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Phenotypic and molecular detection of efflux pumps in *Klebsiella pneumoniae*.

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Abstract

200 samples were collected from patients suffering from different infections and different age groups for both sexes and from different sources distributed on 80 urine samples (Urine), 42 exit samples (Stool), 62 samples (Sputum) and 26 samples from burns and wounds from patients Patients and patients in Al-Diwaniyah Governorate hospitals, whose ages range between 1-60 years, and for a period of five months, from 9/15/2021 to 01/20/2022. Samples were planted on culture media and isolates were diagnosed using traditional methods (culture, microscopic and biochemical).

The vitek2 device was also used to confirm the diagnosis of K. pneumoniae isolates, and the confirmation was reinforced by using the molecular diagnosis of the 16 srRNA gene of K. pneumoniae, and the diagnostic results showed a return of 36 K. pneumoniae isolates with a percentage of 22.1%.

It was detected phenotypically (using the wooden wheel method) and molecularly the Pcr technique to investigate some genes

The results of phenotypic detection of efflux pumps in 18 bacterial isolates showed that they possessed these Acr ABR pumps, and this was confirmed by the molecular detection of efflux pumps, as the results showed that the percentage of the acrA gene was 100%, while the acrB gene was found at 55.5%, and the acrR gene was found in a percentage 100%.

KEYWORDS: Phenotypic ; molecular detection ;efflux pumps ; *Klebsiella pneumoniae*

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INTRODUCTION

Klebsiella pneumoniae is a member of the Enterobacteriacae family, which lives normally in the human intestine, but when present in other parts of the body, it causes many diseases (Siu et al.,2011). It also lives widely in surface water, sewage, soil, plants and mucous membranes of mammals (White et al.,2002). It is one of the most important opportunistic causes of infection in hospitals and society (Togawa et al.,2015).

These bacteria possess many mechanisms of resistance to antibiotics, including their possession of efflux pumps (EPs), which are proteins located in the bacterial plasma membrane. Reaching the goal as well as getting rid of harmful substances for bacteria such as antibiotics and putting them outside (Pagès et al.,2011). These pumps are widely present in Gram-negative bacteria as they pump water-soluble substances such as antibiotics out of the bacterial cell as a method of bacterial resistance to antibiotics (Poole , 2007). Which plays a major role in the pathogenesis of these bacteria, as efflux pumps pump antibiotics out of the cell (Blair et al., 2014). An increase in the number of efflux pumps was observed in K. pneumoniae with a type of (MDR) multi drug resistance (Pages et al.,2009).

One of the important pumps in K. pneumoniae is the AcrABR pumps, which when lost, the bacteria become more sensitive to a group of antibiotics such as erythromycin, chlorophenicol, fluoroquinolones, gentamycin, and others (Padilla et al., 2010). This mechanism of resistance increased significantly among isolates of K. pneumoniae bacteria and to a large group of antibiotics, especially fluoroquinolones (Schneiders et al., 2003).

MATERIALS AND METHODS

200 samples were collected from different sources, distributed on 80 urine samples, 42 exit samples (stool), 62 sputum samples, and 26 samples of burns and wounds from patients attending and in Diwaniyah Teaching Hospital and Women's and Children's Hospital in the governorate. Al-Diwaniyah, whose ages ranged between 1-60 years and for a period of five months from 9/15/2021 to 01/20/2022. Samples were taken using sterile cotton swabs to collect samples and the dishes were incubated at a temperature of 37°C for 24 hours. On the center of the Maconkey agar and blood agar.

Diagnosis of isolates / The API 20E system was used to diagnose bacterial isolates, as the instructions of the supplied company were followed, and then the diagnosis was made using the Vitek 2 device to diagnose bacterial isolates with a high degree of accuracy after confirming them by preliminary biochemical tests.

Phenotypic detection of effluent pumps / Decimal dilutions of all bacterial isolates were prepared using sterile physiological salt solution and its turbidity was compared with a standard turbidity constant solution (McFarland). This detection was carried out on bacterial isolates that have antibiotic resistance by adopting the modified EtBr-agar cartwheel method and by using soybean agar agar and ethidium bromide dye with different concentrations according to what was mentioned (Martins et al.,2013). As it comes:

• Different concentrations of ethidium bromide dye (5, 10, 15, 20, 25) mcg/ml were prepared by adding different amounts of the above dye to the medium of trypton agar soybeans after sterilizing and slightly cooling it.

• The media were shaken well and after sterilization, they were poured into sterilized dishes that were previously divided radially and kept at refrigerator temperature until use.

• Sterile cotton swabs were passed over the diluted bacterial suspension, then pressed on the inner wall to get rid of the excess culture, then they were planted in a radial line from the edge of the dish to its center

for each isolate and for each concentration, then the dishes were incubated at a temperature of 37 °C for a period of 16 hours.

• Bacterial isolates were tested using a UV source to observe the fluorescence intensity.

