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Original Article

Detection of AcrA and AcrB Efflux Pumps in Multidrug-Resistant *Klebsiella pneumonia* that Isolated from Wounds Infection Patients in Al-Diwaniyah Province

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Abstract

Many infections produced by multidrug-resistant (MDR) *Klebsiella pneumoniae* are the main cause of death and treatment restrictions worldwide. In *K. pneumoniae*, the efflux pump system is dangerous in drug resistance. Therefore, this study was designed to investigate the involvement of the AcrA and AcrB efflux pumps in antibiotic resistance in *Klebsiella pneumoniae* isolated from wound patients. During June 2021-February 2022, 87 clinical isolates of *Klebsiella pneumonia* bacteria were obtained from wound samples patients consulted to the hospitals in AL-Diwaniyah province, Iraq. The disc diffusion method performed an antibiotic susceptibility test after microbiological/biochemical identification. The polymerase chain reaction (PCR) technique was used to examine efflux genes' prevalence (*acrA* and *acrB*). The results showed that resistance to Carbenicillin 72 (82.7%), Erythromycin 66 (75.8%), Rifampin 58 (66.6%), Ceftazidime 52 (59.7%), Cefotaxime 44 (50.5%), Novobiocin 38 (43.6%), Tetracycline 32 (36.7%), Ciprofloxacin 22 (25.2%), Gentamicin 16 (18.3%), Nitrofurantoin 6 (10.3%) in *Klebsiella pneumoniae* isolates. The PCR procedure revealed that the occurrence of the *acrA* and *acr*B *genes* is 55 (100%) and 55 (100%), respectively. The findings of this investigation show that the AcrA and AcrB efflux pumps play a crucial character in antibiotic resistance in multidrug-resistant *Klebsiella pneumoniae* bacterial isolates. As a result of the unintentional transmission of antimicrobial resistance genes, precise detection of resistance genes using molecular approaches is required to switch the extent of resistant strains.

Keywords: Efflux pumps, *AcrA*, *AcrB*, Multidrug resistance, *Klebsiella pneumoniae*

1. Introduction

Klebsiella pneumoniae is one of the most common bacteria isolated from wounds (1). *Klebsiella* is the most prevalent member of the Enterobacteriaceae family and is known to produce horizontally transferable plasmid-mediated extended-spectrum beta-lactamases (ESBLs) (2). In recent years, multidrug-resistant *Klebsiella* has become a severe obstacle in treating infections (3).

Efflux pumps are protein transporters found in the cell membrane. The efflux pumps are essential in transporting and placing various materials outside the cell to eliminate their detrimental effect, essential to bacterial resistance to antibiotics. Many compounds, such as hydrophilic poisons, and hydrophobic or amphipathic chemicals, are transported outside the cell via these transporters. As well as dyes like acriflavine, crystal violet, ethidium bromide, disinfectants, antiseptics, fatty acids, heavy metals, organic solvents and antibiotics such as β-lactams, macrolides, tetracycline, chloramphenicol and novobiocin (1). Three criteria are used to classify efflux pumps

depending on the followings: 1) substrate specificity, which may be general efflux pumps that can release compounds with various chemical structures outside the bacterial cell or specific efflux pumps which are specialized in transporting specific substances and liberating them outside the cell, like pumps that specialize in dispensing a single type of antibiotic (4). 2) Source of energy involves pumps that rely on the chemical energy source to complete their roles, that is, rely on active transport to eject substances out. 3) Phylogenic relationship: these pumps are divided into two chief groups based on genetic origin, A) chromosomal efflux pumps, which carry the encoded genes on the chromosome of the cells and give the cells a feature of intrinsic resistance. It also enables bacteria to thrive in a certain environment, such as when there is a high concentration of antibiotics, and B) plasmid efflux pumps. The genes encoding it carry inherited elements such as transposons, integrons and plasmids; cells confer a characteristic acquired resistance, which is found in prokaryotic cells, especially bacterial cells (5), As in the pumps systems of the family (MFS) that includes (TetA/B/E, CmlA, Flo) and pump system OqxAB-TolC that belong to family RND.

