Whole Genome Sequence Analysis of Beta-Lactamase Producing *Klebsiella pneumoniae* Isolates from Diyala/Iraq

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Abstract: *Klebsiella pneumoniae* multidrug-resistant (MDR) infections represent a global health concern and present significant clinical hurdles in both community as well as hospital settings, the development of resistant in Gram-negative strains (G -) occurs more rapidly in comparison with Gram-positive strains (G+). The present study aims to genetically investigate Beta-lactamase enzymes coding genes in isolates of *K. pneumoniae* MDR producing Extended spectrum β- lactamase enzymes (ESBLs), Metallo β- lactamase enzymes (MBLs) and (AmpC) using whole genome sequencing (WGS Isolates) were identified, and antibiotic sensitivity test for ten antibiotics was conducted using VITEK2.Whole Genome Sequencing (WGS) was conducted on two isolates resistant to Cephalosporins and Carbapenems utilizing the Illumina MiSeq technology. The sequences were compared to the antimicrobial resistance (AMR) gene database following the annotation of the assembled draft genomes. Results: Many bacteria show several resistance genes have been identified that facilitate the manufacture of β-lactamases through WGS, which include (ESBLs, MBLs, and AmpC) genes, as (*blaSHV- 79/28, blaTEM1-B, blaCTX-M-15, blaCMY-6, blaNDM-1/2, blaMOX-1, blaDHA-1)*. Furthermore, Quinolone resistance includes *(OqxA, OqxB ,qnrS1, qnrB4, parC, acrR*Moreover), genes that encoding aminoglycosides modification enzymes *(rpsL, aac-(3)lla, aac-(6)Ib3, aadA5, rmtC, aph-(3)la),* trimethoprim- sulfamethoxazole (*dfrA17 ,sul1*), Tetracycline (*tetA ,tetD ,ramR*), Fosfomycin *(fosA6),* chloramphenicol *(catA2*) resistance genes, while other genes that endow Macrolide resistance *(mphA*), and carbapenem resistance genes (*OmpK35, OmpK36,OmpK37).* The molecular characterization of isolates can be reconstructed using WGS thorough analysis.

1 INTRODUCTION

The spread of β- lactam antibiotics resistant *Klebsiella pneumoniae* bacteria is a global health concern, the development of resistant in Gram-negative strains occurs more rapidly in comparison with Gram-positive strains, particularly in multi-resistant variants, due to the presence of numerous resistance genes within their genome [1]. β-lactam antibiotics are among the most potent agents employed to combat severe infections resulting from intestinal bacteria that produce β-lactamase enzymes. [2]. In the Ambler classification scheme beta-lactamase is classified into 4 main classes A, B, C, D. The basis of this classification is the similarity of the protein or the similarity of amino acids to these proteins, phenotypic characteristics are not considered in this type of classification. Classes A ,C and D are Serine β-lactamase, while the enzymes in class B are Metallo β-lactamase [3]. Class A enzymes known as ESBLs are capable of breaking down penicillin, Monobactam, Aztreonam and third-generation antibiotics Cephalosporins, it is a derivative of the genes of TEM-1, TEM-2 or SHV-1 [4]. Class B enzymes are known as Metallo β-lactamase (MBLs), it can effectively decompose carbapenem antibiotics and needs Zn^{+2} as an adjunct to increase its analytical activity, as it uses Zn^{+2} in the active site ,so it was called by this name, usually encoded genes carried on the bacterial chromosome, and others encode them plasmid. MBLs contain several genes, including (*VIM*, *IMP*, *NDM* ([5]. Ampicillinase Class C Beta Lactamase (AmpC) is one of the β-lactamase enzymes that characteristically decompose narrow and broad-spectrum cephalosporins that resist inhibition by Clavulinic acid, Sulbactam and Tazobactam, many Gram-negative bacilli ,including *K . pneumoniae* , produce AmpC enzymes that are genes carried on chromosome or plasmids [6]. Commonly reported

AmpC genotypes can be divided into six groups based on sequence*: CIT ,EBC ,DHA ,ACC ,FOX* and *MOX* [7].One of the of modern and high-throughput sequencing methods is the Next Generation Sequence (NGS), Which provided a lot of data and very quickly in the field of sequencing ,unlike the serialization methods based on the Sanger method, NGS methods can read many sequences in parallel instead of long readings, it produces millions of short sequences with lengths ranging from 21 to 400 base pairs(bp) depending on the system used.Several platforms have been introduced for high-throughput sequences with different mechanisms starting with pyrosequencing 454 [8], [9].

