

Journal of Applied and Natural Science

16(2), 820 - 829 (2024)

ISSN: 0974-9411 (Print), 2231-5209 (Online)

journals.ansfoundation.org

Research Article

CRISPR-Cas system in multi drugs resistant Klebsiella pneumoniae different clinical from samples correlation and its antibiotic-resistant genes in Mosul city / Iraq

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Article Info

https://doi.org/10.31018/ jans.v16i2.5529

Received: March 9, 2024 Revised: May 29, 2024 Accepted: June 05, 2024

How to Cite

AL-Yozbakee, Z.M. & Mohammad, K.O. (2024). CRISPR-Cas system in multi drugs resistant Klebsiella pneumoniae from different clinical samples and its correlation with antibiotic-resistant genes in Mosul city / Iraq. Journal of Applied and Natural Science, 16(2), 820 - 829. https://doi.org/10.31018/jans.v16i2.5529

Abstract

Clustered, regularly interspaced short palindromic repeats (CRISPRs) and their related genes (Cas) are prevalent in the genomes of several bacteria and serve as a defense mechanism against external attackers, such as plasmids and viruses. This study aimed to examine the frequency of the CRISPR/Cas system in naturally occurring strains of Klebsiella pneumoniae sub spp pneumoniae confirmed by Vitek 2 biochemical test, in the hospital setting and determine its correlation with antibiotic resistance both phenotypically and genetically (antibiotic-resistant genes, namely blaTEM and AcrA efflux pump gene). The research was conducted at Medical College/ Mosul University 23 multi-drug resistant K. pneumoniae sub spp. pneumoniae that were obtained from 230 clinical samples from infected patients with different types of infections attending Al-Salam and Al-Jumhoree Teaching hospitals. PCR was used to detect blaTEM, AcrA genes, and CRISPR/Cas system genes (CAS1A and CAS1B) among the clinical isolates. The correlation between the CRISPR/Cas system and antibiotic-resistance was determined. All the isolates were multiple drug-resistant strains, and the blaTEM gene was detected in all clinical isolates, whereas AcrA gene was detected in 94% of the isolates. The frequency of CAS1A and CAS1B was 21.73% and 86.95% respectively. There was an inverse correlation between the CAS1A gene and phenotypic antibiotic resistance Disc diffusion test results, so isolates carrying CAS1A gene were less resistant to different antibiotics studied in this research. In contrast, there was no significant correlation between CRISPR / Cas genes, blaTEM, and AcrA genes at the genetic level.

Keywords: AcrA gene, blaTEM gene, CRISPR / Cas genes, Klebsiella pneumoniae sub spp. pneumoniae

INTRODUCTION

The rise and dissemination of antibiotic resistance poses a danger to human well-being. The primary mode of transmission for antimicrobial resistance is horizontal gene transfer (HGT), which results in the dissemination of bacterial resistance to drugs (Tao, 2022). Klebsiella pneumoniae is a major cause of nosocomial infections, highlighting its importance in healthcare-associated infections. The World Health Organization has designated K. pneumoniae as a "priority pathogen," emphasizing its crucial role in the increasing challenge of antibiotic resistance (Efah, 2020). Considering its widespread frequency and involvement in a variety of disorders, treating Multiple Drug Resistant (MDR) K. pneumoniae infections with the proper therapy is essential to

limiting the spread of antibiotic resistance (Ragupathi et al, 2020). K. pneumoniae is a type of gram-negative bacteria characterized by a highly selective permeability barrier. The limited permeability of their outer membrane is a significant factor contributing to their resistance against numerous antibiotics (Farrag, 2019). Two of the main resistance mechanisms developed by K. pneumoniae are the production of extendedspectrum β-lactamase (ESBL) like blaTEM and efflux pump genes like ArcA gene (Abid, 2022; Raouf, 2023). Bacteria have evolved different ways to immune itself against evading pathogens like bacteriophage and plasmids by what is called the CRISPR-Cas system (Clustered Regularly Interspaced Short Palindromic Repeats-CRISPR associated proteins) 2020). This system serves as a natural immune system