There are five families of stream systems in the prokaryotic cells (6): Major Facilitator Super (MFS) Family, Small Multidrug Resistance (SMR) Family, Multidrug and Toxic Efflux (MATE) Family, ATP-Binding Cassette (ABC) Family and Resistance - Nodulation - Division (RND) Family.

ABC pumps family depends on the lysis of ATP molecules as an energy source. As for other families, it depends on the energy of emitting the proton, Proton motive Force (PMF) as a source of energy. One of the most famous and widespread families is the family of RND, Which predominate in gram negative bacteria like *Proteus spp*. *Klebsiella pneumoniae*, *Salmonella spp*. (7).

The family is divided of RND It is divided into three sections based on its components and includes single-component efflux pumps that are represented by the protein AcrB that present in the inner membrane of the cell, Which is coded by a gene called *acrB*, Which transports hydrophilic antibiotics, and two-component pumps represented by a protein AcrB In the inner membrane and lipoproteins AcrA that present in periplasmic space encoded by a gene called *acrA* and Three-component pumps (tripartite) which protein AcrB represents In the inner membrane and proteins AcrA in periplasmic space and a funnel-like protein channel is called Toic that present in the outer membrane of the cell which It transports most of the hydrophilic antibiotics (8).

Therefore, the goal of this study was to phenotypically and molecularly investigate the possible role of AcrA and AcrB efflux pumps in antibiotic resistance in *Klebsiella pneumonia* isolated from various wound samples, as well as to determine the prevalence of *acrA*/*acrB* efflux pump genes in the studied isolates.

2. Materials and Methods

2.1. Sample Collection

Samples were collected from patients suffering from acute and chronic wounds with purulent discharge or painful spreading erythema around wounds, including continuous abscesses, traumatic wounds, foot ulcers and burns. A total of 450 patients from all age groups attended Al-Diwaniyah teaching hospital, Maternity and children Teaching Hospital and private clinics in AL-Diwaniyah province between 1/6/2021 to 1/2/2022 were included in the study.

2.2. Isolation and Identification

Pus was collected from wounds by sterile disposable cotton swabs and immediately inoculated onto blood agar, Mac Conkey agar, and brain heart infusion broth (Merck Co., Germany) plates before being incubated at 37 ºC for 24 hrs. Bacteriological tests like colony morphology on the medium and biochemical tests, like, Citrate utilization, TSI agar, MR-VP, motility, urease, oxidase, and Sulfide Indole Motility (SIM), as well as the API 20E identification kit (Analytab Products, Inc., Plainview, N. Y.) were used to identify suspected grown colonies as *K. pnumoniae.*

2.3. Susceptibility Testing for Antibiotics

Antibiotic susceptibility patterns of *Klebsiella pneumonia* on Mueller-Hinton Agar isolates were identified using the Kirby-Bauer disc diffusion method (Merck Co., Ger.) medium. For the following antibiotics, according to the Clinical and Laboratory Standards Institute (CLSI) methods (Mast, Merseyside, UK): Carbenicillin PY (25µg), Cefotaxime CTX (30 µg), Ceftazidime CAZ (30 µg), Ciprofloxacin CIP (10 µg), Erythromycin E (10 µg), Gentamicin CN ((10 µg), Nitrofurantoin F (300 µg), Novobiocin NV (30 µg), Rifampin RA(5 µg), Tetracycline TE (10 µg). In summary. Fresh cultures were used to produce a bacterial suspension. Each bacterial suspension was adjusted to a 0.5 turbidity standard of McFarland before being grown on Mueller-Hinton agar (Oxoid, UK). The diameter of the inhibition zone was determined after incubation at 37°C for 18-24 hrs. Susceptible, intermediate, and resistant isolates results were reported. As a quality control, *K. pneumonia* ATCC 7006603 was employed.

