

Brief Report

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Brief report

Genomic analysis of Carbapenem-resistance conferring genes in *Klebsiella pneumoniae*-an *in silico* study

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Abstract: Carbapenems are considered for treating *Klebsiella pneumoniae* and other *Enterobacteriaceae* infections, especially if they are not susceptible to other generally prescribed antibiotics, i.e., if they show resistance. In such cases, antibiotic activity decreases, and most patients succumb to the infection. A better understanding of the disease pattern and resistance mechanisms could be gained by magnifying the genes that confer resistance to antibiotics. Therefore, studying the genes that confer resistance to carbapenems and any other antibiotics for that matter is indispensable for coming up with improved treatment options. This study included the analyses of co-resistance patterns between resistance genes- between drug classes and within the carbapenem-resistant genes, genomic context analysis of highly expressed carbapenem-resistant genes, and phylogenetic study of OXA-producing genes, plasmid incompatibility identification, and sequence type identification using MLST. The presence of ESBLs, MBLs, and SBLs across the downloaded genomes was studied. SHV-producing genes were found to co-occur with most of the resistant genes belonging to different drug classes. The plasmid incompatibility type IncFIB was found to be common among the highly expressed genes, and most of these genes were flanked by different families of insertion sequence (IS) elements. MLST study suggested that the presence of sequence types ST-11, ST-14, and ST-147 was common in the downloaded set of genomes.

Keywords: Carbapenem; Antimicrobial Resistance; *Klebsiella pneumoniae*; genomic context

INTRODUCTION AND LITERATURE REVIEW

Resistance to antibiotics is a growing concern and a threat to human health. Carbapenems are generally considered last-line agents to treat antibacterial-resistant infections¹. Annually, approximately 10 million deaths worldwide are predicted due to antibacterial-resistant infections². Carbapenem-resistant *Enterobacteriaceae* (CRE), especially Carbapenem-resistant *Klebsiella pneumoniae* (CRKP), pose a serious problem to clinicians due to its increasing prevalence in hospital settings.

These pathogens are acquired nosocomially, especially in Intensive Care Unit (ICU) patients. If the prescribed antibiotic course is not completed or if it is overprescribed, the patient may develop CRKP infections.

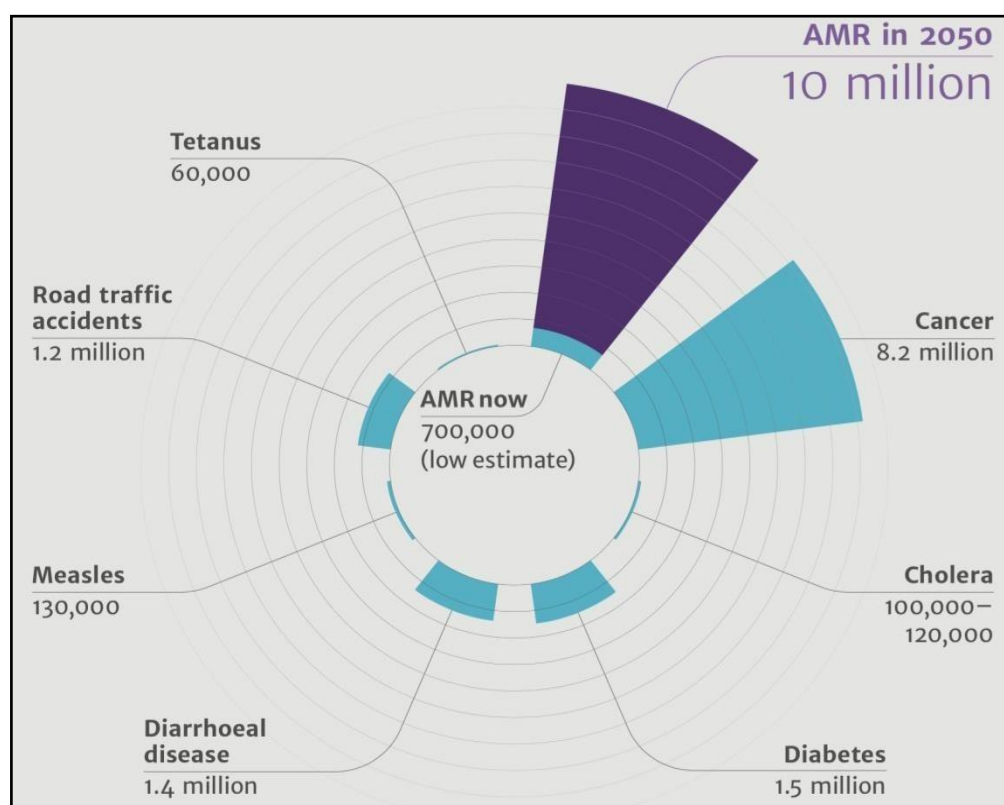


Figure 1. AMR in 2050, Band et al.

In the past, there were a lot of anomalies in coming up with a standard definition for antimicrobial-resistant phenotypes. Consensus terms were proposed by Magiorakos et al. in 2012, which are Multiple Drug Resistance (MDR), extreme Drug Resistance (XDR), and Pan-drug Resistance (PDR). All currently encountered carbapenem-producing *Enterobacteriaceae* come under MDR and a substantial set under XDR. Generally used terms to denote *Enterobacteriaceae* that show resistance to carbapenems are Carbapenemase-producing carbapenem-resistant *Enterobacteriaceae* (Cp-CRE), Carbapenem-resistant Organisms (CRO), carbapenem-producing Organisms (CPO). Pathogens that are acquired nosocomially pose a serious threat to human health. The most common nosocomially acquired pathogens belong to the ESKAPE organisms³. The acronym ESKAPE pathogens exhibit MDR with high virulence. ESKAPE pathogens are *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter spp*⁴.

The hunt for β -lactamase inhibitors began in the late 1960s⁵ when bacterial β -lactamases developed and threatened the use of penicillin. The first β -lactamase inhibitors were identified in 1976, and they were olivanic acids, which are natural products of the Gram-positive bacterium *Streptomyces clavuligerus*. Olivanic acids are broad-spectrum β -lactams with a "carbapenem backbone."

Two β -lactamase inhibitors⁶ were developed then,

- (i) Clavulanic acid from *Streptomyces clavuligerus*, which was the first clinically available inhibitor,
- (ii) Thienamycin from *Streptomyces cattleya*, which became the parent compound for all carbapenems.

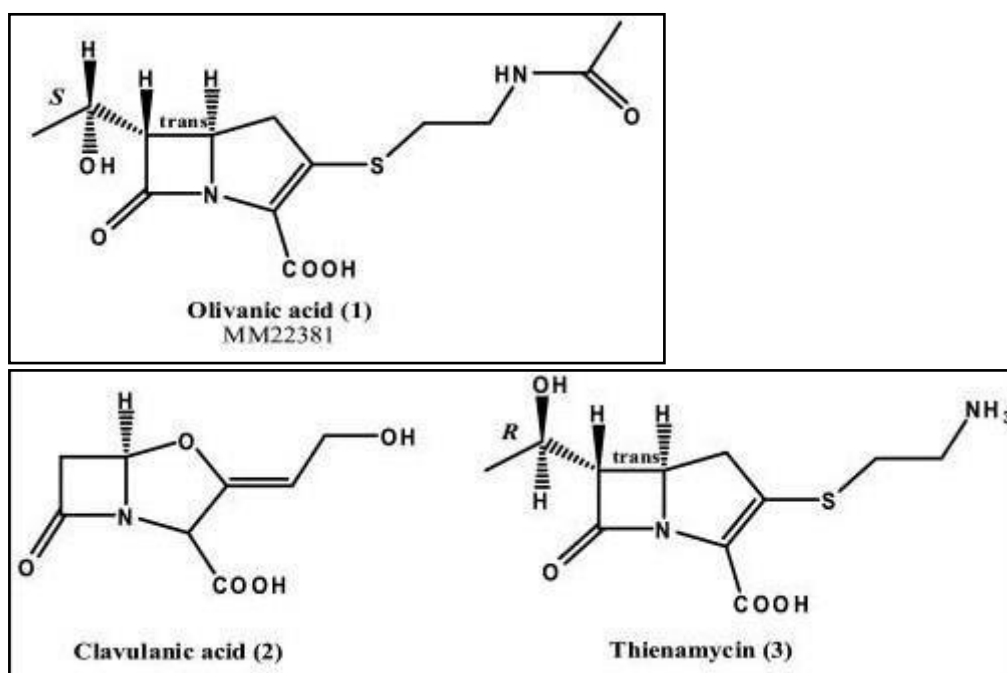


Figure 2. Thienamycin, olivanic acid, and clavulanic acid structure, Krisztina M. Papp-Wallace et al.

Although thienamycin was a natural product, after purification, the yields were low. It also exhibited instability in aqueous solutions, sensitivity to hydrolysis by mild bases, and high reactivity to nucleophiles, which all prompted a better alternative⁷. Therefore, its synthetic preparation gained importance, and this gave rise to imipenem.

Imipenem, an N-formimidoyl derivative⁸, and panipenem, a closely related carbapenem that was identified a little later, were found to be less sensitive to base hydrolysis and more stable than thienamycin. Imipenem became available in 1985, exhibiting great stability against β -lactamases and a high affinity for Penicillin-binding proteins (PBPs). Imipenem and panipenem later became susceptible to dihydropeptidase-1 (DHP-1) mediated deactivation in the human renal brush borders⁹. Therefore, inhibitors like cilastatin and betamipron were used for coadministration¹⁰. Carbapenems exhibiting more stability were later introduced, and some of the compounds that are currently in use include ertapenem, doripenem, meropenem, and biapenem and are also found to be stable towards hydrolysis by DHP-1⁶.