for more than 50% of bacteria and most of the archaea against invading genetic elements (Brooker, 2018; Ishino, 2018). These foreign invading genetic elements are recognized, captured, and preserved as pieces of foreign genetic material within the bacterial chromosomes by CRISPR-Cas system. Medina-Aparicio (2018) states that the CRSPR-Cas system consists of two main components: the CRISPR array which is the first part consists of brief DNA repetitions, usually spanning from 22 to 56 nucleotides, that are interspersed with unique sequences, derived from mobile genetic elements (MGEs) like bacteriophages, plasmids, or transposons (Medina, 2018; Alkompoz, 2023). Transcription of the CRISPR arrays leads to, the generation of CRISPR RNA (crRNA). The crRNA functions as a navigational tool for the second part of the CRSPR-Cas sysem which is one or more Cas operons which regulate Cas proteins productions (Hille, 2018). The Cas proteins, namely Cas1, Cas3, and Cas9, are pivotal in recognizing and cleavage of foreign DNA (Brooker, 2018).

The CRISPR/Cas system is classified into two separate main classes, which are further organized into six types and around forty subtypes. Within this theoretical framework, it has been noted that individuals in Class 1 employ a combination of multi-Cas effector proteins to disrupt invading DNAs, while members of Class 2 rely on a single effector protein for the same purpose (Newsom, 2020). The categories are based on the distinct Cas proteins that carry out the crucial function of cutting invading nucleic acids. This wide range is categorized into distinct classifications: Class 1 of CRISPR/ Cas encompasses types I, III, and IV, whereas Class 2 comprises types II, V, and VI (Makarova, 2017). Each type is distinguished by a specific gene that encodes the Cas protein associated with its respective function. The signature and almost universal protein in almost all bacteria within each class is CAS1 with its two subtypes CAS1 A and CAS 1 B (Makarova, 2015), other effector proteins and their genes were discovered later and are present in different subtypes of CRISPER-Cas system and include, CAS3 protein in type I, CAS 9 protein in type II, CAS10 protein in type III, CSF1 protein in type IV, CAS12 protein in type V, and CAS13 protein in type VI (Makarova, 2018). The taxonomy further extends to subtypes based on the intricate architecture of the CRISPR/Cas locus, a categorization that has evolved to capture the intricacies of this versatile system (Newsom, 2021).

Several studies conducted in the past decade have shown a negative relationship between the occurrence of the CRISPR/Cas system in *K. pneumoniae*, and the development of antibiotic resistance (Kamruzzaman, 2020; Wang, 2020; Jwair, 2023). Nevertheless, the findings in this domain have occasionally been contradictory, thereby highlighting the evident requirement for further investigation in this area

(Touchon, 2012; Shabbir, 2018; Alkompoz, 2023). Exploring the relationship between the presence of CRISPR genes and antibiotic resistance factors, such as ESBLs and drug efflux genes, in K. pneumoniae, could offer valuable knowledge on potential therapeutic targets for combating infections caused by this resistant bacterium. Therefore, the objective of this work was to establish the correlation between the CRISPR/Cas systems and the development of antibiotic-resistance, as well as the synthesis of extended range β-lactamase genes, namely blaTEM gene and efflux pump ArcA gene among K. pneumoniae isolates, obtained from hospitalized patients in Mosul city / Iraq.

MATERIALS AND METHODS

Study area

A cross-sectional study was conducted at Al-Salam and Al-Jumhoree Teaching Hospital in Mosul city during six -month period from February 2023 to August 2023.

Patients

All ages and both sexes were included in this study, and the history of each patient, including their name, age, gender, and the type of specimen, was obtained. Additionally, it was ensured that infected patients included in the study were not under treatment, specifically no antibiotic intake for 3 days prior to specimen collection for culture.

Ethical approval

Ethical approval for this study was obtained from the Iraqi Ministry of Health/Mosul Health Department, with an assigned approval letter, No. 9295, dated 19th February 2023.

