# Chapter 3:

## Sample collection, identification, screening of antibiotic resistant (ABR) isolates, and whole genome sequencing

### 3.1 Introduction:

*Klebsiella pneumoniae* is a facultatively anaerobic, Gram-negative, rod-shaped, encapsulated, non-motile bacterium (Ashurst & Dawson, 2023). *Klebsiella pneumonia*e is frequently detected in the upper and lower gastrointestinal tracts of numerous nonhuman primates as part of the normal microbiota. Upper respiratory tract-associated pathogenic isolates are typically densely encapsulated (Lau et al., 2008). As a major opportunistic pathogen, *Klebsiella pneumoniae* belongs to the ESKAPE multidrug resistance bacterial family. It can cause critical hospital-acquired infections, such as skin and soft tissue infections in immunocompromised people, pneumonia, meningitis, urinary tract infections, liver infections, and septicemia (Pu et al., 2024). It is unknown why *Klebsiella* species cause infections more often than other Gram-negative opportunistic bacteria. The bacteria may be able to withstand starvation (Baker, 2015), resist antibiotics naturally (Wand et al., 2015), outcompete other bacteria (P.-F. Hsieh et al., 2019), easily exchange DNA with other microbiome members (Dunn et al., 2019), and pick up mobile genetic elements encoding a variety of virulence-enhancing and antibiotic resistance genes (Wyres et al., 2020).

Even though *K. pneumoniae* is one of the most prevalent nosocomial organisms in the world (Pendleton et al., 2013), its population structure is distinguished by a high degree of genetic variety as well as the existence of a comparatively limited number of very effective clonal genetic lineages (Wyres et al., 2020). Unlike other bacteria acquired in healthcare settings, the most troublesome clones of *K. pneumoniae* may be readily categorized into two separate groups based on their illness severity. These groups are defined by either having resistance to many drugs or being very virulent (David et al., 2019; Russo & Marr, 2019b). This greatly hampers the effectiveness of therapeutic care, leading to unfavorable treatment results. Tracking antimicrobial resistant bacteria is essential for managing the spread of drugs resistance. This practice has been identified as one of the four main tasks in the CDC antimicrobial resistance action plan (Holt et al., 2015). The use of multilocus sequence typing has shown that the population of multidrug-resistant *K. pneumoniae* is mostly composed of a few closely related clones, and some epidemic/endemic clones have been observed.

Resistance to antimicrobials presents a substantial and imminent threat to worldwide public health (Asokan et al., 2019). The exponential growth and global dissemination of antimicrobial resistance pose a significant threat to the progress made in contemporary medicine, jeopardizing the efficacy

of treating prevalent infections such as urinary tract infections, pneumonia, and tuberculosis (Baker, 2015). Additionally, it compromises the provision of healthcare to patients requiring organ transplantation, intricate surgical procedures, cancer chemotherapy, and intensive care.

Surveillance plays a fundamental role in the management of infectious illnesses (J. Murray & Cohen, 2017; C. J. L. Murray et al., 2022). In this regard, Whole genome sequencing's ultimate molecular resolution has given rise to previously unheard-of insights on the development and dissemination of antibiotic resistance (Grad et al., 2014). Whole-genome sequencing offers a substantial quantity of data and the most precise method for categorizing pathogens. Utilizing whole-genome sequencing for worldwide monitoring may provide insights into the early onset and dissemination of antimicrobial resistance, hence facilitating the creation of timely policies for AMR management. Sequencing data derived from AMR surveillance may provide crucial insights to facilitate the creation of efficient diagnostic tools for improved and expedited identification of AMR. Consequently, this can enhance and supplement phenotypic techniques (Asokan et al., 2019).

Research using whole-genome sequencing has shown a significant ability to differentiate between conserved and changeable genetic material, enabling more accurate species categorization. Whole genome sequencing is employed as a tool for the identification and typing of microorganisms using techniques such as multilocus sequence typing, core genome multilocus sequence typing (cgMLST), CRISPR-Cas, serogrouping, and single nucleotide polymorphisms (SNPs). Additionally, WGS is used to detect genes that provide resistance to antibiotics and/or virulence, enabling more accurate molecular epidemiological studies (Leopold et al., 2014). Hence, the examination of whole genomes and their comparison may unveil the function of antibiotic resistance genes and virulence genes in the pathogenicity of the organism (Jaradat et al., 2014).

NGS technology may be used to get comprehensive genomic information of an organism (M. W. Khan et al., 2016; Nasser et al., 2018), and concurrently identify several infections in clinical samples (Mustafa et al., 2023). Information derived from Whole Genome Sequencing can be examined using a range of bioinformatics tools (Singh et al., 2022). These tools offer insights into the accuracy of sequenced genomes and can identify the species, strains, and genotypes of the infecting organisms. Additionally, they can make predictions about drug susceptibility/resistance and aid in epidemiological investigations (Harris et al., 2013; M. W. Khan et al., 2016; Leong et al., 2018; Safar et al., 2023). This knowledge is crucial for promptly identifying, treating, and

studying both familiar and unfamiliar/emerging infections (Asokan et al., 2019; Ferdinand et al., 2021). Hence, the application of whole-genome sequencing could significantly influence the domain of clinical bacteriology by assisting in the detection, therapy, prevention, management, and surveillance of bacterial illnesses.

## 3.2 Materials and methods

### 3.2.1 Collection of clinical samples of *K. pneumoniae*

Clinical isolates of *K. pneumoniae* ($n$ = 30) were collected from different pathology labs (Metropolis Pathology Lab and Toprani Advanced Lab System) in Gujarat, of which 17 samples (M2, M3, M6, M10, M17B, M25, M27, M33, M34a, M35, M36, M39, M40, M44, M46, DJ, ST1) were collected, identified as *K. pneumoniae*, along with drug susceptibility profile in 2016-17 by Dr. Siddhi Desai (Senior Lab Member), and the rest 13 (M47, M48, M49, M50, M51, M52, M53, M54, M55, M56, M57, M58, M59) were collected in 2020 by myself. Isolates were obtained from several sources, including urine, blood, endotracheal swab, respiratory specimens, etc. All isolates were subcultured on MacConkey agar, and after an overnight incubation period, glycerol stocks were made and preserved at -20 °C for further use in future.

### 3.2.2 Sample Identification

The identification of cultures that were collected before has already been completed. The freshly obtained samples were pre-identified using the Vitek 2 system. Subsequently, prior to whole genome sequencing, additional identification was conducted using 16s rRNA sequencing.

#### *3.2.2.1 Extraction of genomic DNA*

Using a modified phenol-chloroform technique, genomic DNA (gDNA) was isolated from a 16–18-hour old culture that was cultured in Lucia broth (Sambrook et al., 2012). A volume of 1 milliliter of a culture that had been cultivated overnight was subjected to centrifugation at a speed of 1000 revolutions per minute for a duration of 10 minutes. The pellet was reconstituted in 345µl of T.E. buffer and carefully mixed. 40µl of Lysozyme solution with a concentration of 10mg/ml was added to the tubes. The tubes were gently mixed by inverting them and then incubated at a temperature of 37 ºC for a duration of 1 hour. Following the incubation period, 100µl of a 1% SDS

solution, 10 µl of a 0.5 M EDTA solution (pH=8), and 5µl of proteinase K (100µg/ml) were added to the mixture and gently mixed by inverting. The tubes were placed in a controlled environment at a temperature of 50ºC for a duration of 2 hours until the solution achieved a transparent appearance. A mixture of phenol, chloroform, and isoamyl alcohol in equal proportions was thoroughly inverted and subsequently centrifuged at a speed of 10000 revolutions per minute for a duration of 10 minutes. An equal amount of chloroform: isoamyl alcohol was added to the upper aqueous phase. The mixture was gently mixed by inverting and then centrifuged at 10000 rpm for 10 minutes. The upper aqueous layer was meticulously removed into a separate tube, and 2.5 times the volume (2.5X) of absolute ethanol and one-tenth of the volume (V/10) of sodium acetate (0.3M) were added. The tubes were refrigerated at a temperature of -20 ºC overnight to enhance the process of isolating gDNA by causing it to precipitate more effectively. The next day, the liquid portion was removed and disposed of after being spun at a speed of 10,000 revolutions per minute for 10 minutes. The pellet was rinsed with a solution of 70% ethanol and then allowed to dry in the air. A 200 µl amount of sterile nuclease-free high-grade water was added, followed by storing the DNA at a temperature of 4 $^{0}$C.