2.4. DNA Preparation

All genomic DNA was extracted from *Klebsiella pneumoniae* colonies cultivated in LB broth. (Merck Co., Germany) for molecular diagnosis using the Mini genomic DNA Kit kit (Geneaid, Thailand) according to the manufacturer's instructions. A Nanodrop spectrophotometer (NanodropTechnologies, D.E, USA) and electrophoresis on an agarose gel were used to determine the purity of DNA and concentrations. The isolated DNAs were kept at -70°C right away.

2.5. PCR Assay for *acrA* and *acrB* Genes Detection

PCR amplification detected two efflux pump genes (*acrA* and *acrB*) in *K. pneumoniae* clinical isolates. Table 1 shows the primer sequences and chosen genes used in this study. In each PCR test, the total volume of the reaction was 25 µl in a PCR tube. 1.0 µl DNA sample, 0.5 µl primers (25 pmol each), 2.5 mM $MgCl_2$, 1.5 µl Taq DNA polymerase (1.5 U), 1 µl dNTPs (200 M), 2 µl 10x PCR buffer (pH 9.0, 2 mM $MgCl_2$, 75 mM Tris-HCl, 50 mM KCl, 20 mM $(NH_4)_2SO_4$) and sterile distilled water up to 25 µl. For gene amplification, the following thermal cycling settings were used: The *acrA* gene was amplified in a thermocycler (Eppendorf, Germany) using 34 cycles of denaturation at 94°C for 45 seconds, annealing at 52°C for 45 seconds, extension at 68°C for 1 minute, and a final step of extension at 72°C for 10 minutes. For the *acrB* gene, these conditions were 94°C for 45 seconds (denaturation), followed by 32 cycles of 64°C for 45 seconds, and 72°C for 60 seconds (9), with the following primers:

Table 1. *Klebsiella pneumonia*-specific primer sequences used for the detection of Efflux Pumps genes

NO	Gene	Primer sequence(5′-3′)	Product (bp)	Ref
1	*acrA*	F-CTCTCAGGCAGCTTAGCCCTAA R-TGCAGAGGTTCAGTTTTGACTGTT	107	(10)
2	*acrB*	F-GGTCGATTCCGTTCTCCGTTA R-CTACCTGGAAGTAAACGTCATTGGT	105	(10)

The PCR products were run on 1.0% agarose gels with 1µ g/mL power-safe dye and electrophoresed for 1 hour at 95 V and 30 mA in a 0.5X TBE buffer. Under UV light, DNA bands were put on the gel and detected utilizing Bio-imaging document systems (VisiDoc-ItTM system). A DNA marker with a molecular weight of 100 bp increments was used as a DNA standard. The positive control strain was *K. pneumonia* ATCC 700603, and the negative control strain was *P. aeruginosa* ATCC 27853.

2.6. Statistical Analysis

A chi-squared test in SPSSversion 16 was used to evaluate the data (SPSS Inc., Chicago, IL, USA). A statistically significant *P*-value of 0.05 was used.

3. Results

An overall of 450 patients samples that suffered from various accident wounds refers to AL-Diwaniyah teaching hospital, Maternity and children teaching Hospital and private clinics. As shown in figure 1, 50% of the samples were from traumatic wounds, while the rest were from burns (32%) and foot ulcers (18%).

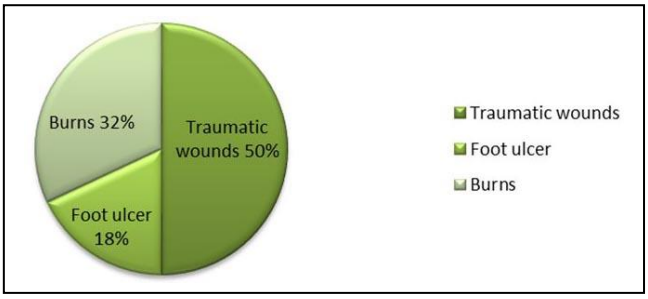

Figure 1. Frequency of cases isolated from a culture of wound infection patients

Generally, 87 isolates of *K. pneumoniae* were generally isolated from 450 (19.33%) wound samples. The uppermost number of *K. pneumoniae* isolates (50%) were isolated from traumatic wounds, and the lowest quantity (32%) was obtained from burns samples and (18%) from foot ulcer samples.