Sample collection

A total of 230 different clinical samples were collected. All samples were cultured on MacConkey, Eosin Methylene Blue, Blood agar, and Brain Heart Infusion broth (BHI), then incubated overnight at 37 °C. Colonies that appeared were tested for oxidase, catalase, and urease production, as well as biochemical reactions for exact strain identification, as confirmed by the VITEK®2 GN ID card.

Antimicrobial susceptibility test

The test was carried out by using the Kirby- Bauer antibiotic disc diffusion technique and performed on Muller-Hinton agar. An inoculum isolate was generated by emulsifying the colonies from an overnight culture in sterile normal saline until they reached the same level of cloudiness as the 0.5 McFarland solution standard. The bacterial suspension was evenly spread on the Muller-Hinton agar using a sterile swab and let to dry. The antibiotic discs have been placed on the plate using sterile forceps. The plates were placed in an incu-

bator set at a temperature of 37°C for a duration of 24 hours. The width of the zone that inhibited growth was measured, and the results were analyzed using the guidelines provided by the Clinical and Laboratory Standards Institute (CLSI) in 2022. These antibiotic discs came in the following types and concentrations: $10\mu g/disc$ of imipenem, $30 \mu g/disc$ of augmentin, $30 \mu g/disc$ of amikacin, $30 \mu g/disc$ of tetracycline, $10 \mu g/disc$ of ceftriaxone, and $10 \mu g/disc$ of gentamicin, chloramphenicol $10 \mu g/disc$, cefotaxime $10 \mu g/disc$, ceftazidime $30 \mu g/disc$, and colistin $10 \mu g/disc$.

DNA extraction for conventional PCR detection for antibiotic-resistance genes (blaTEM and ArcA efflux pump gene) and CRSIPR-Cas genes (CAS1A and CAS1B)

DNA was extracted by a commercial kit (Geneaid company, Taiwan) according to the manufacture procedure. The PCR conditions were as follows: Initial denaturing at 94 °C for 5 min followed by 30 cycles, each cycle contained 1 min at 94 °C for denaturation, 30 s for annealing and the annealing temperature as in Table 1 and 60 S for extension steps and finally one cycle for the final extension at 72 °C for 10 min. All primers information's are given in Table 1.

Data analysis

The statistical analyses were conducted using IBM SPSS statistics software version 25.0 (IBM Corp., Armonk, NY, USA). Data were described as tables, charts and diagrams. Statistical significance was defined as p-values less than 0.05. The t-test values were utilized to compare categorical variables. The Pearson correlation coefficient factor (r) was computed for correlation analysis.

RESULTS AND DISCUSSION

Frequency of *Klebsiella* spp among different clinical samples

Of the 230 clinical isolates investigated in this study, 33 samples contained klebsiella spp. Specifically, there were 11 isolates (33.3%) from urine, 7 isolates (30.3%) from pus, 10 isolates (21.2%) from sputum, 2 isolates (6.06%) from tracheostomy, 1 isolate (3.03%) from

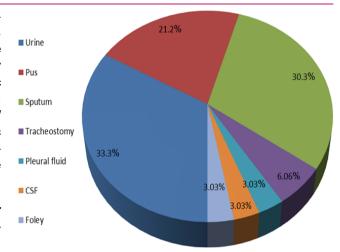


Fig. 1. Percentage of Klebsiella spp isolates from different clinical samples

pleural fluid, 1 isolate (3.03%) from CSF, and 1 isolate (3.03%) from Foley's catheter, as illustrated in Fig. 1. The most frequent sample that contains K. pneumoniae in this study was urine 33.3% this result is generally consistent with numerous studies conducted locally in Iraq, as in a study done in Baghdad City by Al-Saady (Al-Saady, 2023), showed that the most frequent sample that contained Klebsiella spp. was from urine 37 (37%) out of 108 isolates. Other local studies done in Duhok City and Erbil agreed with this study, in which the most frequent sample containing Klebsiella was urine samples 66% and 56% respectively (Naqid, 2020; Nawaz, 2009), whereas other studies done in different countries like China, Iran and Indonesia by (Wang, 2019; Karimi, 2021) showed that upper respiratory tract samples were the most frequent sites of K. pneumoniae infections.