*3.2.2.2 Amplification of the 16S rRNA gene and analysis using the NCBI BLAST tool*

The Polymerase Chain Reaction (PCR) method was used to amplify 16S rRNA gene sequences from 13 isolates. The amplification was performed in a Thermocycler (Bio-Rad Laboratories, CA, USA). The 16S rRNA region was amplified using universal 16S rRNA primers, specifically 27F Forward and 1492R Reverse (De Lillo et al., 2006). The primer sequences that were used were 27F, which was 5'-AGAGTTTGATCMTGGCTCAG-3', and 1492R, which was 5'-GGTTACCTTGTTACGACTT-3. A total of 12.5µl of SapphireAmp® Fast PCR Master Mix (TaKaRa Bio Inc., Tokyo, Japan) with dNTPs, MgCl2, and Taq polymerase was included in each 25µl reaction system. Additionally, 3µl of each primer (10 pmol) and 2µl of appropriately diluted (90-100 ng/µl) template DNA were included. To finish the amplification, the following procedures were implemented: An initial denaturation period of 7 minutes at 95 °C, followed by 30 seconds at 94 °C, 1 minute at 55 °C for annealing, 1 minute at 72 °C for primer extension, and finally 7 minutes at 72 °C for final extension. Gel-Doc (Bio-Rad Laboratories, CA, USA) was used to visualize the presence of around 1.5 kb amplicons under UV light after the PCR product was electrophoresed with a 100 bp DNA ladder (TaKaRa Bio Inc., Tokyo, Japan) marker on 1% agarose gel containing 0.5 µg/ml of EtBr in 1x TBE buffer. For Sanger's sequencing, the amplicons

were sent to Genexplore Diagnostics & Research Centre Pvt. Ltd., Ahmedabad, India. Following sequencing, the amplified gene sequence was saved in FASTA files, which were then compared to the National Center for Biotechnology Information database (NCBI) database. The isolates exhibiting > 98% identity and query coverage were determined to be *Klebsiella pneumoniae*.

### 3.2.3 Screening of Antibiotic resistance isolates.

A microbroth dilution technique and the VITEK-2 Compact system (BioMérieux, France) were used to conduct the antimicrobial susceptibility test in compliance with CLSI guidelines.

#### *3.2.3.1 CLSI's microbroth dilution method*

Broth microdilution technique was used to determine the minimum inhibitory concentration (MIC) of tigecycline and colistin, following the guidelines provided by the Clinical and Laboratory Standards Institute (CLSI, 2016). The culture that had grown overnight was adjusted to a turbidity level with equivalent to that of the 0.5 McFarland standard at OD600 using phosphate-buffered saline, and then diluted by a factor of 10. A suspension of bacteria which was prepared earlier is utilized as the inoculum for the test. It was then diluted with an antibiotic solution to achieve a final inoculation density of around $10^5$ colony-forming units per milliliter (CFU/ml) in each well. A suspension of antibiotics was prepared in sterilized glass test tubes using the appropriate solvent. The drug tigecycline exhibited solubility in dimethyl sulfoxide (DMSO), while colistin shown solubility in sterile molecular grade produced water. A stock solution of tigecycline with a concentration of 512 µg/ml was made in a sterile glass tube. Then, 50 microliters of the stock solution was introduced into the wells, which were already filled with 50 microliters of MHB solvent. Following that, a 50 µl amount of bacterial inoculum was added to the wells that previously had broth and antibiotics. A sterility control group consisting of a single set of triplicate wells was kept, containing only MHB and bacterial inoculum, without antibiotics. A controlled environment was established for the microtiter-plate, maintaining a temperature of 37 °C for a period of 24 hours. The minimal inhibitory concentration (MIC) was established by determining the lowest dosage of antibiotics at which no visible growth was observed after incubation. The turbidity at 600 nm was measured using a reader for micro plates (Multiskan Go, Thermo Fisher Scientific, Waltham, MA, USA). MIC analysis of colistin was performed using the same methodology. Colistin was tested at concentrations between 0.25 and 64 mg/L with sterile distilled water serving as the solvent.

*3.2.3.2 Antibiotic susceptibility test using Vitek 2 system.*

The VITEK-2 System utilizes an automated test approach called antibiotic susceptibility testing (AST), which is based on the minimum inhibitory concentration (MIC) technique developed by (MacLowry & Marsh, 1968; Barry et al., 1979). The Antibiotic sensitivity Testing (AST) procedure for the Vitek 2 system comprises many phases that evaluate the sensitivity of bacterial isolates to different antibiotics. First, a homogeneous culture of the organism is grown and standardized to a turbidity of 0.5 Macfarland units to guarantee precise outcomes. Subsequently, the bacterial solution is introduced into the Vitek 2 AST card, which has wells holding different antibiotics at predetermined concentrations depending on drug's breakpoint. Subsequently, the card is inserted into the Vitek 2 apparatus, where it undergoes automated incubation, growth surveillance, and result interpretation. Throughout the incubation period, the equipment consistently monitors the rate at which the bacteria grow in the presence of antibiotics, resulting in a growth curve for each well. The equipment uses these growth curves to determine the MIC of each antibiotic for the organism being tested. Lastly, based on recognized clinical breakpoints, the Vitek 2 program evaluates the MIC results and offers an appropriate assessment of sensitivity (e.g., susceptible, intermediate, or resistant) of each drug tested.

### 3.2.4 Isolate categorization based on resistance patterns.

Based on the results of antimicrobial susceptibility testing (using MIC and Vitek 2 system), isolates were categorized according to the criteria outlined by (Magiorakos et al., 2012). The isolates were classified into three distinct categories: Multidrug-resistant (MDR), Extreme drug-resistant (XDR), and Pandrug-resistant (PDR). The criteria used to define MDR, XDR, and PDR for *Enterobacteriaceae* are as follows: MDR refers to a microorganism that is resistant to at least one agent in three or more antimicrobial categories. XDR refers to bacterial isolates that are resistant to at least one agent in all but two categories of antimicrobial agents. This means that these isolates are resistant to at least one agent in each category, but they remain susceptible to all agents in no more than two categories. PDR: Resistant to all antimicrobial agents indicated in **Table 3.1** (Magiorakos et al., 2012).

**Table 3.1 Classification of antimicrobial agents and antibiotics used for drug susceptibility testing.**

| Antimicrobial category | Antimicrobial agent |
|---|---|
| Cephalosporins (non-extended spectrum); 1st and 2nd generation cephalosporins | Cefuroxime, Cefazolin, |
| Cephalosporins (extended spectrum); 3rd and 4th generation cephalosporins | Cefotaxime, Cefepime, Ceftazidime |
| Aminoglycosides | Tobramycin, Gentamycin, Amikacin |
| Phenicols | Chloramphenicol |
| Antipseudomonal penicillins + β-lactamase inhibitors | Ticarcillin/clavulanic acid, Piperacillin/tazobactam |
| Tetracyclines | Minocycline, Tetracycline, Doxycycline |
| Folate pathway inhibitors | Trimethoprim / Sulfamethoxazole |
| Fluoroquinolones | Ciprofloxacin |
| Carbapenem | Meropenem, Imipenem, Ertapenem, Doripenem |
| Monobactams | Aztreonam |
| Penicillins | Ampicillin |
| Penicillins + β-lactamase inhibitors | Ampicillin-sulbactam, Amoxicillin/Clavulanic acid |
| Phosphonic acids | Fosfomycin |
| Glycylcyclines | Tigecycline |
| Polymyxins | Colistin |

### 3.2.5. Whole genome sequencing (*n* = 30) isolates of all categories (Susceptible, MDR, XDR, and PDR).

DNA extraction process was carried out on 30 isolates using the XpressDNA Bacteria kit from MagGenome Technologies Private Limited, India. The library preparation was performed for *n* = 12 (M2, M3, M6, M10, M17B, M25, M27, M33, M35, M36, M39, and DJ) isolates using the Ion Xpress™ Plus gDNA Fragment Library Preparation Kit (ThermoFisher Scientific) according to

the provided directions in the handbook, and the sequencing was done by using Ion S5TM system (Iontorrent, Thermo Fisher Scientific). On the other hand, NGS libraries for rest *n* = 18 (M34a, M40, M44, M46, M47, M48, M49, M50, M51, M52, M53, M54, M55, M56, M57, M58, M59, and ST1) isolates were prepared using the Illumina Nextera XT DNA Library Prep Kit (San Diego, CA, USA) according to the instructions provided by the manufacturers, and the sequencing was carried out using the Illumina MiSeq platform (Illumina, CA, USA) using a 2 x 150 nucleotides (nts) paired-end method.

We used FastQC to analyze the raw data and assess its quality, which was followed by the FastX toolkit and Trimmomatic tool v0.40 to remove and filter all contaminations, adaptors, and low-quality sequences. We generated the de novo assembly employing SPAdes v3.14.1 (http://cab.spbu.ru/software/spades/), which employed 32 threads in read error correction and assembly mode. We used QUAST v5.0.2 (http://quast.sourceforge.net/quast) to assess the quality of the assembled sequences. We annotated the genomes and generated multiple different file formats utilizing Prokka v1.13.3, available at https://github.com/tseemann/prokka. We submitted the assembly data to the National Center for Biotechnology Information and assigned the bioproject number PRJNA694019.

## 3.3 Results:

### 3.3.1 Sample collection and Identification

*3.3.1.1 Colony morphology*

After incubation overnight at 37 $^{0}$C, the colonies on MacConkey agar with a pink color, round form, and mucoid texture were observed in **Figure 3.1**. The colonies had a smooth edge and were clearly visible due to the existence of neutral red indicator.