Molecular detection of effluent pump

Chromosomal DNA extraction The chromosomal DNA of selected colistin-resistant bacterial isolates was extracted using a special kit (Genomic DNA extraction kit) provided by Geneaid Corporation of America.

Preparation of the polymerase chain reaction mixture / The reaction mixture was prepared using the AccuPower® PCR PreMix kit supplied by the Korean company (Bioneer) and using the primers in Table (1) designed with the Efflux Pump operon gene primers and the DNA sequencing initiator in this The study was carried out using the genetic sequence of these genes in the gene bank on the National Biological Information website of the NCBI, using the Primer 3 plus primer design program, and these primers were supplied by the Korean company Bioneer.

Table 1. The primers used in the study with the sequence of nitrogenous bases and the size of the primer.

Primer	Nitrogen Bases		Product
AcrA	**F**	**ATG AAC AAA AAC AGA GGG TTA ACG-3`5-**	**1194**
	R	**5-TTA AGA CTT GGT TTG TTC TGA TGG-3`**	
AcrB	**F**	**ATG CCT AAT TTC TTT ATC GATCGC-35-**	**3147**
	R	**TTA ATG ATG CTC AAC CTG ATG GC-35-**	
AcrR	**F**	**ATG GCA CGA AAA ACC AAA CAA C-35-**	**651**
	R	**TTA AGC TGA CAA GCT CTC CGG-35-**	

RESULTS AND DISCUSSION

The current study dealt with the collection of a number of pathological samples in order to obtain isolates of K. pnumoniae bacteria with multiple resistance to antibiotics, as 200 clinical samples were collected from different pathological cases from Al-Diwaniyah Teaching Hospital and Women and Children Hospital in Al-Diwaniyah Governorate for the period from 15/9/2021 until 1/22022 And based on biochemical tests and Vitek2 diagnosis, the results showed that K.pneumoniae bacteria constitute 36 isolates with a rate of 22.1%.

Phenotypic detection of efflux pumps

The detection of efflux pumps in 18 bacterial isolates was carried out using the EtBr CW wheel method (Gressler et al., 2014). Relying on ethidium bromide stain as a proxy for phenotypic detection. The results showed that 18 isolates (100%) were positive for phenotypic detection (Table 2), depending on the lowest concentration in which the isolates did not appear to shimmer under ultraviolet (UV) rays (Gawad et al., 2018). Thus, our results came close to (Al-Saadi et al.,2019) whose isolates showed a positive percentage of phenotypic detection of effluent pumps at a rate of 92%.

Table 2. Results of phenotypic detection of efflux pumps in K. Pneumoniae bacteria using different concentrations of ethidium bromide dye.

Ethidium bromide dye concentration (µg/ml)					
	5	10	15	20	25
1	-	-	-	+	+
2	-	-	-	-	+
3	-	-	+	+	+
4	-	-	-	+	+
5	-	-	+	+	+
6	-	-	-	+	+
7	-	-	-	-	+
8	-	-	-	-	+
9	-	-	-	+	+
10	-	-	-	+	+
11	-	-	-	-	+
12	-	-	-	+	+
13	-	-	+	+	+
14	-	-	+	+	+
15	-	-	-	-	+
16	-	-	-	-	+
17	-	-	+	+	+
18	-	-	-	+	+

Molecular detection of the AcrAB efflux pump in K. pneumonia

The results of the molecular detection of the acrA gene with a size of 3147 base pairs using PCR technology for 18 bacterial isolates under study showed its presence in 100%, by comparing the doubled bundles with the bundles belonging to the DNA ladder. The resulting bundles were also found to have a molecular weight of 3147 base pair. Figure (1)

This study agreed with the findings (Pakzad et al.,2013), as the percentage of bacterial isolates that possessed the acr A gene was 100%. Our results (Maleki et al.,2017; Lafta et al.,2022) also confirmed that all isolates of K. pneumoniae resistant to ciprofloxacin contained the acrA gene, while (Razavi et al.,2020; Margiana et al.,2022) the frequency of the acrA gene was found, reaching 82.90%. acraA is 52.72%.

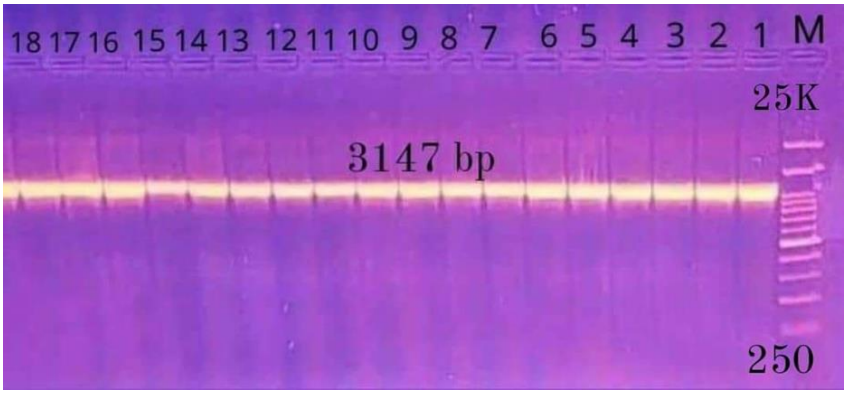

Figure 1. Electrophoresis of the results of the acrA base pair polymerase chain reaction (3147) of isolates of K. pneumoniae on 1.5% agarose gel, voltages 70, 80 amps, 1 hour, column M represents DNA ladder