Antibiotic-resistant pattern of Klebsiella pneumoniae

All *K. pneumoniae* isolates in this investigation exhibited multidrug resistance, the highest level of resistance observed against tetracycline, colistin, and ceftriaxone, with a 100% resistance rate, and the lowest resistance to imipenem. The antibiotic-resistance pattern of the isolates was determined by performing disc diffusion using the Kirby Bauer method on Muller Hinton agar. The diameter of the inhibition zone was measured and

Table 1. Informations about primers used, including their sequences and amplicons' size

Name of the gene	Sequence	Annealing tem- perature	Amplicon size	Reference
CAS1 type A	F CGAAACGCTACGGTGTGAAA R CGGAATCAATTTGCCTGTCA	49 °C	341	Li H Y,2018
CAS1 type B	F CGGCTGGAAATTGATGACAG R ATCCGGAAAGCGTTTAGCAA	50 °C	309	Li H Y, 2018
blaTEM	F GCCAACGTCTACGTTAACCTG R ATATTTCACGGTGCCTGAAAA	63°C	180	This article
AcrA	F ATCAGCGGCCGGATTGGTAAA R CGGGTTCGGGAAAATAGCGCG	56 °C	312	Wasfi R, 2016

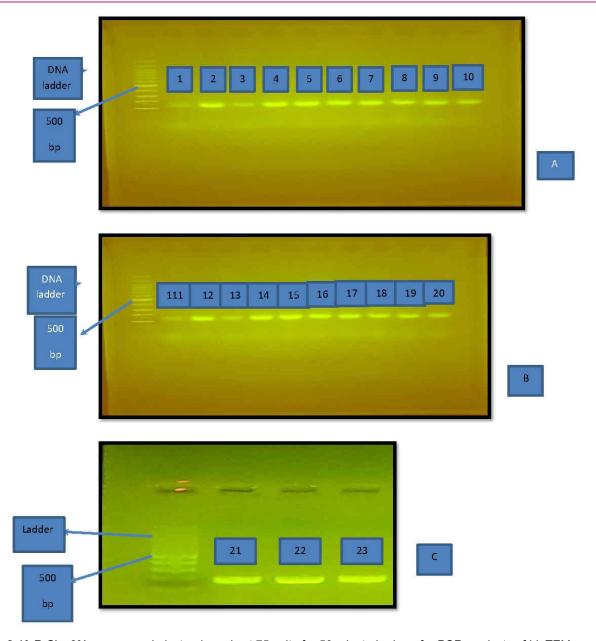


Fig. 2 (A,B,C). 2% agarose gel electrophoresis at 75 volte for 50 minute had run for PCR products of blaTEM gene in 23 isolates of Klebsiella pneumoniae sub spp pneumoniae with its length 180 bp. A) showing the PCR products of blaTEM in first 10 isolates, B) showing the PCR products of blaTEM in last 3 isolates

compared to the CLSI (2022) guidelines. The results were as follows: The bacteria exhibited complete resistance (100%) to tetracycline, colistin, and ceftriaxone. There was a resistance rate of 82.61% to augmentin, 65.21% to amikacin, 39.13% to cefotaxime, 34.78% to ceftazidime, and 13.04% to both chloramphenicol and ciprofloxacin. However, no resistance (0%) was observed to imipenem, as indicated in Table 2.

In the present study, all clinical isolates were resistant to more than three classes of antibiotics; this was in agreement with other studies on *K. pneumoniae* resistant to multiple drugs (Farhadi, 2021; Vaez, 2019; Moghadas, 2018; Odari, 2022; Zhu, 2023). While other

studies showed lesser prevalence of MDR *K. pneu-moniae* like that done on 100 clinical isolates of *K. pneumoniae* showed only 58% of the isolates were MDR (Farhadi, 2021), and the reason for this discrepancy may be due to differences in geographical areas, strain types, hygiene level, specimens collected, study date, sample size, and antibiotic usage limitations.