2 MATERIALS AND METHODS

2.1 Bacteria Diagnosis with Antibiotics Sensitivity

The study was conducted for the period from January -March 2024, for genetic investigation of β-lactamase genes in *Klebsiella pneumoniae*, on 2 isolates of *K. pneumoniae* isolates MDR and confirmed resistance to β-lactam antibiotics carbapenem and cephalosporins, from spinal fluid from a patient with K13 meningitis and sputum smears from a patient with K43 pneumonia were admitted to different hospitals in Diyala. The bacteria were identified by the VITEK2 ID system (bioMérieux, France), and the minimum inhibitory concentration (MIC) in µg/mL against 10 different antibiotics was determined through the use of VITEK2 in the investigation of sensitivity to antibiotic The clinical laboratory standard Institute's (CLSI) breakpoints were used to interpret the findings of the antibiotics sensitivity test [10].

2.2 Whole Genome Sequencing

The WGS Libraries were prepared following the protocol for Preparing Illumina DNA Prep Libraries genomic DNA from *K. pneumoniae* were extracted using Kit ABIO pure Total DNA (ABIO Pure USA) following the manufacturer's protocol. genomic DNA was quantified by the Quantus Florometer (Promega, USA) for each isolate according to the manufacturer's recommendations. This includes whole genome reconstruction, resistance to antibiotics genes typing, annotation using the MiSeq sequencer (Illumina, USA), and whole genome sequencing paired-end of DNA extracts using Illumina data (2×150 bp reads). I followed the manufacturer's instructions and used the Illumina DNA Prep library (Illumina, USA) to prepare DNA libraries. All of the libraries was targeted with 1 ng genomic DNA. Utilizing the Bead-based transposome (Illumina DNA Prep Library Kit, Illumina, USA), genomic DNA was fragmented and tagged with adaptor sequences all at once (tagment genomic DNA). Using the PCR program a limited-cycle (12-cycle) the tagged DNA was amplified. Amplified DNA was purified with Sample Purification Beads (SPB). Then, Qubit was used to quantify Illumina DNA Prep Libraries. Selected libraries were pooled 1 Nm for sequence. To pool libraries, a single tube is used to aggregate equal volumes for normalized libraries. Next, the (MiSeq reagent Kits V2-300 cycles) were used to dilute and heat-denature the constructed libraries before loading them onto the MiSeq sequencer.

2.3 Library Quality Control

After library preparation, agarose gel electrophoresis was adopted to confirm the presence of amplification as a quality control step (Fig. 1).

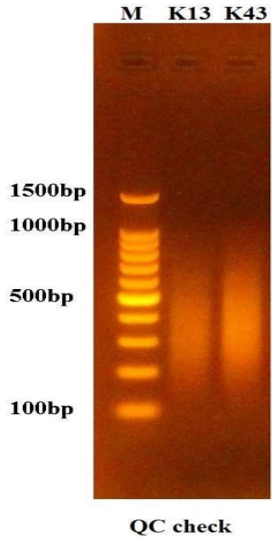

Figure 1: Findings of the Quality control check. M: 100bp ladder marker. Lanes K13, K43 resemble

2.4 Preparation of Agarose

Agarose gel was prepared according to standard molecular biology procedures as follows:

- 2 grams of agarose (for a 2% solution) were added to the buffer.
- The solution was heated to boiling in a microwave until all gel particles were disintegrated

- 1µl of Ethidium Bromide (10mg/ml) was incorporated into the agarose.
- The agarose was agitated to ensure thorough mixing and to prevent bubble formation.
- The solution was allowed to cool to 50-60°C.

2.5 Preparation of Agarose Gel

The agarose solution was poured into the gel tray after sealing both ends with cellophane tape, and the agarose was allowed to harden at ambient temperature for 30 minus. The comb was meticulously extracted, and the gel was positioned in the gel tray. The tray was filled with 1X TAE electrophoresis buffer until the buffer level was 3-5 mm above the gel surface.

2.6 Amplified Library Loading

One microliter of loading dye was applied to each 5µl of the amplified library, and samples were added carefully to the individual wells. Electrical power was turned on at 100v/m Amp for 60min. DNA moves from Cathode to plus Anode poles. The Ethidium bromide-stained bands in gel were visualized using Gel imaging system [11].