Genetic detection of the acrB gene

The results of molecular detection of the acrB gene with a size of 1194 base pairs by PCR technique showed for 18 bacterial isolates under study. That 55.5% of the isolates possessed the acrB gene and by comparing the doubled bundles with the bundles belonging to the DNA ladder) (it was found that the resulting bundles had a molecular weight of 1194 base pairs. Figure 2). Thus, our results were almost identical to (Li et al.,2022; Abbas et al.,2022) who used the PCR technique In order to investigate the acrB gene and they obtained 52.72% of it among their isolates, either) (Pakzad et al.,2013; Hussein et al.,2022) recorded the percentage of appearance of this gene amounted to 100%, and thus our results were inconsistent with them, as (Razavi et al.,2020) violated our results in recording this gene at a rate of 95.90% and in a study conducted by (Schneiders et al.,2003; Al-Jassani et al.,2022) as They recorded the presence of this gene among their isolates by 95%.

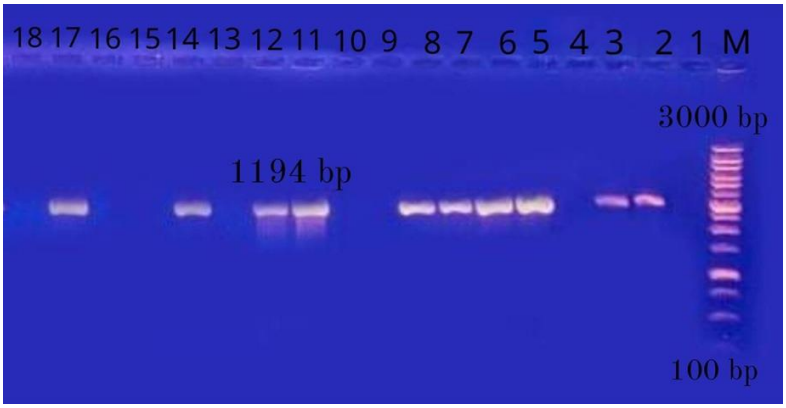

Figure 2. Electrophoresis of the polymerase chain reaction results of acrB 1194 (base pair) gene of isolates of K. pneumoniae on 1.5% agarose gel, voltages 70, 80 amps, for 1 hour, column M represents the DNA ladder

Genetic detection of the acR gene

The results of molecular detection of the acrR gene, which has a size of 651 base pairs, by PCR technique for 18 bacterial isolates under study, showed a 100% appearance rate (Togawa et al., 2015; Zadeh et al.,2022; Arif et al.,2023)

Studies of some Enterobacter family members indicate that mutations within the AcrR repressor lead to increased expression

of acrA and acrB. Complementation of mutated acrR with a gene of the non-mutated type has been shown to reduce the level of antibiotic resistance, suggesting a role of the functional inhibitor in controlling the highly drug-resistant phenotype (Padilla et al.,2010;Rohmah et al.,2023). It was found that deletion of the acrR gene led to a moderate increase in MICs for most of the antibiotics tested compared to the parent strain.

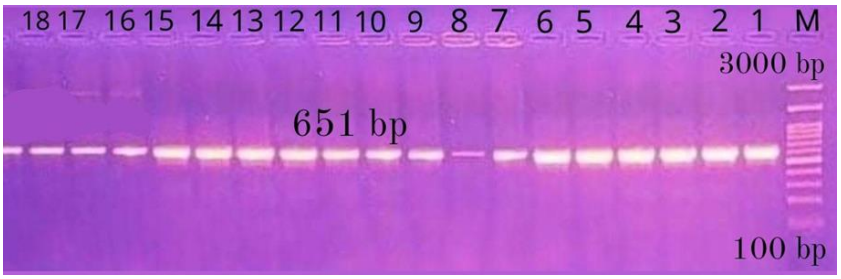

Figure 3. Electrophoresis of acrR(651) base pair polymerase chain reaction results of isolates of K. pneumoniae on 1.5% agarose gel, voltages 70, 80 amps, 1 hour, column M represents DNA ladder.

Compliance with Ethical Standards statements

Ethical approval:

The manuscript is written in original and all the data, results pertaining to this manuscript are original according to the research performed. The authors followed academic integrity and have not copied any content/results from another source.

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Informed Consent: The authors of the manuscript agrees to publish this research in the journal if it's considerable by the editors of the journal. The authors provide full consent for reviewing and publishing this manuscript.

V. All the authors of this study contributed equally in terms of performing the research as well as in preparing the manuscript. All the authors of the study followed the guidelines of the corresponding author. Any query/suggestion related to the manuscript can be reached to the corresponding author

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