Molecular method for detection of the prevalence of blaTEM and AcrA efflux pump genes among the Klebsiella pneumoniae clinical isolates

The study showed that all isolates were positive for blaTEM antibiotic-resistant gene, as shown in Fig. 2

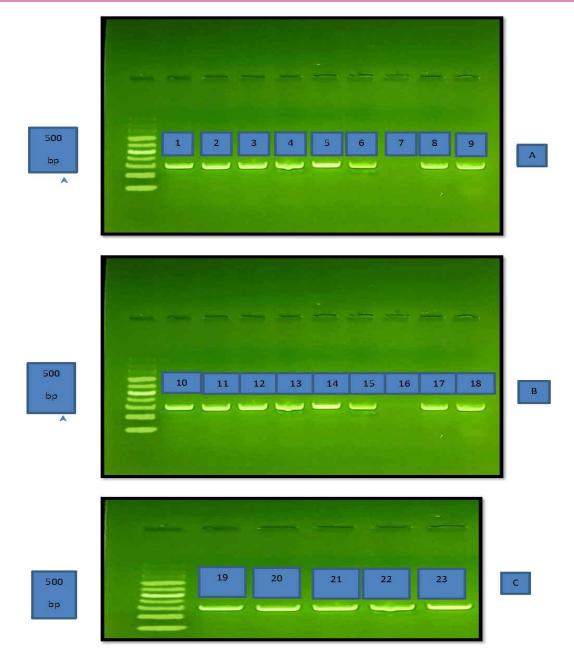


Fig. 3. A, B and C. 2% agarose gel electrophoresis at 75 volte for 50 minute run for PCR products of AcrA gene in 23 Klebsiella pneumoniae sub spp pneumoniae isolates its length is 312 bp. A) showing the PCR products of AcrA in first 9 isolates, B) showing the PCR products of AcrA in second 9, and C) showing the PCR products of AcrA in last 4 isolates.

(A,B, C) and for *ArcA* efflux pump gene 21(91%) were positive and 2 (8.69%) were negative, as in Fig. 3 (A, B, C).

The *blaTEM* was positive in all isolates; this was in agreement with other studies as in other cities in Iraq (Pishtiwan, 2019), in Sudan (Dirar, 2020) and in Iran (Kashefieh, 2021). In contrast, other global studies done in different countries showed *blaCTX-M* was the most common ESBL enzymes (Al-Garni, 2018; Sewunet, 2021; Carvalho, 2021)

The AcrA efflux pump is a periplasmic protein encoded

by *AcrA* gene, which in this study was positive in 91% of isolates. This result was approximate to other studies done on MDR *K. pneumoniae* in Iraq, like that of Abid (2023) in Diwaniyah province and another study done by Khalid (2022) in Baghdad, which showed 100% presence of *AcrA* in all clinical isolates of *K. pneumoniae*. The efflux pump system is a highly significant mechanism of antibiotic resistance in different bacterial species. Numerous global research studies have verified the significance of hese pumps in augmenting the resistance of *K. pneumoniae* to various antibiotic clas-

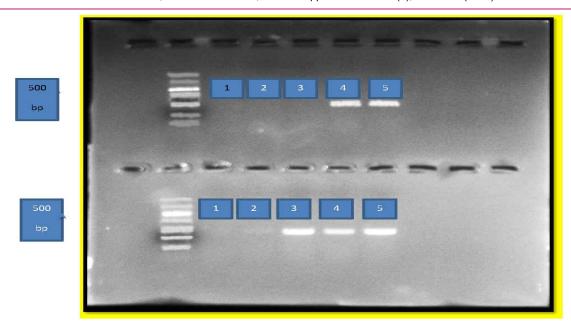


Fig. 4. 2% agarose gel electrophoresis at 75 volte for 50 minute run for PCR products of CAS1A and CAS1B gene in first 5 Klebsiella pneumoniae sub spp pneumoniae isolates. CAS1A gene PCR products size was 341 bp and CAS1B gene PCR products size was 309bp