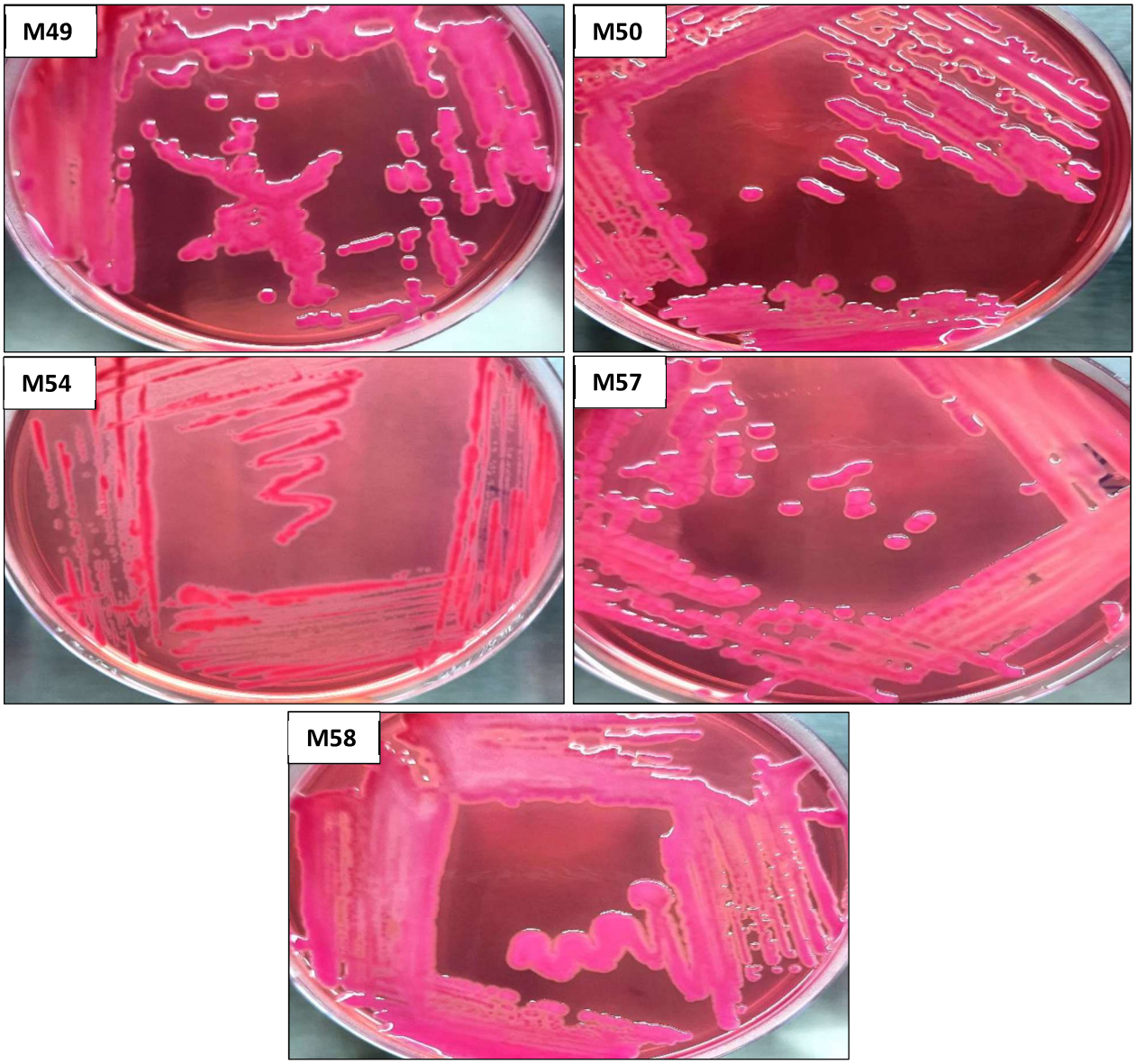


**Figure 3.1 Representative images of isolates of *K. pneumoniae* grown on MacConkey agar plates.** Isolates M49, M50, M54, M57 and M58. There were no notable variations in the morphology of the isolates. However, isolate M54 exhibited a much smaller colony size with flat and rough surface, while isolate M49 and M58 had a larger colony with more mucus.

**Table 3.2** describes the colony features and morphology. We observed the isolates to have a round shape with a smooth edge. We identified the colonies as translucent pink in color, accompanied by a mucoidal layer.

**Table 3. 2 Colony characteristics of all clinical isolates**

| Colony Characteristics | Observations |
|---|---|
| Shape | Round |
| Margin | Entire |
| Size | Varies |
| Elevation | Convex |
| Opacity | Translucent |
| Consistency | Mucoid |
| Pigmentation | Pink |

*3.3.1.2 16s rRNA gene amplification and gene sequencing for identification*

*3.3.1.2.1 Extraction of genomic DNA*

DNA isolated from the isolates was loaded onto a 0.8% agarose gel, and the band containing the genomic DNA was retrieved after completing gel electrophoresis as shown in **Figure 3.2**.

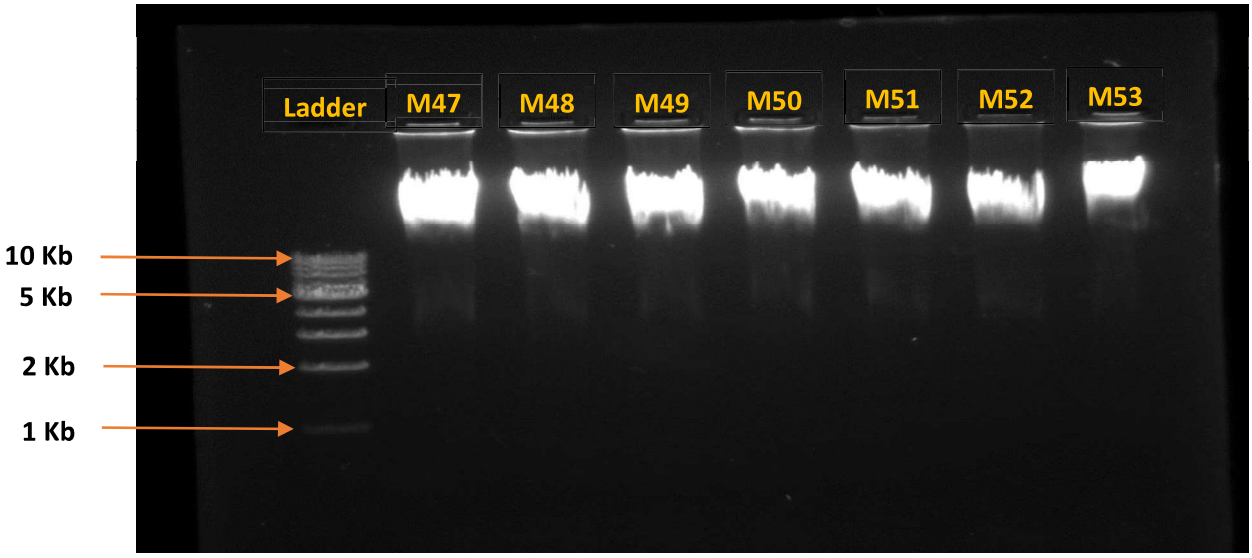


**Figure 3.2 Genomic DNA obtained from the representative clinical isolates.** A 0.8% agarose gel was stained with SYBR Safe DNA Gel Stain. The top of each lane is labeled with the DNA samples that were used to load the gel.

*3.3.1.2.2 PCR amplification of 16s rRNA gene and Shot gun sequencing (Sanger sequencing)*

Amplicons of approximately 1 kilobase in size were obtained by polymerase chain reaction (PCR) targeting the 16s rRNA gene as shown in **Figure 3.3.**

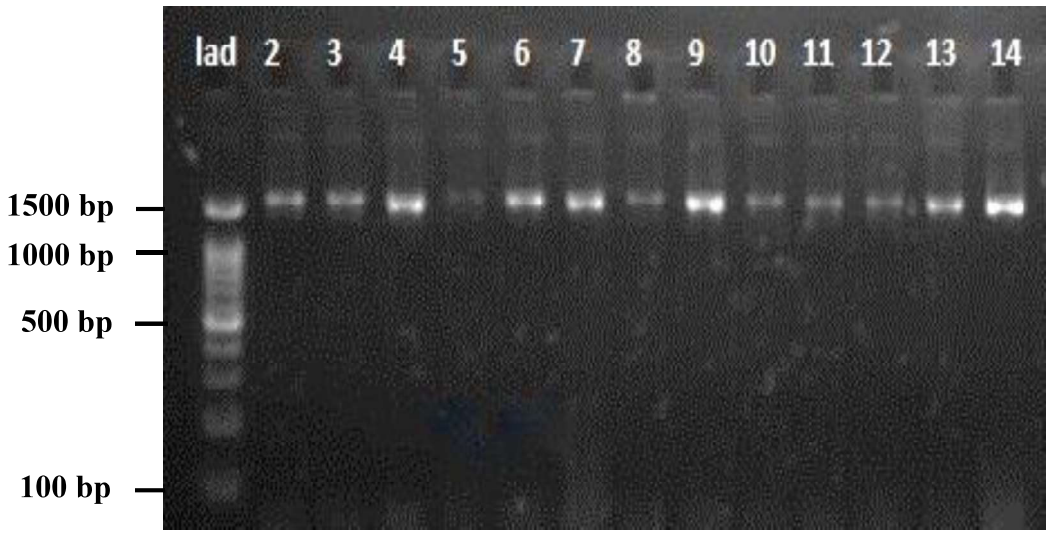


**Figure 3.3 The 1% agarose gel electrophoresis shows discrete bands of PCR amplified 16S rRNA gene.** The template genomic DNA (gDNA) used for the amplification process is as follows: Lane1: Ladder (100bp), Lane2: M47, Lane3: M48, Lane4: M49, Lane5: M50, Lane6: M51, Lane7: M52, Lane8: M53, Lane9: M54, Lane10: M55, Lane11: M56, Lane12: M57, Lane13: M58, and Lane14: M59.