2.7 Bioinformatics Evaluation

Paired-end reads from isolates of K. *pneumoniae* were adapted, subjected to control of quality, as well as trimmed utilizing the BBduk instrument from BB Tools [12] to eliminate low-quality bases, ambiguities, and adapters. Utilizing the Spades technique, trimmed raw data were subsequently assembled de novo to generate a draft genome sequencing for every single isolate [13] as well as data quality was assessed utilizing the FastQC program [14]. The WGS data were submitted to the center for genomic epidemiology. Identify virulent genes [15]. Utilizing ResFinder at [16] the existence of insertion sequences was identified and their identity was confirmed. The sequence types for all isolates were ascertained based on WGS data.

3 RESULTS AND DISCUSSION

3.1 Antibiotic Sensitivity Test

Table 1 show the Resistance pattern of *K. pneumoniae* isolates against 10 antimicrobial agents. The K13 and K43 isolates were resistant against 10 antibiotics as follows; β-lactam group (Ampicillin ، Aztreonam ،Ceftazidime ،Imipenem ،Meropenem); Aminoglycosides group (Gentamicin ،Amikacin ، Tobramycin); Quinolones (Ciprofloxacin); Sulfonamide (Trimethoprim/ sulfamethoxazole).

3.2 Genome Accession Numbers

Raw genomic data for isolates numbered K13 and K43 were submitted to NCBI accessible on 15 June 2024), as well as the genomic sequences of Iraqi *K. pneumoniae* isolates were stored under Bio-Projects designated as ZMT13 and ZMT43 (accession numbers PP892203.1 and PP892204.1, respectively).

Table 1: Resistance pattern of *K. pneumoniae* isolates against antimicrobial agents.

Antimicrobial agents	Bacterial isolates (Minimum Inhibitory Concentrations Breakpoint in µg/ µl) K13, K43	
Ampicillin	32	32
Aztreonam	64	64
Ceftazidime	64	64
Imipenem	16	16
Meropenem	16	16
Gentamicin	8	16
Amikacin	64	64
Tobramycin	16	16
Ciprofloxacin	4	4
Trimethoprim/Sulfamethoxazole	320	320

3.3 Antimicrobial Resistance Pattern

The resistance study reveals the existence of numerous resistant genes, as illustrated in Tables 2 and 3. *K. pneumoniae* isolates exhibited multidrug-resistant phenotypes and harbored several β-lactamase genes. The MIC testing for cephalosporins with carbapenem antibiotics identified the existence of resistant genes for these agents. K13 isolate carried the following β-lactamase genes (*blaSHV-79),* K43 isolate harbored the following (*blaSHV-28, blaTEM-1B, blaCTX-M-15, blaCMY-6),* giving it resistance to the broad-spectrum, β-lactam group, for family ESBLs encodes for the enzyme penicillinase (Cephalothin ,Ampicillin ,Piperacillin ,Amoxicillin , Ticarcillin) [17], [18].

In accordance with findings from an Italian investigation, the characterization of β-lactamase in *K. pneumoniae* resistance to β-lactam isolates revealed the presence of extended-spectrum β-lactamases (ESBLs), including Cefotaximase-Munich (CTX-M), Temoneria (TEM), and Sulfhydryl Variable (SHV) families [15].K13

harbored (*blaNDM-2),* K43 (*blaNDM-1)* New Delhi Metallo-β-lactamase (NDM) giving it resistance to the β-lactam group Carbapenem (Imipenem, Meropenem) encodes for the enzyme Carbapenemase [20].