Table 2. Antibiotic susceptibility test results against Klebsiella pneumoniae

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Antibiotics	NO. / %	NO. / %	NO. / %
Augmentin AMC (30) μg	19 (82.61%)	2 (8.69 %)	2 (8.69%)
Amikacine AK (30) µg	15 (65.21%)	1 (4.34%)	7 (30.43%)
Ciprofloxacin CIP (10) µg	3 (13.04%)	2 (8.69%)	18 (78.26%)
Tetracycline TE (30) µg	23 (100%)	0 (0%)	0 (0%)
Chloramphenicol C (10) µg	3 (13.04%)	4 (17.39%)	16 (69.56 %)
Colistin CL (10) µg	23 (100%)	0 (0%)	0 (0%)
Cefotaxime CTX (10) µg	9 (39.13%)	5 (21.73%)	9 (39.13 %)
Imipenem (10) μg	0 (0%)	0 (0%)	23 (100%)
Ceftriaxone CRO (10) µg	23 (100%)	0 (0%)	0 (0%)
Ceftazidime CAZ (30) µg	8 (34.78%)	9 (39.13%)	6 (26.08%)

ses (Szabo, 2018; Abid, 2022; Khalid, 2022).

Frequency of *CAS1* (A andB) genes in the isolated *Klebsiella pneumoniae* sub spp. *pneumoniae* and the correlation of CRISPR-Cas system with the type of specimen

The presence of *CAS1A* and *CAS1B* was examined in all isolates of *Klebsiella pneumoniae* sub spp *pneumoniae*, and their correlation with the presence of antibiotic resistance genes in the same isolates was evaluated. *CAS1A* was identified in only 5 out of 23 samples, accounting for 21.73% of the total. The remaining 18 samples, or 78.26%, tested were negative for *CAS1A*. On the contrary, *CAS1B* tested negative in just 2 (8.69%) isolates and positive in the remaining 21 (91%) isolates, as shown in Fig. 4, 5

CAS1 gene is present in all CRISPR/Cas types, (Makarova, 2015). CAS1A was less prevalent than CAS1B in Klebsiella pneumpnaie clinical isolates, con-

sistent with a study done in Baghdad/ Iraq (Ali, 2022). Contrary to other studies which showed a lesser prevalence of CRISPR –Cas system among *Klebsiella pneumonaie* in China with a percentage equal to (14.9 and 21.3%) of their collections (Wang, 2020; Liao, 2020) There was no significant correlation between the CRISPR-Cas system and the type of specimens, which agreed with other studies in Egypt (Alkompoz, 2023) and another study in China (Li, 2018).

CRISPR-Cas system among clinical isolates of Klebsiella pneumoniae and its correlation with antibiotic-resistant

There was a strong inverse relationship between the existence of CRSIPR-Cas system, especially *CAS1A* gene and the resistance to different antibiotics that were tested in this study at the phenotypic level (results of antibiotic resistance by Kirby-Bauer Disc diffusion test), as the Pearson correlation factor (r) value

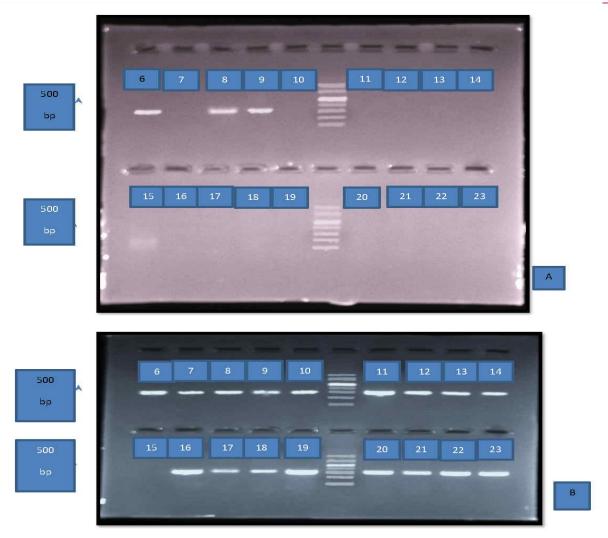


Fig. 5. A , **B.** 2% agarose gel electrophoresis at 75 volte for 50 minute had run for PCR products of CAS1A and CAS1B gene in second 18 Klebsiella pneumoniae sub spp pneumoniae isolates. CAS1A in A) and its PCR products size was 341 bp while CAS1B in B) and its PCR products size was 309bp

was equal to -1 at p level < 0.00001. The isolates which carry CRISPR-Cas system had lower resistance to ciprofloxacin, amikacin, chloramphenicol, cefotaxime, ceftazidime, and augmentin, as shown in Fig. 6

In contrast, there was no significant correlation between antibiotic resistance in both *CAS1B*+ve and *CAS1B*-ve clinical isolates, as *CAS1B* gene was detected in almost all isolates except 2 isolates, which were negative for *CAS1A* too, which indicates the absence of CRISPR-Cas system in these two isolates.