**Table 3.3** presents an overview of the NCBI BLAST results for the Sanger sequenced amplicons of the 16S rRNA. The query coverage quantifies the ratio of the query that corresponds to the hit, whereas % identity represents the percentage of matches that are the same as the hit. The recognized genus/species is the specific genus/species of the highest-ranking sequence that corresponds to the query sequence in a local alignment obtained using NCBI BLAST mapping.

**Table 3.3 Result of the NCBI BLAST of the 16s rRNA gene sequence obtained from sanger sequencing.**

| Isolates | Query coverage | % Identity | Length in bp | Species |
|---|---|---|---|---|
| M47 | 100% | 98% | 1246 | *K. pneumoniae* |
| M48 | 99% | 97.62% | 1292 | *K. pneumoniae* |
| M49 | 100% | 99.25% | 1200 | *K. pneumoniae* |
| M50 | 100% | 99.68% | 932 | *K. pneumoniae* |
| M51 | 99% | 98.56% | 1179 | *K. pneumoniae* |
| M52 | 100% | 99.67% | 909 | *K. pneumoniae* |
| M53 | 99% | 99.89% | 933 | *K. pneumoniae* |
| M54 | 99% | 99.89% | 922 | *K. pneumoniae* |
| M55 | 100% | 99.05% | 1162 | *K. pneumoniae* |
| M56 | 100% | 99.89% | 935 | *K. pneumoniae* |
| M57 | 100% | 99.9% | 1042 | *K. pneumoniae* |
| M58 | 100% | 99.89% | 928 | *K. pneumoniae* |
| M59 | 100% | 99.89% | 932 | *K. pneumoniae* |

Using 16s rRNA identification, it was determined that all isolates were detected as *K. pneumoniae* with a minimum of 98% similarity with more than 99% of query coverage. All discovered isolates were subsequently subjected to additional investigation.

*3.3.1.3 Drug susceptibility testing*

The isolates M47, M50, and DJ exhibited the highest level of resistance in drug susceptibility tests, since they were shown to be resistant to all antibiotics that were tested. In addition, isolates M49, M51, M52, M53, M54, M55, M56, M57, and M59 shown resistance to all antibiotics except for colistin. The isolates M48 and M58 exhibited sensitivity to just two antibiotics. Isolate M48 was sensitive to gentamicin and Trimethoprim/Sulfamethoxazole, while isolate M58 was susceptible

to colistin and Trimethoprim/Sulfamethoxazole. In addition to this, all other isolates showed susceptibility to colistin and tigecycline. However, a few isolates (M2, M6, M17B) exhibited resistance to all carbapenem medicines, but found susceptible against tigecycline and colistin. The tetracycline class of antibiotics was evaluated solely against XDR and PDR isolates, all of which shown resistance to all three tetracycline agents, with the exception of M2, which demonstrated sensitivity to doxycycline and minocycline. All isolates demonstrated resistance to the Ticarcillin-clavulanic acid antibiotic, followed by resistance to ampicillin and Piperacillin-tazobactam as shown in **Table 3.4**. The CLSI recommendations outlined in document M100-Ed33 were used to determine drug susceptibility based on breakpoint standards. The breakpoint for order enterobacterales was considered to define **susceptible (<=)**, **Intermediate (=)**, **Resistant (>=)** based on concentrations of antibiotics (µg/ml) in which for Gentamicin- **2**, **na**, **8**; Amikacin- **4**, **8**, **16**; Ciprofloxacin- **0.25**, **0.5**, **1**; Nalidixic acid- **16**, **na**, **32**; Levofloxacin- **0.5**, **1**, **2**; Ticarcillin-clavulanic acid- **16/2**, **32/2-64/2**, **128/2**; Piperacillin-tazobactam- **8/4**, **16/4**, **32/4**; Ertapenem- **0.5**, **1**, **2**; Imipenem/Meropenem/Doripenem- **1, 2**, **4**; Cefuroxime- **4, 8-16**, **32**; Cefotaxime or Ceftriaxone- **1, 2**, **4**; Ceftazidime- **4, 8**, **16**; Cefepime- **2, 4-8**, **16**; Aztreonam- **4**, **8**, **16**; Ampicillin- **8**, **16**, **32**; Amoxicillin-clavulanic acid- **8/4**, **16/8**, **32/16**; Ampicillin-sulbactam- **8/4**, **16/8**, **32/16**; Chloramphenicol- **8**, **16**, **32**; Cefotelan- **16**, **32**, **64**; Tetracycline/Doxycycline/Minocycline- **4**, **8**, **16**; and Trimethoprim/Sulfamethoxazole- **2/38**, **na**, **4/76**.

**Table 3. 4 Antibiotic susceptibility profile and categorization of isolates (*n* = 30)**

| Drug Class | Drugs {MIC calling range (ug/ml)} | M2 | M3 | M6 | M10 | M17B | M25 | M27 | M33 | M34a | M35 | M36 | M39 | M40 | M44 | M46 | M47 | M48 | M49 | M50 | M51 | M52 | M53 | M54 | M55 | M56 | M57 | M58 | M59 | DJ | ST1 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Aminoglycosides | GT- Gentamicin (1-16) | Res | Res | Res | Res | Res | Sen | Sen | Sen | Sen | Sen | Sen | Res | Sen | Sen | Sen | Res | Sen | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res |
| | AK- Amikacin (2-64) | Res | Sen | Res | Res | Res | Sen | Sen | Sen | Int | Sen | Sen | Sen | Sen | Sen | Sen | Res | Res | Res | Res | Res | Int | Res | Res | Res | Res | Res | Res | Res | Res | Res |
| Fluoroquinolones | CI- Ciprofloxacin (0.25-4) | Res | Res | Res | Res | Res | Sen | Sen | Sen | Sen | Sen | Sen | Sen | Res | Sen | Sen | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res |
| | NA- Nalidixic acid (2-32) | Res | Sen | Res | Sen | Res | Sen | Res | Sen | Sen | Sen | Sen | Sen | Sen | Sen | Sen | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res |
| | LF- Levofloxacin (0.12-8) | Res | Res | Res | Sen | Res | Sen | Sen | Sen | Sen | Sen | Sen | Sen | Res | Sen | Sen | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res |
| Glycylcycline | TG- Tigecycline (0.5-8) | Sen | Sen | Sen | Sen | Sen | Sen | Sen | Sen | Sen | Sen | Sen | Sen | Sen | Sen | Sen | Int | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Int | Res | Res | Sen |
| Antipseudomonal penicillins + β-lactamase inhibitors | TC- Ticarcillin-clavulanic acid (8/2-128/2) | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res |
| | PT- Piperacillin-tazobactam (4/4-128/4) | Res | Sen | Res | Res | Res | Sen | Res | Res | Res | Sen | Sen | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res |
| Carbapenems | ER- Ertapenem (0.5-8) | Res | Sen | Res | Sen | Res | Sen | Sen | Sen | Sen | Sen | Sen | Sen | Sen | Sen | Sen | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res |
| | IM- Imipenem (0.25-16) | Res | Sen | Res | Sen | Res | Sen | Sen | Sen | Sen | Sen | Sen | Sen | Sen | Sen | Sen | Int | Int | Res | Int | Res | Res | Res | Res | Res | Res | Int | Res | Res | Res | Res |
| | MR- Meropenem (0.25-16) | Res | Sen | Res | Sen | Res | Sen | Sen | Sen | Sen | Sen | Sen | Sen | Sen | Sen | Sen | Res | Res | Res | Res | Sen | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res |
| | DR- Doripenem | Res | Sen | Res | Sen | Res | Sen | Sen | Sen | Sen | Sen | Sen | Sen | Sen | Sen | Sen | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res |
| NESC; 1st & 2nd GC | CU- Cefuroxime (1-64) | Res | Sen | Res | Sen | Res | Sen | Res | Sen | Sen | Sen | Sen | Sen | Sen | Sen | Sen | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res |
| ESC; 3rd & 4th GC | CM- Cefotaxime or Ceftriaxone (1-64) | Res | Sen | Res | Res | Res | Sen | Res | Sen | Res | Sen | Sen | Res | Res | Sen | Sen | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res |
| | CZ- Ceftazidime (1-64) | Res | Sen | Res | Sen | Res | Sen | Res | Sen | Sen | Sen | Sen | Sen | Sen | Sen | Sen | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res |
| | CP- Cefepime (1-64) | Res | Res | Res | Res | Res | Sen | Sen | Sen | Res | Sen | Sen | Res | Res | Res | Sen | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res |
| Monobactams | AZ- Aztreonam (1-64) | Res | Res | Res | Res | Res | Sen | Res | Sen | Res | Sen | Sen | Res | Res | Sen | Sen | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Sen |
| Penicillins | Ap- Ampicillin (2-32) | Res | Res | Res | Res | Res | Sen | Res | Sen | Res | Sen | Res | Res | Res | Res | Int | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res |
| Penicillins+ β-lactamase inhibitors | AC- Amoxicillin-clavulanic acid (2/1-32/16) | Sen | Res | Res | Res | Res | Sen | Res | Sen | Sen | Sen | Sen | Sen | Sen | Sen | Sen | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res |
| | AS- Ampicillin-sulbactam (2/1-32/16) | Res | Res | Res | Res | Res | Sen | Res | Sen | Sen | Sen | Sen | Sen | Sen | Sen | Sen | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res |
| Phenicols | CH- Chloramphenicol (4-64) | Res | Sen | Res | Sen | Res | Sen | Res | Sen | Sen | Sen | Sen | Sen | Sen | Sen | Sen | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res |
| Cephamycin | CT- Cefotelan (4-64) | Res | Sen | Res | Sen | Res | Sen | Res | Sen | Sen | Sen | Sen | Sen | Sen | Sen | Sen | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res |
| Tetracyclines | TR- Tetracycline (1-16) | Res | Res | Res | NT | Res | NT | NT | NT | NT | NT | NT | NT | NT | NT | NT | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | NT |
| | DX- Doxycycline (0.5-16) | Sen | Res | Res | NT | Res | NT | NT | NT | NT | NT | NT | NT | NT | NT | NT | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | NT |
| | MN- Minocycline (0.5-32) | Sen | Res | Res | NT | Res | NT | NT | NT | NT | NT | NT | NT | NT | NT | NT | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | NT |
| Polymyxins | CL- Colistin (0.25-16) | Sen | Sen | Sen | Sen | Sen | Sen | Sen | Sen | Sen | Sen | Sen | Sen | Sen | Sen | Sen | Res | Res | Sen | Res | Sen | Sen | Sen | Sen | Sen | Sen | Sen | Sen | Sen | Res | Sen |
| Cephalosporins + β-lactamase inhibitors | CS- Cefoparazone/sulbatam (0.5-16) | Res | Res | Res | Res | Res | Sen | Res | Sen | Res | Sen | Sen | Res | Res | Sen | Sen | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res | Res |
| Sulfanilamides | TS- Trimethoprim/Sulfamethoxazole {20 (1/19)- 320 (16/304)} | Res | Res | Res | Sen | Res | Sen | Sen | Sen | Sen | Sen | Sen | Sen | Sen | Sen | Sen | Res | Sen | Res | Res | Res | Res | Res | Res | Res | Res | Res | Sen | Res | Res | Res |
| Category | | X | X | X | M | X | S | M | S | M | S | S | M | M | M | S | P | X | X | P | X | X | X | X | X | X | X | X | X | P | X |