K13 harbored gene *blaMOX-1,* K43 *blaDHA-1* from AmpC β-lactamase family, gives it resistance to β- lactam antibiotics including the Cephalosporins group (Amoxicillin, Amoxicillin+Clavulanic acid, Ampicillin, Ampicillin+Clavulanic acid, Cefotaxime, Cefoxitin, Ceftazidime, Piperacillin, Ticarcillin, Piperacillin+Tazobactam, Ticarcillin+Clavulanic acid , Ceftriaxone) this genes hydrolysis cephalosporins third generation [7]. Furthermore, K13 and K43 isolates harbored multi-drug efflux (Resistance Nodulation Division) (RND) transporter periplasmic adaptor subunit *oqxA* and multi-drug efflux (RND) transporter permease subunit *oqxB*. gives it resistance to the anti-Trimethoprim belonging to a group folate pathway antagonist, Nalidixic acid and Ciprofloxacin to a group Quinolone and anti-Chloramphenicol to a group Amphenicol. That gives bacteria the characteristic of resistance to many species belonging to different groups [21]. K13, K43 contain the *fosA* gene that gives it resistance to the anti-fosfomycin to the Fosfomycingroup. This gene responsible for modifying antibiotics, It might result from the existence of the chromosome-associated *fos.A* gene present in numerous gram negative bacteria [22]. Were detected aminoglycoside-modifying enzymes with the following gene *rps*L in K13 isolate, as for isolate K43 harbored genes *aac(3')-lla, aac(6')-Ib3, rmtC, aadA5, aph(3")-Ia* that confers resistance to the anti-Streptomycin ,Gentamicin, Tobramycin, Apramycin, Gentamicin, Tobramycin, Dibekacin, Netilmicin, Sisomicin of the Aminoglycoside group. Resistance to aminoglycoside are primarily arises from aminoglycoside-modifying enzymes (AMEs) that exhibit varying capacities for modification aminoglycosides [23]. *rps*L An important gene that is used in the molecular identification of *Klebsiella* bacteria causes a mutation in ribosome proteins, gives bacteria resistance to anti-Tigecycline for intestinal family species [24]. K13 harbored *acrR*, *parC, gyrA, gyrB*, *as for* K43 contain *qnrB4, qnrS1,* gave it resistance of the Quinolone group. *acrR* gene from the flow pump coding genes in *Klebsiella* bacteria, which acts as a local repressor and controls the gene coding area, specially the pump genes [24]. *ramR* gene were detected in K13*,* both *tetA*, *tetB* genes in K43 respectively, encoding tetracycline resistance. These genes is related to modifying enzymes for antibiotics such as Tigecycline [25].

Table 2: Displays the varieties of resistant genes and the processes of the K13 isolate to various antibiotics with genetic backgrounds by (WGS).

Genetic background	WGS-predicted phenotype	Class	Antimicrobial
OqxA (EU370913) *OqxB* (EU370913*)*	Resistant	Folate pathway antagonist, Quinolone Amphenicol	Trimethoprim. Ciprofloxacin, nalidixic acid. Chloramphenicol
fosA6 (KU254579)	Resistant	Fosfomycin	Fosfomycin
rpsL (p.G88R)	Resistant	Aminoglycoside	Streptomycin
acrR (AJ318073.1) *parC* (CP152063.1) *gyrA* (CP043562.1) *gyrB* (CP031849.1)	Resistant	Quinolone	Fluoroquinolone Ciprofloxacin Nalidixic acid
OmpK35 (CP133153.1*)* *OmpK36* (CP133223.1) *OmpK37* (p.I128M)	Resistant	Underdevelopment	Carbapenem
*ramR (*CP126622.1*)*	Resistant	Tetracycline	Tigecycline
*blaMOX-1(*D13304)	Resistant	β-lactam	Ceftriaxone Ceftazidime
blaSHV-79 (AM176554)	Resistant	β-lactam	Ampicillin Cephalothin Ticarcillin Amoxicillin Piperacillin
*blaNDM-2 (*FN396876)	Resistant	β-lactam	Imipenem, Meropenem

K13 contain *OmpK35,OmpK36,OmpK37* genes confers *K. pneumoniae* resistance to carbapenem antibiotics, these genes encode for outer membrane purines that allow small molecules like anti-carbapenem to diffuse across the bacterial cell membrane ,so they give them resistance to those antibiotics [26]. The following genes were found in K43 isolate, *mph (A)* encoding macrolide resistance. of genes that regulate the flow pump [27]. conferring dihydrofolate reductase genes *dfrA*17 encoded trimethoprim resistance enzyme. *sul1* gene encoded sulfonamide resistance, one of the genes responsible for replacing the target site of the antibiotic ,which gives the bacteria resistance to the anti- sulfonamide [28]. *catA2* gene was detected in K43 isolate that confers resistance to the Amphenicol group. of genes that regulate the flow pump [27].

WGS technology is distinguished over traditional technologies in the ability to generate millions of readings in a single run. This study showed that isolates harbord several antibiotic resistance genes , including extended-spectrum β-lactamase genes ESBLs, MBLs, AmpC, other genes that conferred *K. pneumoniae* bacteria resistance to Quinolone Aminoglycoside, Tetracycline, Amphenicol, Trimethoprim, Sulfonamide and Macrolide ,along with multiple flow pumps. *K. pneumoniae* isolates exhibited multidrug-resistant phenotypes and harboured several β-lactamase genes. The MIC testing for cephalosporins and carbapenems antibiotics identified the presence of resistance genes to these agents. The frequency of gram-negative bacteria that produce β-lactamase is particularly concerning due to restricted therapeutic options. *K. pneumoniae* is readily transmitted through contact between people, but its pathogenicity was augmented through the facile acquisition of β-lactamase coding genes. via horizontal gene transfer among Gram-negative strains.