Some important considerations include,

- (i) imipenem and doripenem are more potent than meropenem against *Acinetobacter baumannii*¹¹,
- (ii) low Minimum inhibitory Concentration (MIC) of doripenem than imipenem and doripenem against *A.baumannii* and *P.aeruginosa*, and doripenem are least susceptible to hydrolysis by carbapenemases¹²,
- (iii) limited spectrum of ertapenem against *P.aeruginosa*¹¹,
- (iv) MDR *Mycobacterium tuberculosis* can be treated with meropenem in combination with clavulanic acid¹³.

Klebsiella pneumoniae, when found in their natural intestinal residence, are harmless. But if found anywhere else in the body, they could turn into superbugs which could render them undefeatable by antibiotics. Some of the diseases caused by the bacterium are meningitis, urinary tract infections (UTIs), endocarditis, etc. Since certain strains of the

Enterobacteriaceae family harbor genes that encode Extended Spectrum β -Lactamases (ESBLs), misuse of antibiotics could lead to bacterial resistance to carbapenems. The mechanisms by which bacterial strains mediate resistance are porin modifications or loss (ampC overexpression), efflux pumps, and hydrolyzing enzymes.

Carbapenems present a broad spectrum of antimicrobial activity. The presence of carbapenem (4:5 fused ring lactam) with a β -lactam ring, a double bond between C-1 and C-2, and sulfur at the C-1 structure give them more stability even against ESBLs. They inhibit the cell wall formation in bacteria by binding to the penicillin-binding proteins (PBP)¹⁴. For treating pan-drug-resistant bacteria, dual or synergistic therapy like carbapenem (doripenem) with colistin is considered¹⁵. This helps in fighting bacteria that are resistant to carbapenems but susceptible to colistin.

Carbapenems, like other β -lactams, do not pass through the cell wall of the bacteria¹⁶. They enter the Gram-negative bacteria through Outer Membrane Porin (OMP). Once they are inside the bacteria, they acylate the PBPs, which include transpeptidases, carboxypeptidases, and transglycosylase, and they help in the formation catalysis of peptidoglycan in the cell wall. Cell wall formation is a dynamic process where cell wall formation and autolysis occur at the same time, and when the PBPs are acylated, the autolysis continues, peptidoglycan weakens, and due to osmotic pressure, the cell bursts. The ability of carbapenems to bind to multiple different PBPs confers efficacy to this antibiotic (Hashizume et al.).

Certain enzymes called β -lactamases hydrolyze most β -lactam antibiotics, including carbapenems. According to the Ambler classification, β -lactamases can be classified as follows, class A (*K. pneumoniae* carbapenemase, KPC), class B Metallo- β -lactamases, MBL (New Delhi Metallo- β -lactamase, NDM) and class D (OXA-48-like carbapenemases)¹⁷. Class A carbapenemases contain serine at their active site that facilitates ring opening. Thus, they are called serine β -Lactamases (SBLs). KPC-2 and KPC-3 (*Klebsiella pneumoniae* Carbapenemases) are the most frequently reported of all variants. The β -lactam inhibitors of SBL are clavulanic acid, sulbactam, and tazobactam¹⁸. Class B β -lactamases contain two zinc cations at their active site and are classified into three groups: B1, B2, and B3. Thus, they are called Metallo β -Lactamases (MBLs)¹⁹. NDM family genes are an example of this class. Inhibitors are dipicolinic acid, EDTA, etc²⁰. Class D β -lactamases contain serine at their active site, and they do not have any inhibitors. An example of this class of β -lactamases in the OXA-48 family genes²¹.

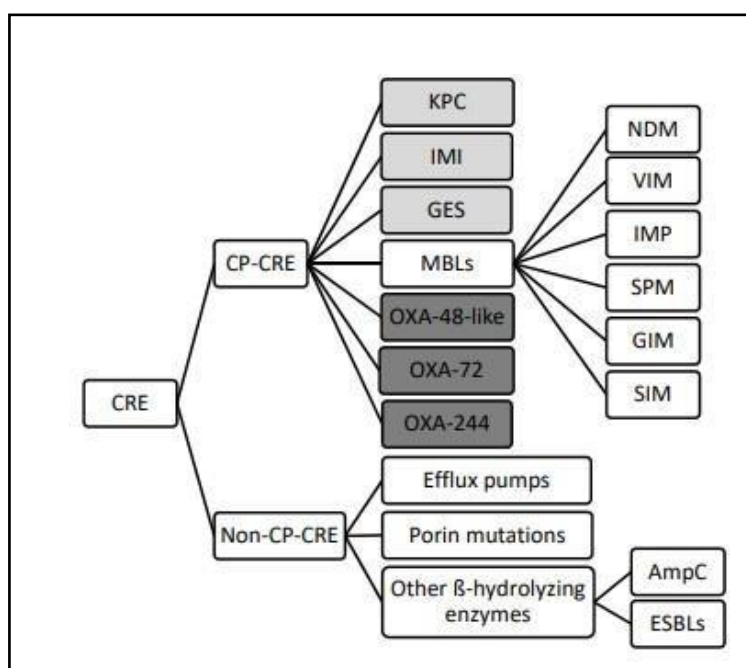


Figure 3. Classification of different mechanisms of drug resistance in CRE, Suay-García et al.

A study conducted by Xiaolin Yu et al. highlights that pathogens that harbor KPC-2 show resistance to all β -lactams and β -lactamase inhibitors like ceftazidime/avibactam, suggesting limited treatment options leading to a high rate of mortality. Apart from that, NDM has become a serious public threat because of the global dissemination of NDM-carrying pathogens and their presence on mobile genetic elements (MGEs) across different species of bacteria.

It has been shown that the alleles are usually carried on plasmids or MGEs, and the plasmid has a toxin-antitoxin system that prevents plasmid loss even in the absence of selective pressure caused by the antibiotic²². The most prevalent carbapenemase genes include *bla*_{KPC}, *bla*_{NDM}, *bla*_{OXA-48}, and *bla*_{VIM}. Clonal expansion is suggested to be responsible for the infection outbreaks; plasmid-mediated transfer is hard to detect and may be underestimated in studies²³. The most common enzymatic cause of carbapenems is because of the KPC enzymes. *bla*_{KPC-2} and *bla*_{KPC-3} are the most common and frequently reported genes that come under the *bla*_{KPC} class. In isolation, OXA-48-like carbapenemases bring about a weak hydrolysis of penicillin and carbapenems but not cephalosporins. This leads to difficulty in detection, and for this reason, they are called "the phantom menace," according to Poirel et al.

Because of the presence of carbapenem resistance genes in pathogenic strains, it has been declared a "global sentient event." Prolonged hospital stay and severe underlying illness are the main risk factors for the isolation of ESBL-producing organisms. This is associated with adverse clinical and economic outcomes like the increased length of stay and mortality rate, delay in the administration of effective therapy, and increased cost of care. According to Kwak et al., previous exposure to cephalosporins and carbapenems is the major cause of acquiring CRKP infections, while exposure to fluoroquinolones is protective. In a study by Flagas et al., 53 patients with CRKP isolation were compared with matched controls, and it was found that prior infection to antipseudomonal penicillins and fluoroquinolones were independent risk factors for CRKP infections²⁴.

On a global scale, the prevalence of carbapenemase genes was studied, and it was observed that the KPC-producing *Enterobacteriaceae* are found to be endemic to countries like the United States, Argentina, and Colombia, Italy, and Greece. MBL NDM-1 is the most prevalent in countries like India, Sri Lanka, and Pakistan²⁵. According to Walsh et al., India is the main reservoir of NDM-producing bacteria. OXA-48-like enzyme producers are seen mainly in the middle eastern countries and North Africa²⁵.

As suggested by the guidelines of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and the Clinical and Laboratory Standard Institute (CLSI), carbapenems continue to be administered for *Enterobacteriaceae* infections. Due to the unavailability of doripenem in most countries, it has been removed from the EUCAST 2019 guidelines²⁵. High virulence in *K.pneumoniae* helps them in invading the host's immune protective mechanisms and infect. High viscosity is noted along with high virulence in such strains but is not an essential factor. The carbapenem antibiotics that are used for treatment not only inhibit the formation of cell walls but also function by inhibiting β -lactamases and protein synthesis (DeJonge et al. & Karaikos et al.).

Studying the carbapenem-resistant genes gives an idea about the genes that confer resistance against different classes of antibiotics. This study aims at understanding the resistance patterns, identifying the plasmid type using Plasmid Database (PLSDB) and sequence type identification using Multiple Locus Sequence Typing (MLST), and studying the upstream and downstream genes of the gene under study.

MATERIALS AND METHODS

For studying the genomic context of carbapenem-resistant *Klebsiella pneumoniae* and analyzing the genes involved in resistance acquisition, 1025 complete, chromosome level assembled genomes were downloaded from the NCBI genome database. This required the installation of the Miniconda Package Manager to ensure that all bioinformatics tools used were up to date and functional. The main packages used for this study are ncbi-genome-download and Resistance Gene Identifier (RGI).

Downloading protein genome sequence from NCBI

A conda environment was created, and the ncbi-genome-download tool was used to download the protein genome sequences from the NCBI database (RefSeq).