**(Footnote: NESC-** Non-extended spectrum cephalosporins, **ESC-** Extended spectrum cephalosporins, **1st & 2nd GC-** 1st and 2nd generation cephalosporins**; Sen-** Sensitive**, Int-** Intermediate**, Res-** Resistant, **NT-** not tested**; S-** Susceptible**, M-** MDR**, X-**XDR**, P-** PDR). Antimicrobial susceptibility testing for tetracycline drugs was conducted exclusively for XDR and PDR strains. The antibiotic susceptibility data for the emphasized isolates were sourced from Dr. Siddhi Desai's thesis (Sidhhi Desai, 2021) and subsequently published in a study report by (Shukla, Desai, et al., 2023). The data was utilized to correlate the whole genome sequencing data (acquired by myself) of these isolates with antibiotic resistance.

In MIC determination using the broth dilution technique for colistin and tigecycline for the isolates, four isolates (M47, M48, M50, and DJ) were detected as colistin-resistant with a ≥16 ug/mL MIC value. Nevertheless, in the case of tigecycline, some isolates were found to be resistant within the MIC range of 2 ug/mL to 16 ug/mL, as shown in **Table 3.5.** The M54 isolate had the highest MIC value of 16 ug/mL for tigecycline. However, the remaining isolates were shown to be sensitive to both drugs.

**Table 3.5 MIC value (ug/ml) of colistin and tigecycline.**

| Isolates | Colistin | Tigecycline |
|---|---|---|
| M47 | **≥16 ug/ml** | **2 ug/ml** |
| M48 | **≥16 ug/ml** | **4 ug/ml** |
| M49 | 0.5 ug/ml | **8 ug/ml** |
| M50 | **≥16 ug/ml** | **4 ug/ml** |
| M51 | 0.25ug/ml | **8 ug/ml** |
| M52 | 0.25ug/ml | **4 ug/ml** |
| M53 | 0.5 ug/ml | **4 ug/ml** |
| M54 | 0.5 ug/ml | **16 ug/ml** |
| M55 | 0.5 ug/ml | **8 ug/ml** |
| M56 | 0.25ug/ml | **8 ug/ml** |
| M57 | 0.25ug/ml | **4 ug/ml** |
| M58 | 0.25ug/ml | **2 ug/ml** |
| M59 | 0.5 ug/ml | **4 ug/ml** |
| DJ | **≥16 ug/ml** | **4 ug/ml** |

(**Footnote:** MIC breakpoint: For colistin: susceptible ≤2 µg/ml, resistant >2 µg/ml (EUCAST and CLSI, 2016); for tigecycline: ≤1 µg/ml is susceptible, 2.0 µg/ml is intermediate, and >2.0 µg/ml is resistant (EUCAST and CLSI, 2016**)**

The Antipseudomonal penicillins + β-lactamase inhibitors drugs class showed the lowest efficacy against all isolates when considering their drug susceptibility profile versus other medicines. All

isolates exhibited resistance to Ticarcillin-clavulanic acid, and with the exception of isolates M3, M25, M35, and M36, all were also resistant to Piperacillin-tazobactam. When examining the efficacy of antibiotics drugs it was found that more than 20 isolates shown resistance to ampicillin. This was followed by resistance to cefoparazone/sulbatam, cefepime, aztreonam, cefotaxime or ceftriaxone, ampicillin-sulbactam, ciprofloxacin, gentamicin, levofloxacin, and amoxicillin-clavulanic acid. However, only colistin shown promise as a treatment since it exhibited susceptibility against 26 isolates. Tigecycline and other drugs in the carbapenem class followed in terms of effectiveness as shown in **Figure 3.4.**

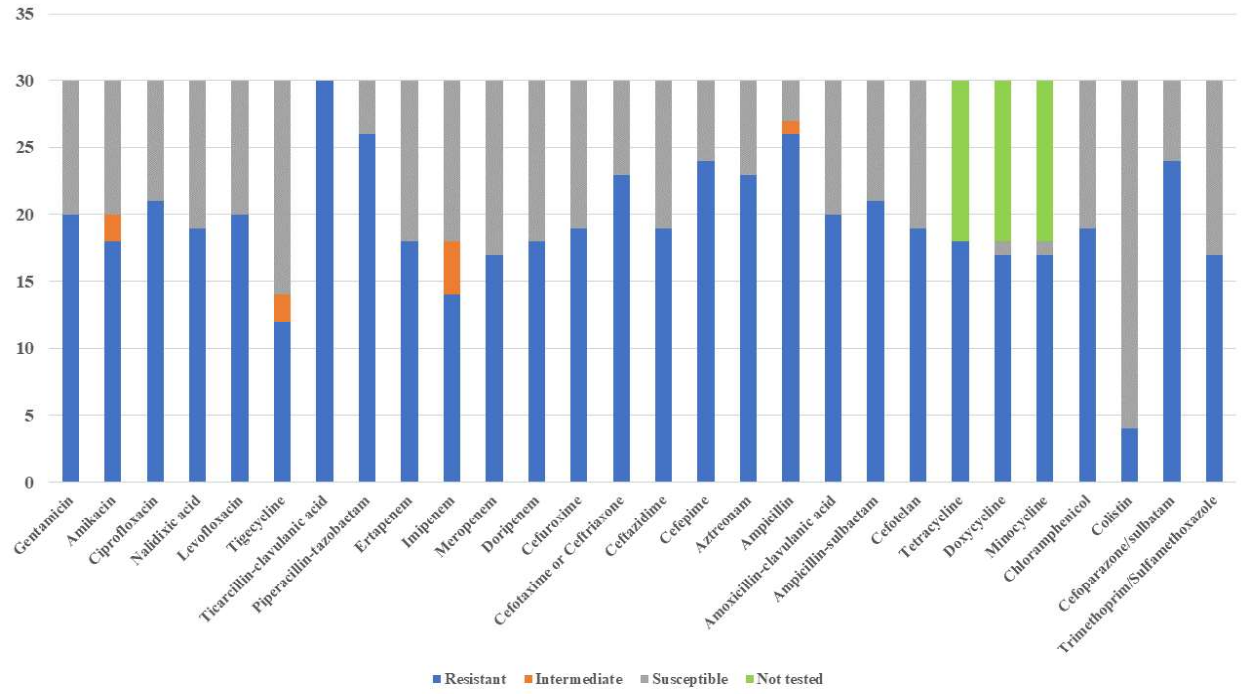


**Figure 3.4 Distribution of isolates concerning drug susceptibility patterns against different antibiotics**. Ticarcillin-clavulanic acid and Piperacillin-tazobactam exhibited the highest level of ineffectiveness against the isolates, as the isolates shown greatest resistance to both medications. Colistin and tigecycline had the highest susceptibility against the isolates, making them the most promising drugs**.**