Disk diffusion method for antibiotic sensitivity tests revealed the rate of resistance to antibiotics as follows: Carbenicillin 72 (82.7 %), Erythromycin 66 (75.8 %), Rifampin 58 (66.6%), Ceftazidime 52 (59.7 %), Cefotaxime 44 (50.5 %), Novobiocin 38 (43.6 %), Tetracycline 32 (36.7 %), Ciprofloxacin 22 (25.2 %), Gentamicin 16 (18.3 %), Nitrofurantoin6 (10.3 %). As shown in table 2.

Table 2. Antibiotic susceptibility profile of *Klebsiella pneumonia* isolated from wound infection patients (N=87)

Antibiotics	Susceptible No. (%)	Intermediate No. (%)	Resistant No. (%)
Carbenicillin	10 (11.4)	5 (5.74)	72 (82.7)
Erythromycin	17 (19.5)	4 (4.59)	66 (75.8)
Rifampin	21 (24.1)	8 (9.19)	58 (66.6)
Ceftazidime	28 (32.1)	7 (8.04)	52 (59.7)
Cefotaxime	30 (34.4)	13 (14.9)	44 (50.5)
Novobiocin	38 (43.6)	11 (12.6)	38 (43.6)
Tetracycline	47 (54.02)	8 (9.19)	32 (36.7)
Ciprofloxacin	55 (63.2)	10 (11.4)	22 (25.2)
Gentamicin	63 (72.4)	8 (9.19)	16 (18.3)
Nitrofurantoin	68 (78.1)	10 (11.4)	9 (10.3)

After examining the antibiogram of the *K. pneumoniae* samples, 55 multidrug-resistant isolates (MDR) were detected and isolated, each of which was resistant to more than three drugs. Because they were resistant to various antibiotics, these bacterial isolates were good candidates for investigating AcrA and AcrB pumps as a broad efflux pumps family in *K. pneumoniae.*

The finding of the target efflux pump genes amplification by PCR in 55 isolates of *Klebsiella pneumonia* revealed that the *acrA* and *acrB* genes were present in 100% and 100% of the isolates, respectively (Table 3). The results of gel electrophoresis of *Klebsiella pneumoniae* efflux pump genes amplified by PCR are shown in figures 2 and 3.

Table 3. Percentage of efflux pump genes in multidrug resistance in *k. pneumoniae* isolates (N=55)

NO	Genes	NO of the isolates have the gene	%
1	*acrA acrA*	55	100
2	*acrB*	55	100

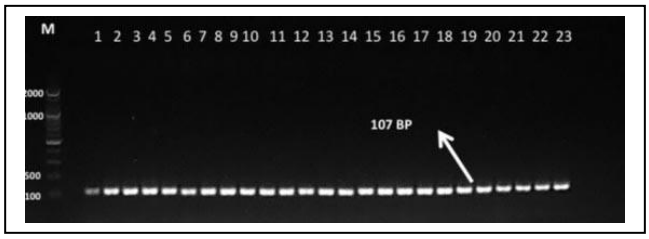

Figure 2. Gel electrophoresis image of *acrA* gene of efflux pump in *K. pneumonia* by PCR procedure. Lanes 2-23: *acrA* gene positive results (band: 107 bp), Ladder of DNA was 100 bp