There was no significant correlation in the distribution of antibiotic-resistant genes (ARGs), namely *blaTEM* and *AcrA* genes, and *CAS1A* and *CAS1B* gene results in studied clinical isolates. The Fisher exact test statistic value was equal to 1 at p value < 0.05. The antibiotic-resistant genes were more found in *CAS1B*+ isolates than among *CAS1A* + isolates, as in Fig. 7

The correlation between CRISPR-Cas system and antibiotic resistance is still under study and varies from one research to another, some researches said there is no significant correlation (Alkompoz, 2023) and some said there is a negative correlation between the presence of CRSPR-Cas system and antibiotic susceptibility (Jwair, 2023; Ali, 2022). Prior research has shown that K. pneumoniae strains possessing type I CRISPR system exhibit a significant abundance of tetracycline resistance genes while displaying increased susceptibility to aminoglycoside and β -lactam antibiotics and a reduced presence of related resistance genes (Tao, 2022). A few resistant strains containing the CRISPR-Cas system were considered to be antibiotic-resistant strains and this may be due to the mutation of the original spacer sequence, the partial or total deletion in the Cas gene cluster, and the presence of anti-CRISPR proteins (Basgall, 2018; Guo, 2017).

Gene sequencing by Sanger sequencing method

After completing gene detection by conventional PCR method for the previously mentioned genes in the studied clinical isolates, PCR products of genes were sent

Correlation of antibiotic resistance and CAS1A

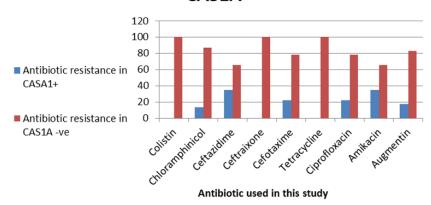


Fig. 6. Correlation of antibiotic resistance and CAS1A gene in all isolates (CAS1A +ve and CAS1B-ve isolates)

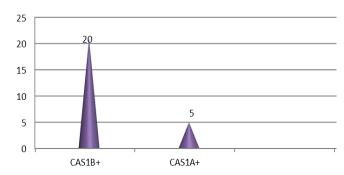


Fig. 7. Distribution of ARGs in CAS1A and CAS1B + isolates

for gene sequencing Sanger method together with forward primer of each of 4 genes and compared with National Center Biotechnology Information (NCBI) gene bank and all were 100 % identical strains, all the tested isolates carrying type I-E CRISPR- CAS system.

Conclusion

Most clinical isolates of K. pneumoniae included antibiotic resistance genes, specifically blaTEM and AcrA genes. The prevalence of blaTEM genes was higher than that of AcrA genes, with percentages of 100% and 91%, respectively. All studied K. pneumoniae isolates exhibited solely the Type I-E CRISPR-Cas system, with the CAS1A gene being less prevalent than the CAS1B gene. In this study, a strong inverse relationship was observed between the presence of the CRSIPR-Cas system, particularly the CAS1A gene, and resistance to various antibiotics tested at the phenotypic level using the Kirby-Bauer Disc diffusion test. However, no significant correlation was found at the genotypic level. These findings suggest the need for further research on the relationship between the CRISPR-Cas system and other antibiotic resistance genes. The practical potential of the system lies in its ability to prevent and control horizontal gene transfer, hence reducing the dissemination of antibiotic resistance. This may potentially lead to the development of methods, such as immunotherapy, to prevent and control horizontal gene transfer.

Conflict of interest

The authors declare that they have no conflicts of interest.

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