Identification of AMR genes

The genomes that were downloaded were analyzed to detect the presence of antimicrobial resistance genes using RGI. It uses the Comprehensive Antibiotic Resistance Database (CARD) to identify the presence of AMR genes. RGI reads the ORFs using Prodigal if nucleotide fasta is submitted to predict the protein sequences²⁶. Protein fasta files were used with the default blast search strategy, which led to the generation of a csv file. Genomes and their respective genes that confer resistance to different drug classes were obtained. The json files obtained as an output of blast (DIAMOND BLAST) search were used to generate a heatmap. A shell script was created that included '-t' (input viz protein), '-p' (algorithm viz DIAMOND BLAST), and '-n' (number of CPU threads-10). The codes used for the generation of the files are,

```
$ wget https://card.mcmaster.ca/latest/data
$ tar -xvf data ./card.json
$ rgi load --card_json /path/to/card.json
$ rgi database --version
$ rgi main --input_sequence /path/to/nucleotide_input.fasta \
--output_file /path/to/output_file --input_type contig --clean
$ rgi heatmap --input /path/to/rgi_results_json_files_directory/ \
--output /path/to/output_file -cat drug_class -clus samples

$ conda activate rgi
$ conda install --channel conda-forge --channel bioconda --channel defaults rgi
$ rgi --help
```

A heatmap in the form of a csv file was generated that showed different drug classes and their respective resistant gene.

Co-occurrence study

Co-occurrence of resistance genes was conducted between carbapenem genes and genes of other drug classes like aminoglycosides, fosfomycin, colistin, etc., and also within the carbapenem-resistant gene classes. The input for this study was the csv file that was generated as an output from the RGI heatmap. The first sheet of M.S. Excel consisted of the drug classes, and a copy was created in the second sheet. The individual carbapenem resistance gene classes in the second sheet were selected and filled (black fill) in order to find out the genes that conferred resistance along with the selected genes. A script or a code was run through macros that carried out the computation. The code (fig. 4) was written in such a way that the computation can be carried out only in the second sheet. Initially, the input (csv) file gave the perfect, strict, and no hits as 2, 1, and 0, respectively. These numbers were used for studying co-resistance patterns among different drug

classes. The percentage of co-occurrence was calculated, and radar charts were created for better visualization of the data.

```
Sub Macro1()
'
' Macro1 Macro
' Range("A1").Select
With Application.FindFormat.Interior
    .PatternColorIndex = xlAutomatic
    .ThemeColor = xlThemeColorLight1
    .TintAndShade = 0
    .PatternTintAndShade = 0
End With
With Application.ReplaceFormat.Interior
    .PatternColorIndex = xlAutomatic
    .ThemeColor = xlThemeColorDark1
    .TintAndShade = 0
    .PatternTintAndShade = 0
End With
Cells.Replace What:="0", Replacement:="", LookAt:=xlPart, SearchOrder:= _
    xlByRows, MatchCase:=False, SearchFormat:=True, ReplaceFormat:=True

Range("A1").Select
With Selection.Interior
    .Pattern = xlSolid
    .PatternColorIndex = xlAutomatic
    .ThemeColor = xlThemeColorLight1
    .TintAndShade = 0
    .PatternTintAndShade = 0
End With

Dim objRange As Range, TargetCell As Range, i As Integer
Set TargetCell = Range("A1")
For Each objRange In ActiveSheet.UsedRange
    If objRange.Interior.Color = Range("A1").Interior.Color Then
        Set TargetCell = Union(TargetCell, objRange)
    End If
Next objRange
TargetCell.Select
```

```
Selection.EntireColumn.Hidden = True
Cells.SpecialCells(xlCellTypeVisible).Select
Application.CutCopyMode = False
Selection.Delete Shift:=xlToLeft
Cells.EntireColumn.Hidden = False
Cells.Select
With Selection.Interior
    .Pattern = xlNone
    .TintAndShade = 0
    .PatternTintAndShade = 0
End With

Range("A1").Select
Range(Selection, Selection.End(xlDown)).Select
Selection.Name = "Esum"
Range("B2").Select
Range(Selection, Selection.End(xlToRight)).Select
i = Selection.Cells.Count
Selection.End(xlToRight).Select
ActiveCell.Offset(0, 1).Range("A1").Select
ActiveCell.FormulaR1C1 = "=SUM(RC[-" & i & "]:RC[-1])"
Selection.AutoFill Destination:=ActiveCell.Range("Esum")
ActiveSheet.Previous.Select
Range("A1").Select
Range(Selection, Selection.End(xlDown)).Select
Selection.Name = "Esum"
Range("B2").Select
Range(Selection, Selection.End(xlToRight)).Select
i = Selection.Cells.Count
Selection.End(xlToRight).Select
ActiveCell.Offset(0, 1).Range("A1").Select
ActiveCell.FormulaR1C1 = "=SUM(RC[-" & i & "]:RC[-1])"
Selection.AutoFill Destination:=ActiveCell.Range("Esum")

Range(Selection, Selection.End(xlDown)).Select
Selection.Copy
```

```

Sheets.Add After:=ActiveSheet
ActiveCell.Offset(0, 1).Range("A1").Select
Selection.PasteSpecial Paste:=xlPasteAllUsingSourceTheme, Operation:=xlNone _
, SkipBlanks:=False, Transpose:=False
Selection.PasteSpecial Paste:=xlPasteValues, Operation:=xlNone, SkipBlanks _
:=False, Transpose:=False
ActiveSheet.Next.Select
Range(Selection, Selection.End(xlDown)).Select
Application.CutCopyMode = False
Selection.Copy
ActiveSheet.Previous.Select
ActiveCell.Offset(0, 1).Range("A1").Select
Selection.PasteSpecial Paste:=xlPasteAllUsingSourceTheme, Operation:=xlNone _
, SkipBlanks:=False, Transpose:=False
Selection.PasteSpecial Paste:=xlPasteValues, Operation:=xlNone, SkipBlanks _
:=False, Transpose:=False
ActiveSheet.Previous.Select
ActiveCell.Offset(0, -7).Range("A1").Select
Range(Selection, Selection.End(xlDown)).Select
Application.CutCopyMode = False
Selection.Copy
ActiveSheet.Next.Select
ActiveCell.Offset(0, -2).Range("A1").Select
Selection.PasteSpecial Paste:=xlPasteAllUsingSourceTheme, Operation:=xlNone _
, SkipBlanks:=False, Transpose:=False
Selection.PasteSpecial Paste:=xlPasteValues, Operation:=xlNone, SkipBlanks _
:=False, Transpose:=False
Range("A1").Select

Range(Selection, Selection.End(xlToRight)).Select
Application.CutCopyMode = False
Selection.Insert Shift:=xlDown
Range("A1").Select
ActiveCell.FormulaR1C1 = "gene"
Range("B1").Select
ActiveCell.FormulaR1C1 = "all"

```

```

Range("C1").Select
ActiveCell.FormulaR1C1 = "selected"
Range("D1").Select
' Keyboard Shortcut: Ctrl+h
End Sub

```

Figure 4. Code for running Macros in M.S. Excel for the analysis of co-occurrence of resistance genes.

Genomic context study

For analyzing the genomic context of selected genes, a software called GeneSpy was used. It is a powerful visualization tool to study functional links between genes²⁷. A database was built by providing the NCBI ftp links of the genomes. Using the ftp links, a MySQL-based database with the gfm files were built. The gene names were given as input, and a search was performed to build the genetic context. The neighboring genes and their assembly in either chromosome or plasmid are the output that was exported as a pdf file. The conserved gene context was manually analyzed from the results.

Phylogenetic analysis

Out of the 14 gene classes that were obtained as a result of the RGI-CARD analysis, a phylogenetic study was carried out for the OXA-48-like family of genes (8 genes). To conduct this analysis, software called Mega (Molecular Evolutionary Genetic Analysis) v.11²⁸ was installed. The protein fasta files of the OXA-48-like genes were downloaded as a batch from NCBI Batch Entrez. The database was set to protein, and the accession numbers were provided. The fasta files were retrieved and were manually copied/pasted in the Mega

software for the alignment of the sequences. Alignment was carried out with the help of ClustalW. A Maximum Likelihood tree was constructed with 1000 bootstraps. For better visualization of the tree, a Newick format of the tree was created and was submitted to the iTOL²⁹ (Interactive Tree of Life) online web tool. A circular tree was generated.

PLSDB

Plasmid Database (PLSDB) is an online database for plasmids gathered from NCBI and INSDC³⁰. Since resistance in microbes is spread through plasmids and other MGEs, this analysis is of importance to characterize the infection-causing pathogens. The metadata includes incompatibility type, date, and geographical location. The Plasmid Finder pipeline determines the incompatibility type. Plasmid types of CMY- 2, CTX-M-27, IMP-4, KPC-3, OXA-1, NDM-1, SHV-11, and VIM-1 producing genes were determined.