In drug susceptibility testing followed by categorization, 53% of the isolates (M2, M3, M6, M17B, M48, M49, M51, M52, M53, M54, M55, M56, M57, M58, M59, ST1) were classified as extensively drug-resistant based on their drug susceptibility profile, followed by the multidrug-

resistant category (M10, M27, M34a, M39, M40, M44), pan-drug-resistant (DJ, M47, M50), and susceptible isolates (M25, M33, M33, M35, and M46) as shown in **Figure 3.5.**

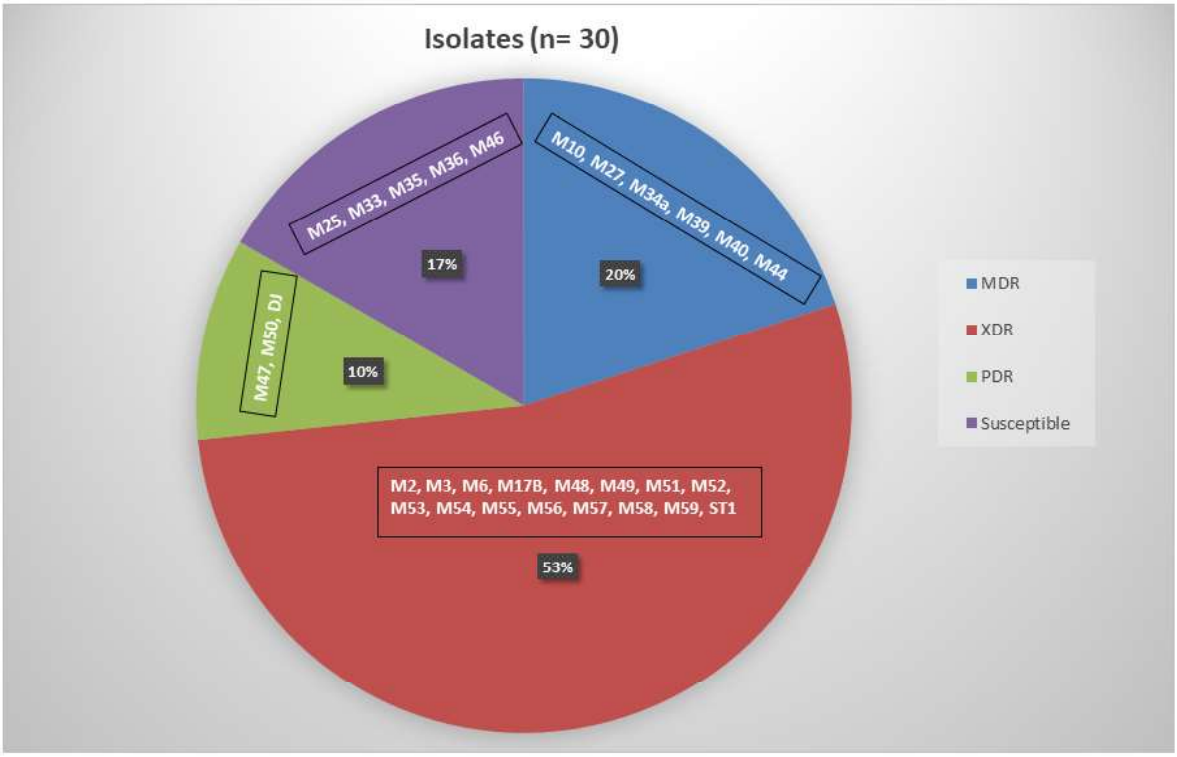


**Figure 3.5 Categorization of isolates in Susceptible, MDR, XDR, and PDR.** Out of the total isolates, 53% were classified as extensively drug-resistant (XDR), followed by 20% classified as multidrug-resistant (MDR), 17% classified as susceptible, and 10% classified as pandrug-resistant (PDR).

The drug resistance patterns of isolates over time revealed that isolates from 2020 had a significantly higher percentage of XDR isolates—which were resistant to more antibiotics—than isolates from 2016–17. Luckily, the XDR and less resistant isolates from 2016–17 were sensitive to tigecycline. However, the XDR isolates from 2020 exhibited resistance to tigecycline, which is now concerning, as shown in **Figure 3.6**.

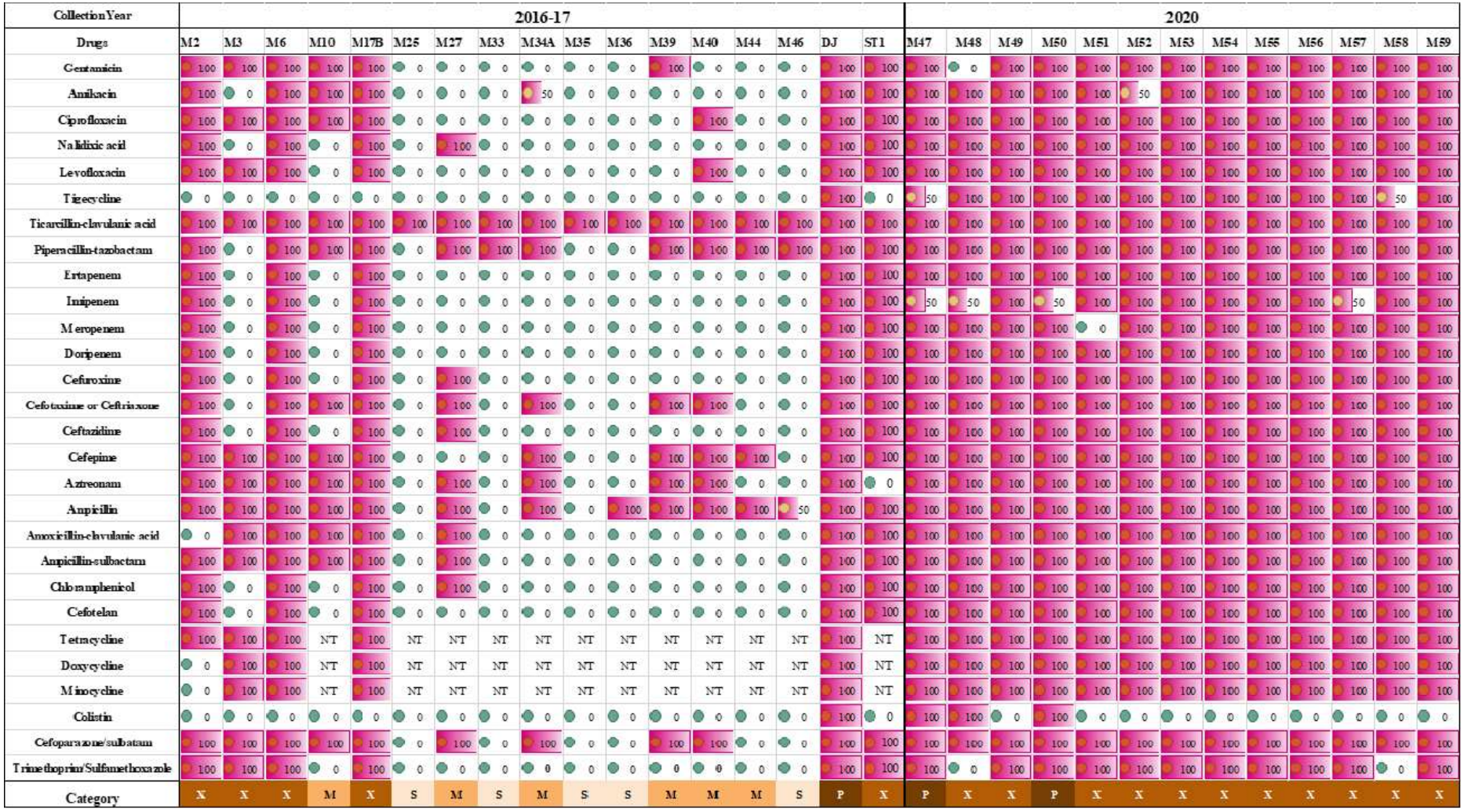


**Figure 3.6 Comparison of drug resistance pattern between isolates of 2016-17 and 2020.** The majority of XDR (extensively drug-resistant) and PDR (pan-drug resistant) isolates were identified in 2020. However, susceptible, MDR (multi-drug resistant), and a small number of XDR and PDR isolates appeared in 2016-17. (Red dot with 100 value indicates- Resistant phenotype, orange dot with 50 value indicates- Intermediate Phenotype, and green dot with O value indicates Sensitive phenotype; **S** denotes- Susceptible, **M** denotes- MDR, **X** denotes- XDR, and **P** denotes- PDR, **NT-** not detected).