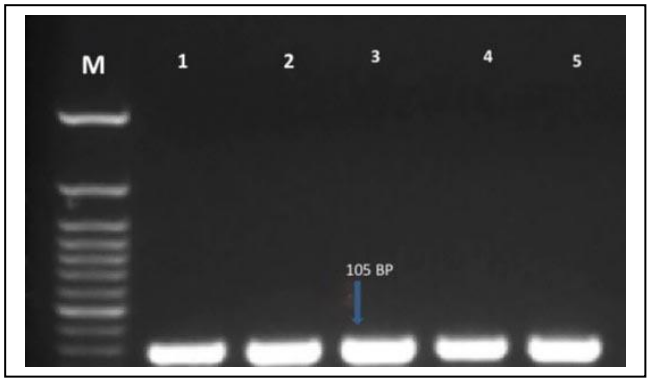

Figure 3. Gel electrophoresis image of *acrB* gene of efflux pump in *K. pneumo*niae by PCR procedure. Lanes 1-5: *acrB* gene positive results (band: 105 bp), Ladder of DNA was 100 b

4. Discussion

Klebsiella pneumoniae is a common pathogen that causes hospital-acquired and community-acquired

complaints, like UTIs, pneumonia, and wound infections (10). The satisfying choices for treating the infection produced by this pathogen have existed problematic in line with the growing development of multidrug resistance (MDR) in *Klebsiella pneumonia*e isolates in clinical cases. Most *K. pneumoniae* isolates have been studied as multidrug-resistant (MDR) strains, according to current investigations in recent years. MDR isolates are known to be challenging to treat, especially for many adults, immunocompromised patients, and youngsters with underdeveloped physiology (11). The efflux pump system is one of the most prominent antibiotic resistance mechanisms in this bacteria. The importance of these pumps in increasing the resistance of *klebsiella pneumoniae* strains to diverse families of antibiotics has been proven in many worldwide research studies (12).

The results of this study for the antibiotic carbenicillin are in agreement with previous studies, including the local results reached by Jabar and Hassoon (13), in which the percentage of isolates of bacteria resistant to the antibiotic carbenicillin reached (100%), as this study showed that many of the β-lactamases that are controlled by the carried genes on the plasmid in the Gram-negative bacteria could degrade each of the antibiotics carbenicillin, ampicillin, cephalothin and other antibiotics of the same group. Also, the findings of this current study align with the results reached by Hinthong, Pumipuntu (14), in which the percentage of bacterial isolates resistant to this antibiotic reached (95%). They explained that the high resistance to most antibiotics is due to the excessive use of antibiotics, mutations that encode enzymes, and the transmission of resistance mediated by the plasmid.

In terms of the current study's findings, for the third-generation cephalosporin antibiotics that include the antibiotics ceftazidime and cefotaxime, they agree with the study carried out by the researcher Herran (15), who found that *Klebsiella pneumoniae* was highly resistant to antibiotics belonging to the β-lactams group, and in Iran, a study of researcher Yousefi Mashouf, Alijani (16) shows *K. pneumoniae* strains were resistant to cefotaxime (95%) and ceftazidime (97%), the highest antibiotic resistance of *K. pneumoniae* as the resistance of these bacteria to these two groups was the two antibiotics (76%) and (75%), respectively, and they showed that the main reason for this high resistance of bacteria is that they have efficient effluent pumps that expel antibiotics from outside the cell and remove their harmful effect on the cell. One of the reasons for Klebsiella bacteria's resistance to β-lactams antibiotics is the production of β-lactamases, which include the enzymes cephalosporins and penicillinase. As these enzymes break down the beta-lactam ring, inhibiting the action of the antibiotics belonging to the groups of penicillins and cephalosporins, one of the causes of resistance is a change in the permeability of the outer membrane of the bacterial cell and its possession of efflux pumps system. The most common is the AcrAB-ToIC efflux pumps of the RND family, a common efflux in *Klebsiella pneumonia* (17).

The results of the current study for the antibiotic ciprofloxacin (25.2%) of the quinolones group were approaching the results of the researcher Tajbakhsh, Ahmadi (18) in Iran that the percentage of bacterial resistance to this antibiotic was (36.3%), one of the motives for the resistance of *klebsiella* bacteria to antibiotics belonging to the quinolones group is a change in the target site, a decrease in the penetrability of the outer membrane of bacteria and its possession of efflux pumps systems that include (AcrAB-ToIC, MdfA, YhiV) or due to a genetic mutation in it (18).