MLST

Sequence Type identification of the RefSeq genomes was done using the tseemann/mlst downloaded from the git repository. To get the results of MLST as a csv file, a bash script was run. Data was generated by the RGI and the pubMLST pipeline. The data from CARD live included 10,030 genomes, out of which 1025 were that of *K.pneumoniae*. A python script (fig. 5) was run in the directory having the json files and was used to parse the data from the downloaded json files. Analysis of data was carried out using M.S. Excel. A bar chart indicating the Sequence Type (ST) and the number of genomes was made.

```
import os

import sys

import json

import glob

import csv

file_directory=os.getcwd()

out=os.path.join(file_directory,"results")

os.mkdir(out)

final_list = []

filename=""

a=0

for filename in glob.glob('*json'):

    temp_list=[]

    print(filename)

    json_file = open(filename)
```

```

json_data = json.load(json_file)

if(len(json_data['mlst']) > 0):

    seq=json_data['mlst'][0]["sequence_type"]

    sch=json_data['mlst'][0]["scheme"]

temp_list.append(seq)

temp_list.append(sch)

gen=""

print(seq)

b=1

for gene in json_data['rgi_main']:

    g = gene["Best_Hit_ARO"]

    gen = gen + g+', '

```

```

temp_list.append(g)

b+=1

print(gen)

a+=1

final_list.append(temp_list)

#after taking input data

os.chdir(out)

```

```
# writing to csv file

with open('parsed_data.csv', 'w') as csvfile:

    # creating a csv writer object

    csvwriter = csv.writer(csvfile)

    # writing the data rows

    csvwriter.writerows(final_list)
```

Figure 5. Python code for MLST.

RESULTS

Carbapenem-resistant genes that were highly expressed were analyzed. They are CMY-2, CTX-M-27, IMP-4, NDM-1, OXA-1, KPC-3, VIM-1, and SHV-11.

Carbapenem-resistance gene detection

Using RGI-CARD software, the genes conferring resistance against the carbapenem class of antibiotics and against other antibiotics were identified. A total of 82 carbapenem-resistance genes were found among 1022 genomes. Among them, 43 genes coding for ESBLs, CMY-2, CTX-M-27, PER-1, PER-7, and 39 coded for SHV. Two genes that code for efflux pumps, i.e., KpnG and KpnH, two genes that code for an outer membrane protein porin, OmpK37, and OmpA, one suspected transcriptional activator of genes involved in multiple antibiotic resistance, and marA, were found. An *lptD* gene that is involved in lipopolysaccharide (LPS) assembly at the outer membrane surface and an SFO-1 gene were identified. Thirty-two genes that encode carbapenemases like NDM, KPC, OXA, IMP, and VIM were detected. The individual gene classes are listed below (Table 1).

Table 1. Carbapenemases of different classes.

KPC	OXA	NDM	IMP	VIM
KPC-1	OXA-1	NDM-1	IMP-1	VIM-1
KPC-3	OXA-2	NDM-3	IMP-4	VIM-27
KPC-4	OXA-9	NDM-4	IMP-38	
KPC-12	OXA-10	NDM-5	IMP-68	
KPC-14	OXA-48	NDM-6		
KPC-31	OXA-181	NDM-7		
KPC-33	OXA-204	NDM-19		
KPC-40	OXA-232			
KPC-74	OXA-244			

The distribution of the carbapenem resistance genes among 1025 *K.pneumoniae* genomes was studied. Among the genomes, NDM expression was found to be the highest with 36%, followed by OXA-48 like family genes with 21% expression (fig.6).

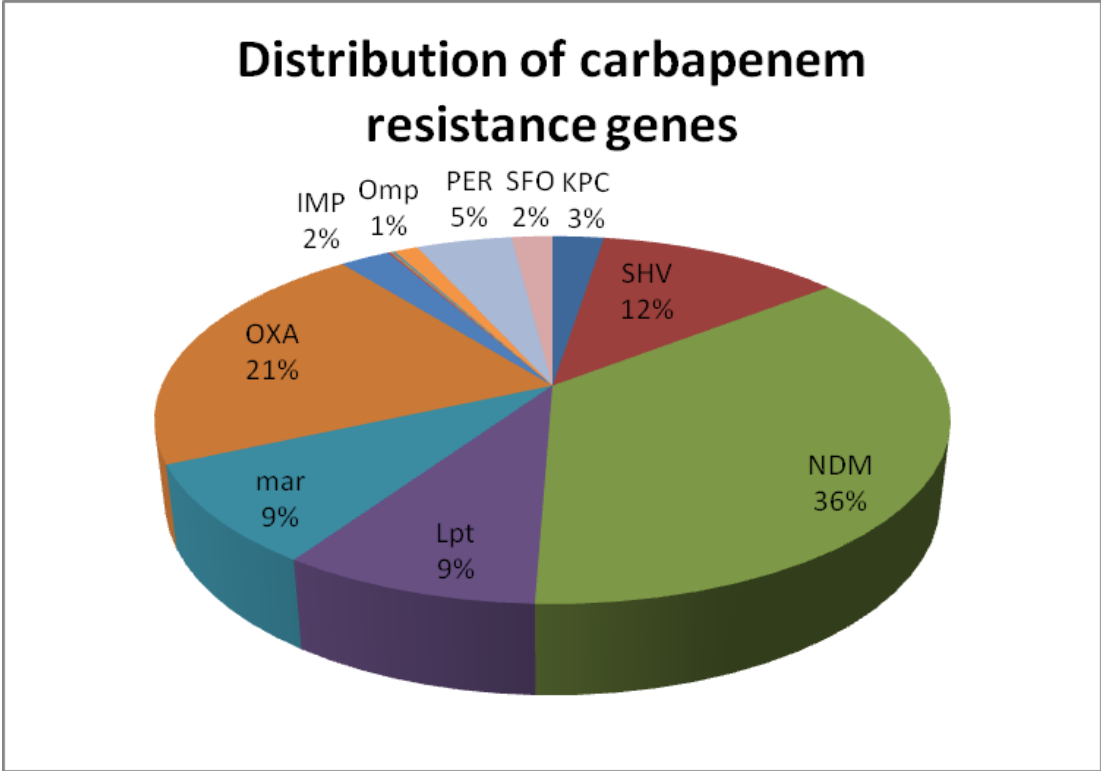


Figure 6. Distribution of carbapenem resistance genes.

Co-occurrence analysis

Two co-occurrence studies were conducted, one with the selected set of carbapenem resistance genes against the resistance-conferring genes of all other drug classes and the other between the selected set of carbapenem resistance genes against other genes belonging to the same class. Genes occurring $\geq 60\%$ are listed in the following sections.

Between drug classes

Co-occurrence of the following carbapenem resistance gene classes with respect to the other drug classes was observed.

KPC

Resistance genes of aminoglycosides, carbapenems, diaminopyrimidine, disinfecting agents and intercalating dyes, fluoroquinolones, fosfomycin, penem, monobactam, phenicol, rifamycin, tetracycline, and triclosan were found to be co-occurring with KPC-producing genomes. A total of 23 genes occurring $\geq 60\%$ were recorded (Table 2). Eighty-one percent of genomes producing *bla*_{KPC} were also found to harbor *fosA3*, a fosfomycin resistant gene, and 80.165% of genomes also harbored *bla*_{SHV-134}.

Table 2. Co-occurrence of resistance genes with KPC class genes.

Drug class	Gene/Protein	Percentage
Aminoglycosides	AAC(3')-IIc	66.66667
Aminoglycosides	AAC(3')-IV	61.01695
Aminoglycosides	APH(4')-Ia	61.01695

Aminoglycosides	LAP-2	67.12329
Aminoglycosides	<i>aadA3</i>	70.16575
Aminoglycosides	<i>rmtB</i>	77.41935
Carbapenem	SHV-134	80.165
Carbapenem	SHV-30	66.66667
Diaminopyrimidine	<i>dfrA25</i>	66.66667
Diaminopyrimidine	<i>oqxB</i>	57

Disinfecting agents and inter-calating dyes		<i>qacL</i>	65.15152
Fluoroquinolones		LAP-2	67.12329
Fluoroquinolones	<i>Salmonella</i> serovars <i>gyrB</i> conferring resistance to fluoroquinolone		79.7619
Fosfomycin		<i>fosA3</i>	81.03448
Monobactam		TEM-214	66.66667
Penem		SHV-134	80.16529
Penem		TEM-214	66.66667
Phenicol	catII from <i>Escherichia coli</i> K-12		75.92593
Phenicol		<i>cmlA1</i>	61.81818
Rifamycin	<i>Escherichia coli</i> <i>rpoB</i> mutants conferring resistance to rifampicin		66.66667
Rifamycin		LAP-2	67.12329
Tetracyclin		LAP-2	67.12329
Triclosan	<i>Salmonella enterica</i> <i>gyrA</i> with mutation conferring resistance to triclosan		72.72727

NDM

Co-occurrence of 3 genes conferring resistance to aminoglycosides, diaminopyrimidine, and glycopeptides with respect to the NDM class of genes was observed (Table 3). Ninety-two percent of the genomes that produced blaNDM were also found to possess *aph(3')-VI*, which confer resistance to aminoglycosides.

Table 3. Co-occurrence of resistance genes with NDM class genes.

Drug class	Genes/Protein	Percentage
aminoglycoside antibiotic	APH(3')-VI	92.85714286
diaminopyrimidine antibiotic	<i>dfrA5</i>	77.77777778

glycopeptide antibiotic	determinant of resistance	bleomycin	98.11320755
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OXA

Twenty-eight genes were found to be co-occurring with the OXA-48-like family genes. blaOXA harboring genomes were also found to possess genes that conferred resistance to aminoglycosides, carbapenems, cephalosporins, cephamycins, diaminopyrimidine, fluoroquinolones, lincosamide, macrolides, penam, nucleoside, rifamycin, and tetracyclines (Table 4). The highest co-occurrence pattern noted was with *aph(3'')-VI*, which confers resistance to aminoglycosides with a co-occurrence rate of 96.42%.

Table 4. Co-occurrence of resistance genes with OXA class genes.