Table 3: Types of antimicrobial resistance genes, resistance mechanisms, and genetic background of *Klebsiella pneumoniae* isolate K43 identified by whole-genome sequencing (WGS).

Genetic background	WGS-predicted phenotype	Class	Antimicrobial
aac(6')-Ib3 (X60321*)* *aac(3')-lla* (CP023555*)* *aadA5* (AF137361*)* *rmtC (*AB194779*)* *aph(3')-la* (V00359*)*	Resistant	Aminoglycoside	Amikacin Tobramycin Gentamicin Spectinomycin Streptomycin Kanamycin Neomycin
OqxA (EU370913*)* *OqxB* (EU370913*)*	Resistant	folate pathway antagonist	Trimethoprim Ciprofloxacin nalidixic acid Chloramphenicol
fosA (ACWO01000079*)*	Resistant	Fosfomycin	Fosfomycin
blaTEM-1B (AY458016*)* *blaSHV-28* (AF299299) *blaCTX-M-15*(AY044436 *blaCMY-6(AJ011293)*	Resistant	β-lactam	Amoxicillin Ampicillin Cephalothin Piperacillin Ticarcillin
blaDHA-1 (Y16410*)*	Resistant	β-lactam	Ceftazidime Ceftriaxone
blaNDM-1(FN396876)	Resistant	β-lactam	Imipenem Meropenem
tetA (AJ517790*)* *tetD (*AF467077*)*	Resistant	Tetracycline	Doxycycline Tetracycline
*qnrB4 (*DQ303921) *qnrS1 (*AB187515*)*	Resistant	Quinolone	Ciprofloxacin
dfrA17 (FJ460238*)*	Resistant	Folate pathway antagonist	Trimethoprim
*sul1(*U12338*)*	Resistant	Folate pathway antagonist	sulfamethoxazole
catA2 (X53796*)*	Resistant	Amphenicol	Chloramphenicol
*mphA (*D16251*)*	Resistant	Macrolide	Erythromycin Azithromycin, Spiramycin Telithromycin

4 CONCLUSIONS

Whole-genome sequencing (WGS) provided a comprehensive characterization of the multidrug-resistant *Klebsiella pneumoniae* isolates by identifying their antimicrobial resistance determinants and genetic backgrounds. To the best of our knowledge, this is one of the first studies conducted in Iraq, and the first in Diyala Province, to apply WGS for the genomic characterization of clinical *K. pneumoniae* isolates.

Phenotypic antimicrobial susceptibility testing demonstrated that isolates K13 and K43 exhibited resistance to multiple classes of antibiotics, including β-lactams, carbapenems, aminoglycosides, quinolones, and trimethoprim/sulfamethoxazole. Whole-genome sequencing confirmed these phenotypic findings by identifying a wide range of resistance genes, including β-lactamase genes belonging to the ESBL (*blaSHV*, *blaTEM*, *blaCTX-M*), metallo-β-lactamase (*blaNDM*), and AmpC (*blaMOX-1*, *blaDHA-1*) families. In addition, numerous genes associated with resistance to aminoglycosides, quinolones, tetracyclines, fosfomycin, sulfonamides, trimethoprim, chloramphenicol, and macrolides were detected.

The presence of multiple efflux pump genes, including *oqxA*, *oqxB*, and *acrR*, together with outer membrane porin genes (*OmpK35*, *OmpK36*, and *OmpK37*), highlights the complex molecular mechanisms contributing to multidrug resistance in these isolates. The coexistence of several resistance determinants within the same bacterial genome demonstrates the ability of *K. pneumoniae* to accumulate diverse antimicrobial resistance mechanisms, thereby limiting available therapeutic options.

Overall, this study demonstrates the value of WGS as a powerful tool for accurately identifying resistance genes, correlating genotypic and phenotypic resistance profiles, and improving the surveillance of multidrug-resistant pathogens. Broader implementation of WGS in clinical microbiology laboratories and epidemiological surveillance programs will enhance the early detection of emerging resistance mechanisms, support infection control strategies, and facilitate more effective antimicrobial stewardship.

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