*3.3.1.4 Next generation sequencing data analysis of all isolates (n = 30)*

Whole-genome sequencing (WGS) was performed on all isolates within each resistance category to analyze the genomic features of PDR, XDR, MDR, and susceptible isolates. A comprehensive study was conducted on the complete genome sequences of 30 isolates. This analysis included determining the number of contigs in each genome, calculating the N50 values, measuring the GC content as a percentage, counting the number of coding sequences, and recording the NCBI accession number for each genome. All the genomes had a GC content of around 57%, indicating their origin from *K. pneumoniae*. The majority of isolates had a contig count < 500, which is a key indicator of high genome quality, with the exception of M3, M33, and M34a. Nevertheless, the M3 sample had almost 4000 contigs and was excluded from further investigations owing to its subpar genome quality. The amount of coding sequences in the genomes varied from 5000 to 8000, with M39 having the highest count of 7794 CDS in its genome. Apart from M3, all genomes submitted to NCBI (Bioproject number: PRJNA694019) have their accession numbers shown in **Table 3.6.**

**Table 3.6 Quality assessment and genome annotation of whole genome sequencing data of all isolates (*n* = 30).**

| Isolates | Strain Name | No. of Contigs | N50 Value | GC (%) | No. of Coding Sequences | NCBI Genome Acc. No. |
|---|---|---|---|---|---|---|
| **M2** | SBS1 | 110 | 166101 | 57.14 | 5168 | JAFFRJ000000000 |
| **M3** | SBS2 | **4118** | **1206** | 56.84 | - | - |
| **M6** | SBS3 | 122 | 155288 | 57.06 | 5258 | JAFFRI000000000 |
| **M10** | SBS4 | 360 | 34708 | 57.42 | 6274 | JAFFRH000000000 |
| **M17B** | SBS5 | 271 | 128395 | 56.56 | 5563 | JAFFRG000000000 |
| **M25** | SBS6 | 128 | 112366 | 57.31 | 5119 | JAFFRF000000000 |
| **M27** | SBS7 | 163 | 50783 | 57.25 | 7088 | JAFFRE000000000 |
| **M33** | SBS8 | 435 | 27428 | 57.12 | 5508 | JAFFRD000000000 |

| | | | | | | |
|---|---|---|---|---|---|---|
| **M35** | SBS9 | 171 | 60203 | 57.33 | 5348 | JAFFRC000000000 |
| **M36** | SBS10 | 362 | 23105 | 57.05 | 5488 | JAFFRB000000000 |
| **M39** | SBS11 | 343 | 30289 | 56.68 | 7794 | JAFFRA000000000 |
| **DJ** | SBS12 | 113 | 156272 | 57.1 | 5394 | JAFFQZ000000000 |
| **M34a** | DGL1 | 798 | 233182 | 57.79 | 6033 | JAJBAN000000000 |
| **M40** | DGL2 | 60 | 477905 | 57.35 | 5083 | JAJBAM000000000 |
| **M44** | DGL3 | 74 | 370620 | 57.32 | 5007 | JAJBAL000000000 |
| **M46** | DGL4 | 71 | 291888 | 57.25 | 5293 | JAJBAK000000000 |
| **M47** | DGL5 | 91 | 325780 | 57.13 | 5198 | JAJBAJ000000000 |
| **M48** | DGL6 | 73 | 345494 | 56.8 | 5325 | JAJBAI000000000 |
| **M49** | DGL7 | 81 | 213958 | 56.95 | 5393 | JAJBAH000000000 |
| **M50** | DGL8 | 77 | 338351 | 57.12 | 5159 | JAJBAG000000000 |
| **M51** | DGL9 | 89 | 338946 | 56.99 | 5284 | JAJBAF000000000 |
| **M52** | DGL10 | 100 | 334333 | 57.15 | 5123 | JAJBAE000000000 |
| **M53** | DGL11 | 82 | 202837 | 57.08 | 5344 | JAJBAD000000000 |
| **M54** | DGL12 | 85 | 310446 | 57.23 | 5262 | JAJBAC000000000 |
| **M55** | DGL13 | 90 | 214738 | 56.54 | 5611 | JAJBAB000000000 |
| **M56** | DGL14 | 82 | 284546 | 57.14 | 5113 | JAJBAA000000000 |
| **M57** | DGL15 | 82 | 238541 | 57.09 | 5176 | JAJAZZ000000000 |
| **M58** | DGL16 | 103 | 190095 | 57.06 | 5271 | JAJAZY000000000 |
| **M59** | DGL17 | 118 | 189363 | 57.05 | 5271 | JAJAZX000000000 |
| **ST1** | DGL18 | 151 | 105757 | 56.87 | 5271 | JAJAZW000000000 |

**3.4 Discussion:**

*Klebsiella pneumoniae* is a prevalent kind of gram-negative bacterium that may lead to infections in both people and animals, causing a range of disorders (Kohler et al., 2007). Specifically, when the host's immune system is weakened or prolonged use of a high quantity of antibiotics disrupts the balance of bacteria in the body, there is an increased likelihood of the host being infected. In such situations, if the therapy is not administered correctly, it might result in fatality (Gonzalez-Ferrer et al., 2021). Microbial communities occupy a wide range of habitats that are continually changing. They experience fluctuations in nutrition availability, which may lead to rapid bacterial growth, as well as challenging situations where individuals develop methods to survive in nutrient-poor conditions (Phillips et al., 2019). Phenotypic variation is one of them, where a population may alternate between different phenotypes to survive in harsh environments imposed, for example, by the host immune system and the pressure of antibiotics (Ahmad et al., 2019).

The morphological analysis of isolates was performed for all isolates, and it is worth noting that while belonging to the same species, *Klebsiella* isolates acquired from different clinical samples exhibit variations in their morphological characteristics. We observed size differences between few isolates. The isolate M54 colony was much smaller compared to the others. On the other hand, isolate M49 and M58 exhibited a very mucoid texture on the MacConkey agar plate. There are few reports showed that *Klebsiella pneumoniae* has the ability to exhibit phenotypic plasticity, transitioning from a mucoid to a non-mucoid appearance in colony morphology (Matatov et al., 1999). The presence of a hypo- or non-mucoid appearance in *Klebsiella* is linked to the absence of a capsule. The level of mucoidity may vary and may suggest distinct mutations that alter the genes responsible for capsule manufacturing (H. Lee et al., 2019; Ernst et al., 2020).

Presently, a significant obstacle faced by clinical practice and public health monitoring is the prompt and precise detection of infectious pathogens. In clinical syndromes like sepsis, it is sometimes difficult to determine the presence of a specific pathogen since fewer than 50% of cases can be confirmed by culturing. As a consequence, a significant majority of patients get empirical therapy (Martin & Bachman, 2018).

In the molecular identification of the newly collected isolates, during the 16s rRNA sequencing and BLAST analysis, it was shown that all the isolates had a minimum of 99% query coverage and an identity of over 97% with *Klebsiella pneumoniae* genomes, which comes under the cut-off

value set for the identification of bacteria. Molecular testing enables the precise and accurate identification of a wide range of pathogens from clinical isolates and tissues. Molecular approaches have the capability to directly identify pathogens from clinical samples, allowing for quick identification without the need for culture (Hallin et al., 2003; L. E. Lehmann et al., 2008; Barbut et al., 2011). A novel technology for microbe detection began to develop in the 1980s. Research conducted in the laboratory of Woese and colleagues shown that analyzing a conserved segment of the genetic code can elucidate the evolutionary relationships among bacteria and, indeed, all organisms (Woese et al., 1985; Woese, 1987). Possible options for this bacterial genetic tract comprised the genes encoding 5S, 16S (referred to as the small subunit), and 23S rRNA, together with the intergenic regions between these genes. The *16S rRNA* gene is currently the most often utilized DNA segment for bacterial taxonomy classification (Garrity & Holt, 2001; Tortoli, 2003; Clarridge, 2004). Based on the suggested criteria for bacterial classification, bacterial strains that have a similarity of less than 97% in their *16S rRNA* gene sequence are considered to be separate species. However, if the similarity is more than 97%, another method should be used for classification (Janda & Abbott, 2007).

The GenBank database, maintained by the National Center for Biotechnology Information, currently holds over 29 million entries of 16S sequences. These sequences come from a wide range of bacteria found in different clinical and environmental sources. The database includes both complete 16S sequences and incomplete or complete genomes that contain 16S sequences (Church et al., 2020). However, although *16S rRNA* gene sequencing is a comprehensive technique, using only the 16S target may not be enough to accurately identify many human clinical pathogens for a variety of reasons. These include the high genetic similarity between specific microorganisms or groups, as well as the existence of varying numbers of 16S rRNA genes with sequence variations in their genomes (Marchandin et al., 2003; Sun et al., 2013; Ibal et al., 2019). Furthermore, there is presently a scarcity of 16S sequencing information of many pathogens in existing public data sources. This is unsurprising, considering many microorganisms or groups of microorganisms remain unidentified or unclassified (i.e., just around 1% of all bacteria have been detected) (Lennon & Locey, 2016; Locey & Lennon, 2016).

In addition, around 10% of these microbes cannot be classified at the level of species using 16S owing to either their similarity or the absence of sequencing data. The *Escherichia-Shigella-Pantoea-Klebsiella-Raoultella-Cronobacter* genera exhibit a high level of similarity (Paradis et

al., 2005), with just a few differences in all 16S variable regions (Petti et al., 2018). And precise identification of bacteria is crucial for clinical treatment and public health monitoring in order to comprehend the pathobiology of infectious clinical syndromes and optimize the use of targeted antibiotic and infection control measures for patients and populations. Typically, this is done in order to provide understanding about the specific cause of an infectious illness, including its pathological correlations and potential treatments with antimicrobial agents. The traditional approach for carrying out this operation relies on comparing the precise morphological and phenotypic characteristics of type or typical strains with those of the isolate to be identified (Clarridge, 2004).