Drug class	Genes/Proteins	Percentage
aminoglycoside antibiotic	AAC(3')-IIe	90.09901
aminoglycoside antibiotic	AAC(6')-Ib-cr5	80.69498
aminoglycoside antibiotic	APH(3')-VI	96.42857
aminoglycoside antibiotic	APH(3')-VIb	83.33333
aminoglycoside antibiotic	<i>aadA</i>	62.26415
aminoglycoside antibiotic	<i>armA</i>	65.33333
aminoglycoside antibiotic	<i>rmtF</i>	84.21053
carbapenem	IMP-4	66.66667
carbapenem	NDM-1	61.84211
carbapenem	SHV-110	83.33333
carbapenem	SHV-30	66.66667
cephalosporin	CTX-M-194	91.66667
cephalosporin	CTX-M-2	100
cephalosporin	CTX-M-55	66.66667
cephamycin	CMY-4	92.30769
diaminopyrimidine antibiotic	<i>dfrA1</i>	67.01031
diaminopyrimidine antibiotic	<i>dfrA14</i>	61.39535
diaminopyrimidine antibiotic	<i>dfrA23</i>	100
diaminopyrimidine antibiotic	<i>dfrA35</i>	100
fluoroquinolone antibiotic	AAC(6')-Ib-cr5	80.69498
fluoroquinolone antibiotic	QnrB1	80.4878
fluoroquinolone antibiotic	QnrB9	66.66667

lincosamide antibiotic	ErmB	75.67568
macrolide antibiotic	EreA2	94.44444
macrolide antibiotic	ErmB	75.67568
penam	CTX-M-15	77.69784
nucleoside antibiotic	SAT-1	96.875
rifamycin antibiotic	<i>arr-2</i>	92.20779
tetracycline antibiotic	<i>tet(B)</i>	66.66667
tetracycline antibiotic	<i>tetR</i>	71.42857

Individual studies were conducted for the CMY and IMP classes of genes, but significant co-resistance patterns were not observed.

Within carbapenem drug class

Co-occurrence analysis between the resistance genes of the carbapenem drug class was carried out, and the results are listed below.

Genomes that harbored KPC were also found to possess SHV-134 (80.1%), SHV-7 (75%), SHV-30 (66.6%), SHV-42 (66.6%), and CMY-2 (60%) (Fig. 7).

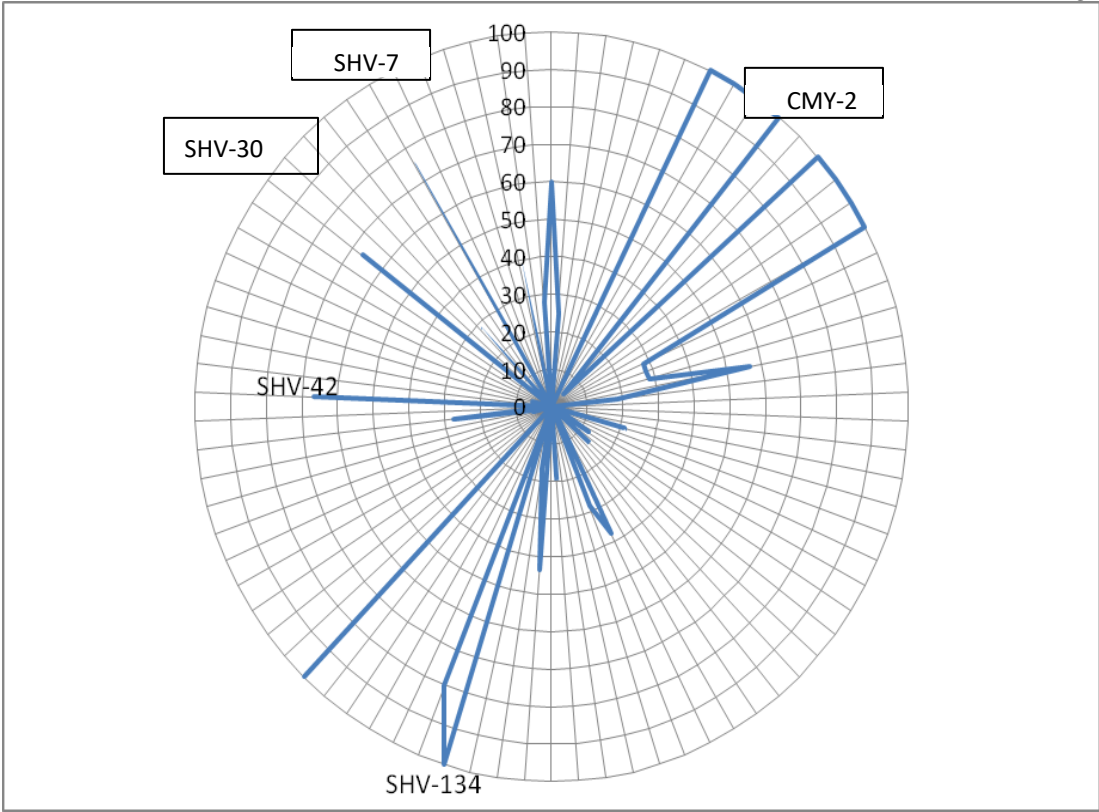


Figure 7. Co-occurrence of carbapenem-resistance genes with KPC gene class.

All OXA harboring genomes possessed NDM-3, NDM-4, and NDM-19. Additionally, 83.3% of them had SHV-110, 66.6% had SHV-30, IMP-4, and 61.8% had NDM-1 (Fig. 8).

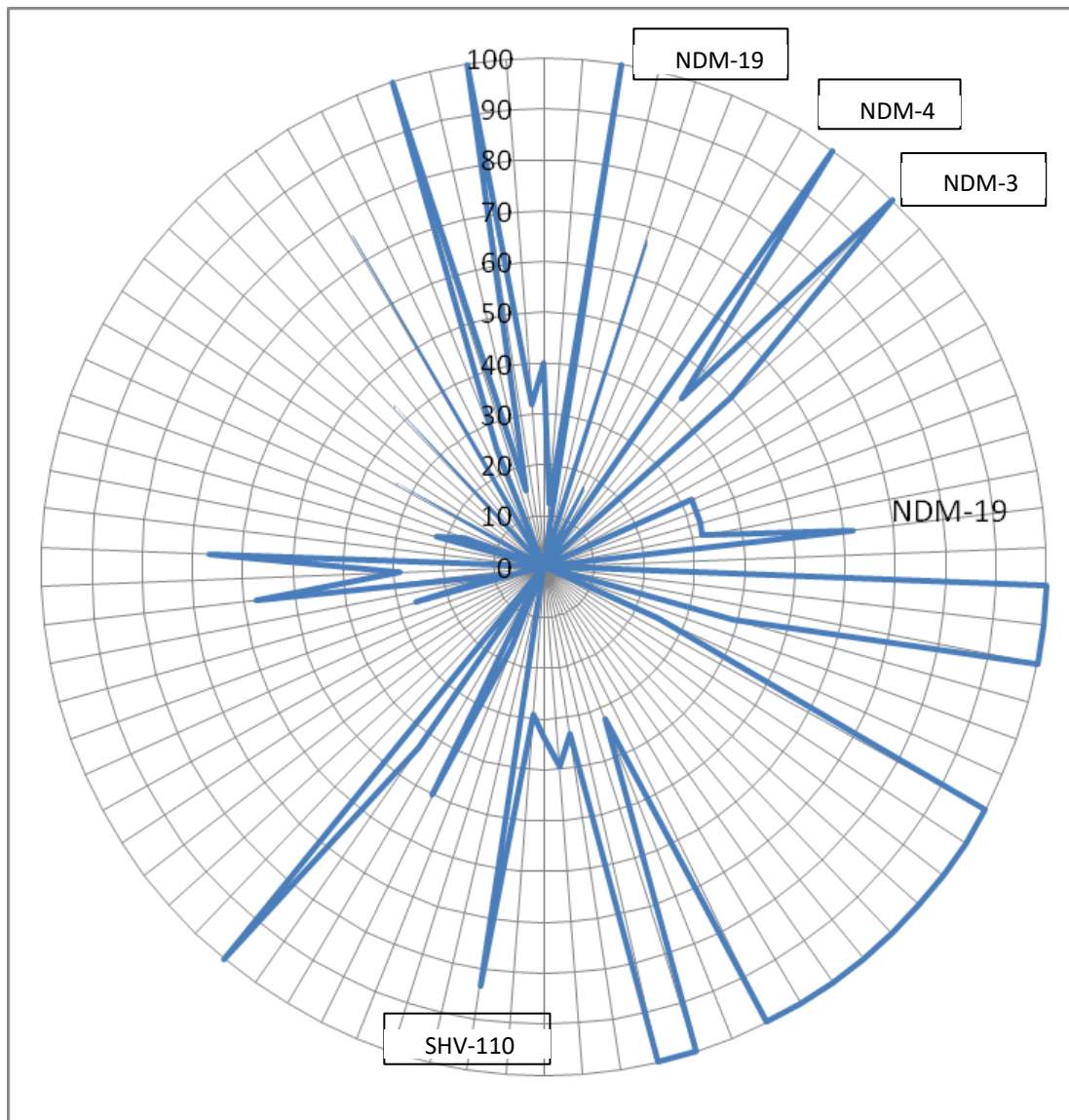


Figure 8. Co-occurrence of carbapenem-resistance genes with OXA gene class.

Individual studies were also conducted for the VIM, CMY, NDM, and IMP against the other carbapenem-resistance genes, but significant co-resistance patterns were not observed.

