated strains are ESBL positive strains in Japan and Singapore, in comparison to higher rates -more than 30 % in Italy and New York.[31]

CTX-M genotype has become the most dominant internationally. On the other hand, the distribution rate of the ctx-m gene differs from one place to another indicating diversity in the epidemiology of these resistance genes.[32] In the present study, (50 %) of ESBL producing *K.pneumoniae* were positive for bla CTX-M gene. This result is close to the study conducted by Ola et al[33] which revealed that (53.3%) of total *K.pneumoniae* carried bla CTX-M gene. On the other side, a higher percentage of ESBL -producing *K. pneumoniae* was found by Parajuli et al[34] where bla CTX-M gene was present on 100% *K.pneumoniae*. The occurrence of CTX-M ESBL differs based on geographical location and difference in sample size.[27]

Another possible mechanism of antibiotic resistance in *K.pneumoniae* is the expression of efflux pump system. The efflux pump acts by reducing the intracellular concentration of antimicrobial agent that is an essential etiology for the bacteria to survive.[7] In this study we used phenylalanine arginine ß-naphthylamide (PAßN) as an efflux pump inhibitor to assess the effects of AcrAB efflux pump in ciprofloxacin resistance among *K.pneumoniae* strains.[35] In our study, the effect of PAßN on ciprofloxacin minimal inhibitory concentration (MIC) value was tested in 45 *K.pneumoniae* strains resistant to ciprofloxacin antibiotic. Thirty- eight (84.4 %) positive strains showed decrease in ciprofloxacin MIC starting from 2 folds and reaching 32 folds, while 7(15.6 per cent) strains showed no change in MIC of ciprofloxacin after

addition of PAßN. In a study made by Serhat et al[36] the effect of efflux pump inhibitor on 21 *K.pneumoniae* strains resistant to ciprofloxacin was that 13(61.9%) strains showed a decrease in MIC of ciprofloxacin. Another study conducted by Hasdemir et al[16] in Turkey, they tested the role of efflux pump in the antibiotic resistance of 18 MDR *K.pneumoniae* strains. They found that (39%) of total strains showed activity for efflux pump system. In our study PCR revealed that acrA gene was detected in (86.8%) of phenotypically positive ciprofloxacin resistant *K.pneumoniae* strains. Another research carried out of forty *K.pneumoniae* isolates were resistant to ciprofloxacin and all these strains harbored acrA gene and hence contain AcrAB efflux system.[37] In the same study, 47.5% of ciprofloxacin resistant strains showed decrease in MIC of ciprofloxacin antibiotic as a result different mechanisms as gene mutation in target proteins of DNA gyrase and topoisomerase IV enzymes propably involved in ciprofloxacin resistance of the strains.[38] While Razavi et al[39] found that the frequency of acrA gene was (52.72%) among clinical strains of *K.pneumoniae* We can conclude that, in our study we have found that there is a high prevalence of MDR *K.pneumoniae* strains among isolated samples, with resistance to multiple antibiotics, these strains could produce extended spectrum β-lactamases and expression of efflux pump system which can be considered as a major problem and a great challenge for finding a treatment of *K.pneumoniae* infections.

CONCLUSION

K.pneumoniae bacteria normally inhabit respiratory tract system but they cause can cause different types of diseases in susceptible individuals, including pneumonia and surgical site infections. Antibiotic resistance plays a major role in increasing virulence of the bacteria and making the host defence mechanisms are un able to eradicate these organisms. *K.pneumoniae* bacteria have developed different mechanisms for increasing their resistance against different antimicrobial agents. We have studied two machanisms which participate in antibiotic resistance produced by *K.pneumoniae* isolates. All positive K.pneumoniae isolates were multidrug resistant strains and a high percentage of them harbored the AcrAB efflux pump system while less number of MDR strains were ESBL producing organisms.We can conclude that there are a higher prevalence of MDR K.pneumoniae that contain acrA gene than ctx-M gene. This reveals that there is an important role of efflux pump and unresponsiveness of bacteria to antibiotics.These findings may help in determining a treatment regimen for these MDR *K.pneumoniae* strains which forma a major challenge facing the medical persons.

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