The results of the current study for the antibiotic gentamicin (18.3%) belonging to the aminoglycoside group showed results consistent with the results reached by the researcher Suresh, Nithya (19), which showed that the percentage of isolates resistant to this antibiotic (37%). The causes of resistant *klebsiella pneumoniae* for aminoglycosides groups own bacteria for the efflux pumps systems of the RND family and change in outer membrane permeability of the bacterial

cell (20) as well as the possession of aminoglycoside modifying enzymes (AMEs) such as phosphotransferase and N-acetyl transferase (21).

The study also showed the resistance of bacterial isolates to tetracycline antibiotics, which was attributed to several reasons, including changing the outer membrane permeability in the bacteria, the presence of efflux pump systems, and a change in the target site (22).

The present study showed very high resistance to the antibiotic erythromycin, where the resistance rate was (75.8%) of the macrolides group. These results have approached the researchers Kibret and Abera (23) results. The rate of resistance of bacteria for this antibiotic is 89%. There are several reasons for bacterial resistance to this antibiotic, such as hydrolysis, which works on the antibiotic's degradation and inhibit its activity and the production of enzymes. Glycosylation and phosphorylation inhibit the action of the antibiotic (24).

Findings of the current study about the antibiotic Novobiocin (43.6%) approached what the researcher Ogawa, Onishi (25) found, where the resistance of bacteria isolates was (56.5%), and one of the most significant causes of antibiotic resistance in *Klebsiella pneumoniae* bacteria to the antibiotic novobiocin is their possession of efflux pumps as well as the transmission of resistance to it through plasmids as well as the occurrence of genetic mutations Randomness that mutates the components of the bacterial cell and turns them into resistant bacteria (26).

Klebsiella pneumoniae isolates showed weak resistance to the antibiotic nitrofurantoin, and the resistance rate was (10.3%); these results were in parallel to the results of the researcher Maina, Makau (27) in Kenya in terms of the percentage (28%) of resistance to bacterial isolates isolated from primary UTI. Among the reasons why bacteria are resistant to this antibiotic are their possession of efflux pump systems, decreased cell membrane permeability, and the occurrence of chromosomal mutations (28). More investigation into the efflux pump gene expression in resistant isolates is required to determine this link. Adopting these pumps by microorganisms increases medication resistance and, as a result, increases pathogenicity in individuals, making these attitudes a severe health threat. As a result, to avoid treatment failure, careful diagnosis of infections, identification of resistant microorganisms and their resistant mechanisms, and the use of appropriate antibiotics are essential (29).

The antibiotics Gentamicin and Nitrofurantoin showed the best effect against *K. pneumoniae* isolates, according to the results of this investigation. Resistance to Carbenicillin, Erythromycin, and Rifampin was shown to be more prevalent in the bacterial isolates examined. Physicians must be cautious when recommending medication and send the clinical material to a laboratory for an antibiogram test so that the most effective treatment pill may be prescribed. Drug resistance mechanisms, such as efflux pumps, should be found in labs using phenotypic and molecular approaches, and the results should be communicated to doctors. To avoid the spread of drug-resistant bacteria, lab personnel, hospital workers, and everyone in dealings with patients in the Hospital should be checked for identifying these microorganisms.

Authors' Contribution

Study concept and design: S. A. F. A.
Acquisition of data: S. A. F. A.
Analysis and interpretation of data: S. A. F. A.
Drafting of the manuscript: S. A. F. A.
Critical revision of the manuscript for important intellectual content: S. A. F. A.
Statistical analysis: S. A. F. A.
Administrative, technical, and material support: S. A. F. A.

Ethics

All ethical standards have been respected in preparation of the submitted article and have been contained from the ethical committee of the AL-Qadisiyah University, Ad Diwaniyah, Iraq.

Conflict of Interest

The authors declare that they have no conflict of interest.

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