Genomic context analysis

A genomic context study was performed using software called GeneSpy to understand the inherent genetic structure. Five upstream genes and five genes downstream of the gene under study were visualized and analyzed. The conserved genetic context of each gene under study is represented.

CMY-2

The presence of Class C beta-lactamase CMY-2 was seen in 11 genomes and four genomes (GCF_019317065.1, GCF_019317185.1, GCF_019317265.1, GCF_019334705.1) had CMY-2 family Class C beta-lactamase that harbored blaCMY-2. The upstream genes were IS1380-like element ISEcp1 family transposase, a hypothetical protein, traV (type 4 transfer system lipoprotein), traB (conjugal transfer), another hypothetical protein, and traL (type 4 conjugative transfer system). The downstream genes were lipocalin family proteins, sugE (quaternary ammonia compound efflux SMR transporter protein), Ig-like domain-containing protein, a Dsbc family protein, and traC (type 4 secretion system protein) (Fig. 9).

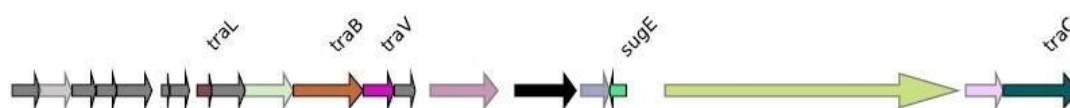


Figure 9. Genomic context of CMY-2-*Klebsiella pneumoniae* IR1251 WP_000976514.1 (GCF_017310565.1).

CTX-M-27

CTX-M-27 genes were observed in 11 genomes. The upstream genes were IS1380-like element *ISEcp1* family transposase, malate/lactate/ureidoglycolate dehydrogenase gene, an *fdhf* (formate dehydrogenase subunit α), an M20 family metalloproteinase, and a *yitZ* MFS transporter. Also, a multidrug efflux transporter *mdtM* gene was observed. In the genome GCF_019334625.1, tetracycline antibiotic transporter *tetR* and *tetA* (fig. 10) were observed. The downstream genes were ISKra4 family transposase, a recombinase family protein, a *qnrS1* (Quinolone-resistant pentapeptide repeat), IS3, and a serine hydrolase (Fig. 11).



Figure 10. Genomic context showing tetracycline transporter-*Klebsiella pneumoniae* JP20 WP_032277257.1 (GCF_019334625.1).

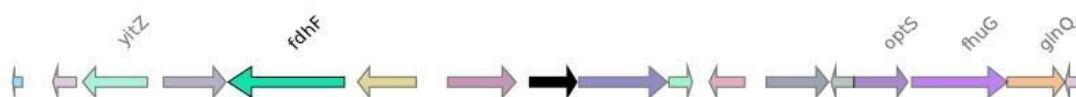


Figure 11. Genomic context of CTX-M-27-*Klebsiella pneumoniae* subsp. pneumoniae WP_032277257.1 (GCF_003037615.2).

IMP-4

Nine genomes were found to harbor the IMP-4 gene. The upstream genes were recombinase family protein, a TN3 family transposase, an IS110/IS4321 family transposase, and a DUF6531 domain-containing protein. The downstream genes were *qacG2* (ammonia compound efflux SMR transporter), aminoglycoside N-acetyltransferase *aac(6')-Ib4*, *catB3* chloramphenicol O-acetyl transferase, NAD⁺-rifamycin ADP-ribosyltransferase Arr-3, and a *qacE* (ammonia SMR transporter). Macrolide-resistant MFS transporter *mrxA* was found extremely downstream (fig. 12).

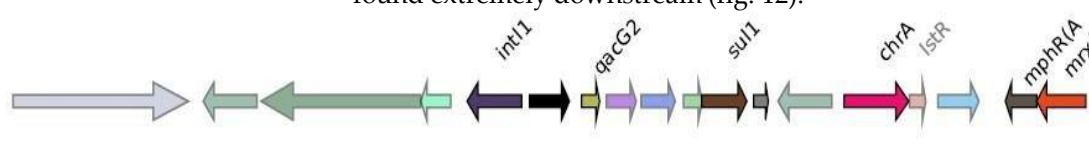


Figure 12. Genomic context of IMP-4-*Klebsiella pneumoniae* INF157 WP_015060105.1 (GCF_002753075.1).

KPC-3

KPC-3 genes were found in 57 genomes. The upstream genes were *istB*, *istA*, *tnpA* (transposase), *tnpR* (resolvase), and a hypothetical protein. The downstream genes were IS1182/ISKpn6 family transposase, a *ycfP* (alpha/beta hydrolase), a *nagZ* (β -N-acetyl hexosaminidase), a *thiK* (thiamine kinase), and a *lpoB* (PBP-activator) (fig. 13).

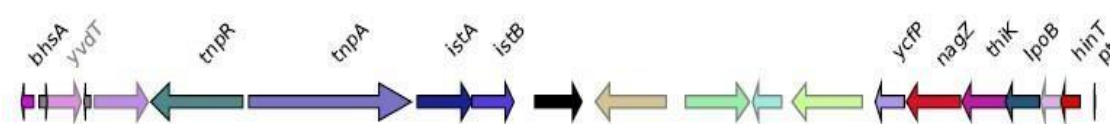


Figure 13. Genomic context of KPC-3-*Klebsiella pneumoniae* KPN1H45 WP_004152396.1 (GCF_002918585.2).

NDM-1

Seventy-nine genomes were found to possess the NDM-1 producing genes. The upstream genes were IS630/ISEc33 family transposase, IS30/ISAbA125, APH(3')-VI family aminoglycoside O-phosphotransferase, IS3, *repM*, and ABC-F type protection protein MSr(E). The downstream genes were Ble-MBL, PRA1, protein disulfide reductase, DsbD N-terminal domain-containing protein, divalent cation tolerance protein CutA, and co-chaperones *groES* and *groEL* (fig. 14).

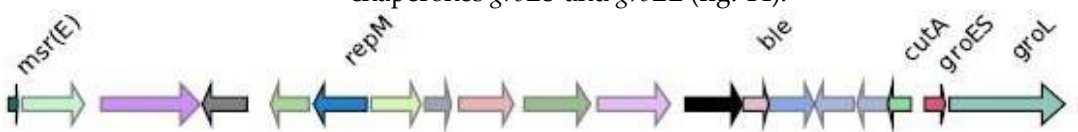


Figure 14. Genomic context of NDM-1-*Klebsiella pneumoniae* AR_0068 WP_004201164.1 (GCF_002055855.1).

OXA-1

OXA-1 was found in 297 genomes. The upstream genes were fluoroquinolone acetylating gene, aminoglycoside 6'-N-acetyltransferase AAC(6'-Ib-Cr5), IS6/IS26 family transposase, Tn3/TnAs1 family transposase, and a *tetR* tetracycline-resistant transcription repressor and a *tetA* efflux MFS transporter. The downstream genes were CatB-related O-acetyltransferase, IS6/IS26 family transposase, a recombinase family protein, *intI1* class 1 integron integrase, and a *dfrA14* trimethoprim resistant dihydrofolate reductase (fig. 15). In *Klebsiella pneumoniae* WP_201507552.1

(GCF_904863295.1), *blaOXA-1* was observed, and upstream to it, *blaCTX* was also observed (fig. 16).

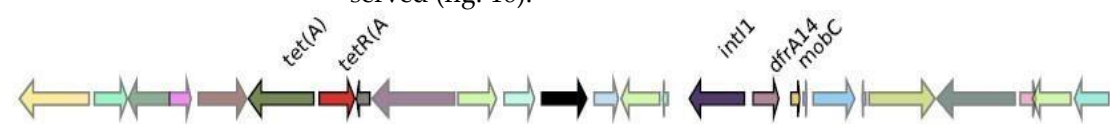


Figure 15. Genomic context of OXA-1-*Klebsiella pneumoniae* WP_001334766.1 (GCF_904863225.1).

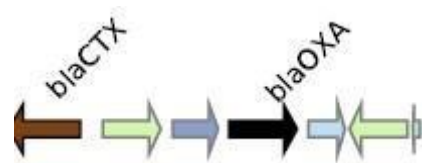


Figure 16. SHV-11.

Five hundred and sixty-two genomes containing SHV-11 were noted. The upstream genes were AAA family ATPase, a *yitz*, a *bgaA*- β galactosidase, Lac1 family DNA-binding transcriptional regulator, and a GNAT family N-acetyl transferase. The downstream genes were DeoR/GlpR family DNA-binding transcriptional regulator, NADP-dependent oxidoreductase, aldolase, and an HPr family phospho carrier (fig. 17).

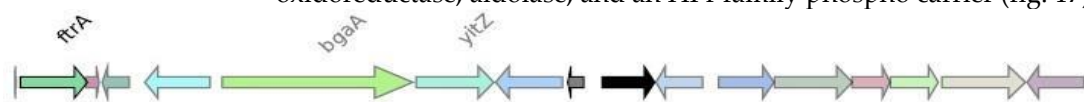


Figure 17. Genomic context of SHV-11-*Klebsiella pneumoniae* WP_004176269.1 (GCF_002211665.1).

VIM-1

VIM-1 was found to be present in 14 genomes. The upstream genes were *intl1* class 1 integron integrase, IS6/IS26 family transposase, DeoR/GlpR family DNA-binding transcriptional regulator, class A β -lactamase SHV-200 (plasmid), and a hypothetical protein. *urtB* urea ABC transporter permease subunit and *urtC* genes were also observed. The downstream genes were aminoglycoside 6'-N-acetyltransferase AAC(6')-Ib4, aminoglycoside O-phosphotransferase APH(3'), ANT(3') family aminoglycoside nucleotidyltransferase, type B-2 chloramphenicol O-acetyltransferase CatB2, and *qacE*. *mer* family of genes and sulfonamide resistant dihydropteroate synthase *sul1* was also observed (fig. 18)

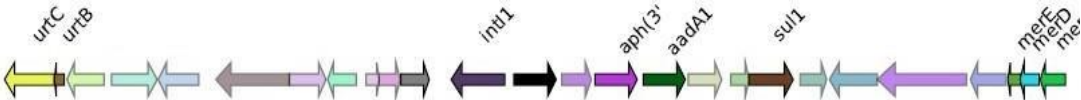


Figure 18. Genomic context of VIM-1-*Klebsiella pneumoniae* AR_0135 WP_013263789.1 (GCF_003571545.1).

Phylogenetic analysis

ClustalW alignment of the OXA-class genes of multiple species, which included *Enterobacteriaceae* and Proteobacteria, was carried out using the Mega software, and a maximum likelihood tree was constructed. The bootstrap was set to 1000 and was run on two threads for the construction of the tree. The protein sequences of OXA-1, OXA-2, OXA-9, OXA-48, OXA-181, OXA-204, OXA-232, and OXA-244 were downloaded as a batch from the NCBI Batch Entrez. The phylogenetic tree was constructed (fig. 19).



Figure 19. Phylogenetic tree of the OXA gene class.

PLSDB

IncFIB type plasmid was observed to be predominant in OXA-1, and IncFII was found in GCF_008065415.1 (NCBI accession ID: NZ_CP041928.1). Among CMY-2, IncC was found to be predominant, with GCF_016903775.1 (NCBI accession ID: NZ_CP069963.1) showing IncI2. IncFIB was found in CTX-M-27 and GCF_019334625.1 (NCBI accession ID: NZ_CP079764.1), showing IncFA type. IncC, IncN, and IncFIBtyoe plasmids were found

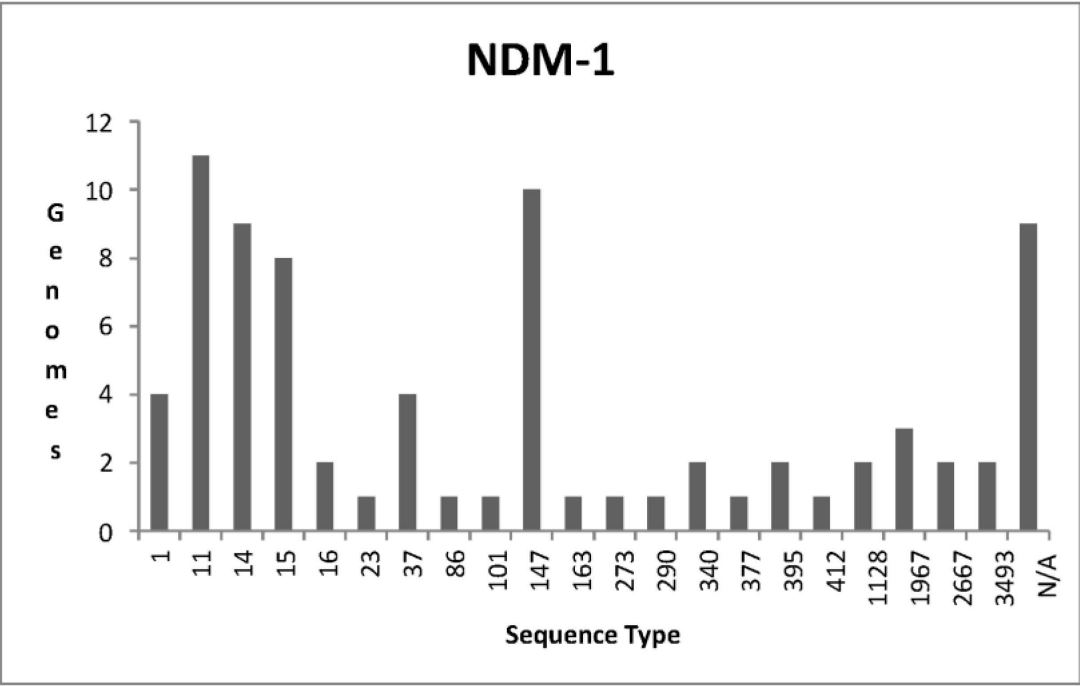
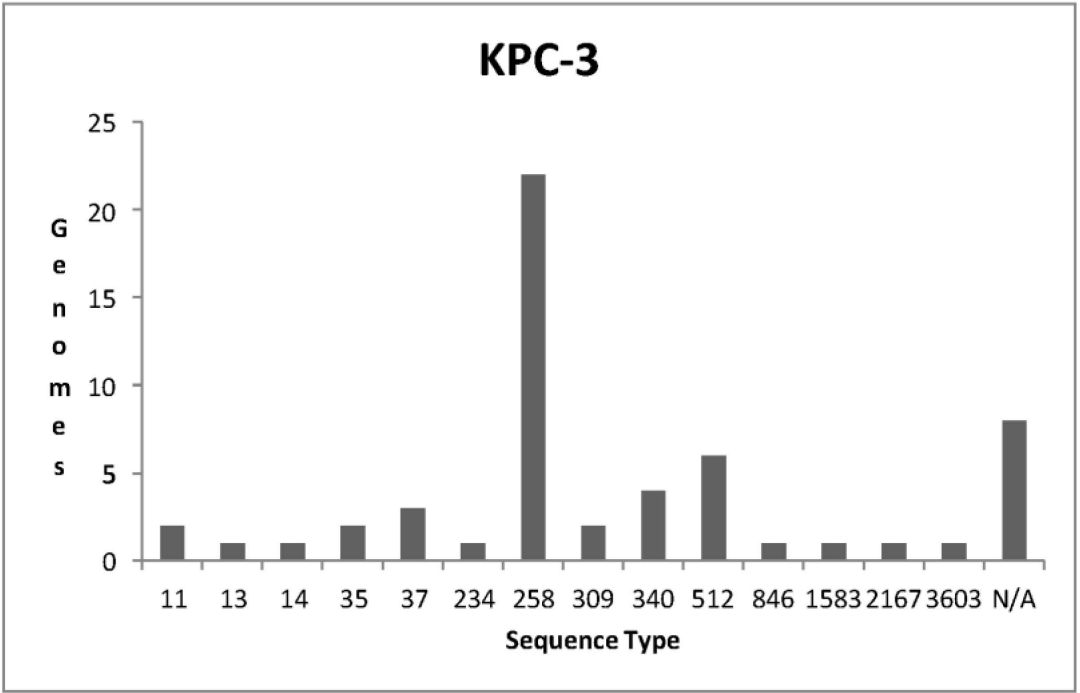
among the IMP-4 genes. IncI, IncFIB, and IncFII were observed in KPC-3. IncHI1B was predominantly found in NDM-1, and GCF_000764615.1 (NCBI accession ID: NZ_CP008933.1) had IncFIB type. IncFIA, IncR, and IncHI1B were found among SHV-11 genes. In VIM-1, IncC was predominant, and GCF_013694425.1 (NCBI accession ID: NZ_CP059314.1) showed type IncR. IncFIB was found to be the most predominant type, occurring in five genes out of the eight understudies, i.e., OXA-1, CTX-M-21, IMP-4, KPC-3, and NDM-1 (Table 5).

Table 5. Plasmid type distribution and AMR genes carried by them.

Plasmid type	Gene
IncFIA	<i>blaSHV-11</i>
IncFIB	<i>blaOXA-1, blaCTX-M-27, blaIMP-4, blaKPC-3, blaNDM-1</i>
IncFII	<i>blaOXA-1, blaKPC-3</i>
IncHIB	<i>blaNDM-1, blaSHV-11</i>
IncI2	<i>blaKPC-3</i>
IncFIA	<i>blaSHV-11, blaCTX-M-27</i>
IncC	<i>blaCMY-2, blaIMP-4, blaVIM-1</i>
IncN	<i>blaIMP-4</i>
IncR	<i>blaVIM-1, blaSHV-11</i>

MLST

Sequence Types of the carbapenem-resistance genes were identified. Predominantly occurring S.T.s among the strains were observed and reported. Four ST-231 and three ST-11 strains were found to harbor the *blaCMY-2* gene. Four genomes containing *blaCTX-M-27* belonged to ST-11 and three to ST-48. Four *blaIMP-4* harboring strains were ST-340. Twenty-two genomes were found to be ST-258 that had KPC-3 genes. Two genomes belonged to ST-101 that had *blaVIM-1*. Eleven strains with *blaNDM-1* belonged to ST-11, ten to ST-147, and nine to ST-14. Thirty-five genomes with *blaOXA-1* were ST-11, and thirty-four were ST-14. One hundred and fifty-six genomes were ST-11 and harbored the *blaSHV-11* gene (fig. 20).



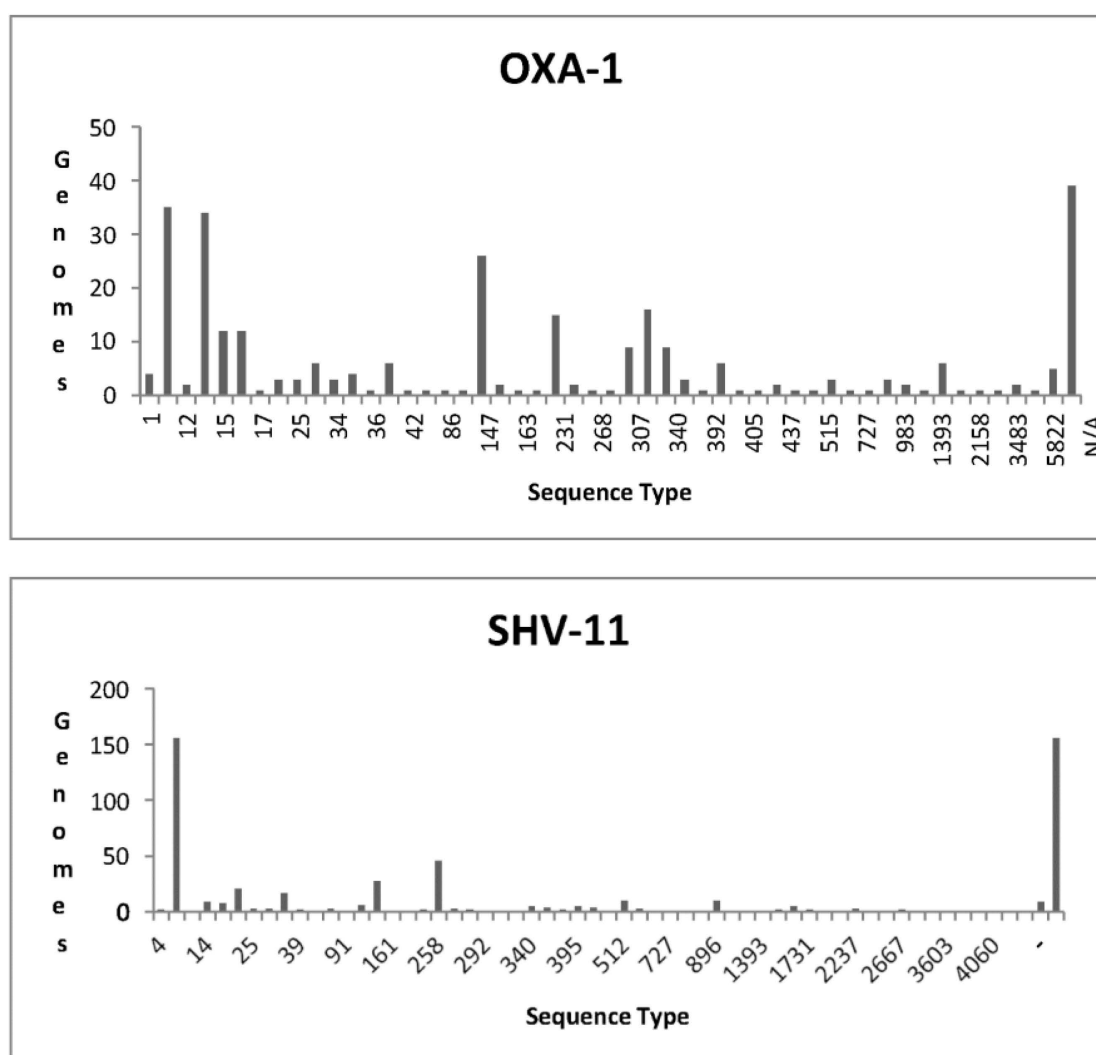


Fig. 20: MLST analysis-bar graphs plotted S.T. Vs. Genomes

DISCUSSIONS

For studying the co-resistance patterns of the genes, those genes (conferring resistance to other drug classes) that were expressed $\geq 60\%$ along with the carbapenem-resistant gene were analyzed. Such probability of occurrence suggests that a particular strain could be resistant to more than one class of antibiotics. Eighty-one percent of the genomes expressed *fosA3* (fosfomycin resistance gene) in KPC-producing strains. This was the highest recorded co-occurrence with the KPC gene class. A similar pattern was observed in a study that was conducted in a Chinese hospital with a 60.8% co-resistance rate³¹. Ninety-eight percent of the genomes that produced NDM-1 carbapenemase also showed resistance to glycopeptide antibiotics. OXA-1-producing bacteria expressed resistance to a wide range of antibiotics along with carbapenems. A hundred percent co-resistant rate was found with cephalosporin-resistant CTX-M-2 and diaminopyrimidine-resistant *dfrA14* and *dfrA23*. *dfrA14* also confers resistance to rifamycin, and 92% of the genomes expressed this gene. A macrolide-resistant gene, *ereA2*, was observed with a co-resistance rate of 94.4%, and 96.4% co-resistant APH(3')-VI of aminoglycoside was observed. Within the carbapenem-resistance genes, OXA-1 was found to be co-occurring with NDM class genes with a co-resistance rate of $>83\%$. This is correlated with a study conducted in Dubai, UAE, showing a similar pattern of resistance³². This study is important, especially for clinicians, because it would help them dispense antibiotics to which the bacteria are susceptible. SHV-producing genes were found to co-occur with most of the other carbapenem-resistant genes and other drug class resistant genes and this can be attributed to their ESBL characteristic.

Certain neighboring genes to the resistance-conferring genes could play a role in the mechanism of resistance. To understand how resistance genes function in inactivating an antibiotic, studying the upstream and the downstream genes is indispensable. By studying the genetic context of CMY-2 and CTX-M-27, IS1380/*ISEcp1* element was found to occur immediately upstream of CMY-2 and CTX-M-27. Previous studies suggest that *ISEcp1* helps in the mobilization of the CMY-2 and CTX-M-27 genes, functioning as a transposon through plasmids in *Citrobacter freundii*³³. *ISEcp1* was also found to be important clinically in the spread of ESBLs. *ISKra4* has a similar function, and it was found to be occurring immediately downstream of the CTX-M-27 gene. The neighboring genes of IMP-4 included *qacG2*, *aac(6¹)-Ib4*, and *catB3*. These genes were found downstream of the IMP-4 gene. The *bla_{IMP-4}-aacA4-catB3* cassette was found in clinical isolates of *Acinetobacter baumannii* in Sydney and Melbourne, Australia, suggesting the movement of this cassette through plasmids mediated by homologous recombination³⁴. *qacG2* codes for an ammonia compound efflux Small Multidrug Resistance (SMR) transporter. In VIM-1 and OXA-1 genes, *intI1*-class 1 integron integrase was found in the upstream and the downstream regions, respectively. Integron consists of integrase or size-specific recombination and attI site and acts as a strong promoter in the case of VIM-1. The antibiotic resistance gene cassette integrates with the integron, and the transfer is mediated by homologous recombination³⁵. *aac(6¹)-Ib-cr5*, a variant of *aac(6¹)-Ib*, found upstream to *bla_{OXA-1}* codes for aminoglycoside N-acetyl transferase. It acetylated fluoroquinolones and aminoglycosides, which were found to be the major cause of resistance to aminoglycosides³⁶. The presence of *ble_{MBL}* downstream to the *bla_{NDM-1}* gene was observed, and it confers resistance against bleomycin, an anti-tumor glycopeptide³⁷. Two insertion sequence elements were found downstream of *bla_{NDM-1}*. Upstream of *bla_{KPC-3}*, *istB*-an IS21 putative ATP binding protein is expressed only when there is a tandem repeat of IS21. Immediately after that, *istA* if IS21 is seen. It cleaves the 3' ends of IS21 tandem repeats elements. Both these genes are required for the formation of cointegration, and the plasmids that carry these cointegrates with other replicons are formed at higher frequencies³⁸. The presence of α/β hydrolase was found immediately downstream.

The phylogenetic study showed that the gene *bla_{OXA-232}* is more primitive than the rest of the genes. The short branch length between *bla_{OXA-48}*, *bla_{OXA-204}*, and *bla_{OXA-244}* suggests that they are more closely related, and the expression pattern could be similar. *bla_{OXA-244}* is a derivative of *bla_{OXA-48}* and is a weak carbapenemase³⁹. The commonly occurring incompatibility groups of plasmids were analyzed. IncFIB was the most commonly occurring plasmid, followed by IncC. IncFIB was found to be a conjugative plasmid and also involved in the dissemination of antibiotic resistance genes⁴⁰. Sequence Type analysis using MLST showed the predominant occurrence of ST-11, ST-14, and ST-147 among the genomes. ST-11 is disseminated worldwide and is a single locus variant of ST-258, which is a hyper epidemic clone and is responsible for the dispersion of carbapenem resistance⁴¹. ST-147 has been characterized as a high-risk CRKP clone, and the resistance genes were found to be located in multiple plasmids⁴².

Analyses of all the 82 carbapenem resistance genes were not conducted which limited the scope of studies. Individual resistance gene analysis could give more insights into the mechanisms by which bacteria evade antibiotics. The gathering of data was done only using bioinformatics tools, whereas *in vitro* characterization and identification of bacteria using Vitek or Minimum Inhibitory Concentrations (MIC) for different antibiotics *in vitro* were not conducted.

CONCLUSIONS

Understanding the resistance determinants like porins, carbapenemases, PBPs, and efflux pumps in order to prolong the lives of infected individuals should be one of the most important goals in combating AMR infections. Synthetic carbapenems that are able to tackle bacterial resistance by bypassing the efflux pumps could be designed. Efflux pump inhibitors have been found to restore the antibiotic activity⁴³, and the focus should

also be on coming up with effective inhibitors of Metallo β -lactamases and serine β -lactamases.

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