*Klebsiella pneumoniae* is classified by the World Health Organization as one of the antibiotic-resistant infections of utmost importance, necessitating the creation of novel antibiotics to effectively tackle them. This concerning superbug is also classified as part of the ESKAPE group of pathogens that includes *E. faecium, S. aureus, K. pneumoniae, A. baumannii, P. aeruginosa*, and all *Enterobacteriaceae* because of their ability to "escape" conventional antibiotic treatment (Peterson, 2009). Moreover, *K. pneumoniae* is a well-recognized infection that is often acquired in healthcare settings and has become increasingly resistant to many drugs and even all available drugs (Bhagirath et al., 2019).

All the isolates we obtained were identified as *Klebsiella spp.* during the initial identification process. Additionally, their resistance profiles were determined using the Vitek 2 system. The susceptibility profile of all isolates, with the exception of colistin and tigecycline, were taken into account for the subsequent phase. Further the MIC of tigecycline and colistin was determined by broth microdilution method as per CLSI guidelines. The rationale for doing manual minimum inhibitory concentration testing for colistin and tigecycline was the potential for mistakes in detection using Vitek-2, as shown by a literature search. In a recent study conducted by (Főldes et al., 2022), the Vitek 2 approach was shown to be the second most erratic method for detecting drug resistance in *Klebsiella pneumoniae*. However, the error rate associated with this method was just 10%. The previously published statistics indicated that the Vitek 2 Compact had insufficient performance testing results when evaluated against the usual criteria: category agreement (CA) $\geq$ 90%, essential agreement (EA) $\geq$ 90%, Very major discrepancy (VMD) $\leq$ 3%, and MD < 3% (Chew et al., 2017; Lellouche et al., 2019; Pfennigwerth et al., 2019; Khurana et al., 2020).

Based on the drug susceptibility profile, it was interesting to see that isolates M47, M48, M50, and DJ were not affected by colistin, as their minimum inhibitory concentration was higher than 16. We identified all these isolates as pan-drug-resistant, except for M48. On the other hand, among the 14 isolates found for tigecycline resistance, the MIC ranged from 2 ug/mL to 16 ug/mL. M47 and M58 had the lowest MIC of 2 ug/mL, while M54 had the highest MIC of 16 ug/mL. According to recent research from Europe, 11% of the entire population of *K. pneumoniae* examined showed a minimum inhibitory concentration value of >2ug/ml, indicating resistance to colistin (Braspenning et al., 2024). In addition, a publication from India has shown that the minimum inhibitory concentration value for five *K. pneumoniae* isolates is more than 128ug/ml (Azam et al., 2021). Another study by Sodhi et al., (2020), it was shown that 8.75% of the total 533 *K. pneumoniae* isolates were found to be resistant to colistin. Singh-Moodley & Perovic, (2016) observed a minimum inhibitory concentration exceeding 2 µg/ml for colistin in *K. pneumoniae*.

In the past decade, the worldwide incidence of resistance to colistin in carbapenem-resistant *K. pneumoniae* has steadily increased from under 2% to 9% (Marchandin et al., 2003; Gupta et al., 2011; Ah et al., 2014). For tigecycline, from north India reported a lower level of resistance (Intermediate) for tigecycline, with a MIC value of 2ug/ml (Khare, 2017). In another investigation conducted by (Mohanty & Mahapatra, 2021), the minimum inhibitory concentration value for tigecycline was detected up to 48ug/ml in six *K. pneumoniae* isolates from India. Both colistin and tigecycline are known as last resort drug for the treatment of chronic bacterial diseases. Colistin is a very toxic substance used only for treating life threatening illness caused by highly drug-resistant Gram-negative bacteria (Rodrigues et al., 2022). It is important to prevent incorrect test findings and delays in reporting in the clinical setting (Satlin, 2019).

In further analysis, 18 out of 30 isolates were found to be resistant to all four tested carbapenem drugs (Ertapenem, Imipenem, Doripenem, Meropenem), indicating carbapenem resistance. All of these isolates belonged to either the extensively drug-resistant or pandrug-resistant categories, except for M3. Curiously, a few isolates (M47, M48, M50, and M57) exhibited intermediate sensitivity to imipenem. Surprisingly, out of these four isolates, three were belonged to colistin resistant isolates ($n = 4$). The research indicates a potential association between colistin resistance and a lower degree of resistance to imipenem.

Similarly, a study from Thailand has shown that out of the 26 clinical isolates of colistin-resistant *K. pneumoniae*, seven of them remained susceptible to carbapenems with imipenem < 2 µg/ml and

meropenem ≤ 2 μg/ml (Abe et al., 2022). Also, there has been few research that have examined the combined impact of imipenem and colistin. These studies have shown that the combination of imipenem and colistin methanesulfonate enhances the effectiveness of imipenem against multidrug-resistant *Acinetobacter baumannii* strains that are not sensitive to imipenem alone (Leu et al., 2014).

Based on the result of drug susceptibility testing and further categorization of isolates, it is noteworthy that XDR and PDR have superseded the previously sensitive MDR resistance patterns over a very short span of 4-5 years. All the isolates from the 2016-17 period, namely M2, M3, M6, M10, M17B, M25, M27, M33, M34a, M35, M36, M39, M40, M44, and M46, as well as ST1, were determined to be susceptible to tigecycline, except for isolate DJ. Nevertheless, each recently obtained isolate exhibited a resistance trait against the tigecycline drug. A similar trend was seen for the carbapenem, aminoglycosides, and fluoroquinolones medication classes, where the majority of isolates in 2016-17 were susceptible, while the isolates in 2022 had full resistance.

According to our data, the number of colistin-resistant isolates has somewhat grown over time. Previously, only 1 out of 18 isolates was resistant to colistin, but lately there are 3 out of 13 isolates that show resistance to colistin.

From India, Sharma et al., also made a similar finding. In contrast to 2018, the 2022 group exhibited no susceptibility to the strain (Sharma et al., 2023). Nine strains (21.4%) were classed as resistant, three strains (7%) as MDR, and 30 strains (93%) were classified as XDR. In another research conducted by (Karuna et al., 2024), it was shown that 80.7% of the *K. pneumoniae* isolates in India were resistant to third generation cephalosporins, 72.8% were resistant to piperacillin-tazobactam, 63.3% were resistant to carbapenems, and 14% were resistant to colistin.

The emergence of highly efficient next-generation sequencing (NGS) technology has allowed for the thorough analysis of microbiomes to an unprecedented extent that was not possible with earlier techniques (Duran-Pinedo & Frias-Lopez, 2015). The area of microbiome research has been profoundly transformed by Next Generation Sequencing (Metzker, 2010; J. Zhang et al., 2011; K. Wu et al., 2013). In the last ten years, Next-Generation Sequencing has emerged as a more efficient and precise method for studying intricate microbial communities, offering improved speed, accuracy, and cost-effectiveness (Venter et al., 2003). The utilization of whole genome sequencing in examining pathogen genomes has presented unparalleled prospects for comprehending the

epidemiology of healthcare-associated infection, ultimately resulting in the management and prevention of infections (Eyre, 2022).

In the analysis of whole genome sequencing data, all the isolates that were sequenced had contigs below 500, except for M3 and M34a. Specifically, the isolates sequenced using illumina miseq technology had higher genome quality, with the number of contigs below 200, except for the M34a isolate. On the other hand, the isolates sequenced using ion torrent technology had more contigs in their genomes after the assembly preparation. The N50 value for the isolates sequenced with Illumina MiSeq ranged from 105757 (lowest for isolate ST1) to 477905 (highest for isolate M40). In comparison, the N50 value for the isolates sequenced with Ion Torrent ranged from 1206 (lowest for M3) to 166101 (highest for isolate M2). The coding sequences in all isolates ranged from 5000 to 5500, except for a few isolates: M10 (6274), M27 (7088), M39 (7794), and M34a (6033), and the isolates also exhibited GC content ranging from 56.54% to 57.79%, like other reported genomes of *K. pneumoniae* (X. Yu et al., 2019; Du et al., 2021). Interestingly, all the isolates with higher coding sequences belonged to the multidrug-resistant (MDR) group. Similar results were seen in terms of a higher number of coding sequences; however, the isolate belonged XDR group of bacteria included 7414 coding sequences in its 6.68 megabases (mb) draft genome of *K. pneumoniae*, which originated in Pakistan (Lahens et al., 2017; Rahmat Ullah et al., 2020).

The *K. pneumoniae* genome is around 5.5 Mbp in size and has around 5500 genes. Of these genes, over 3,500 are accessory, demonstrating the genome's dynamic and adaptive characteristics (Bialek-Davenet et al., 2014; Holt et al., 2015). Flores-Valdez et al., conducted a recent study using the HiSeq 2000 platform (Illumina) to analyze 38 *K. pneumoniae* isolates (Flores-Valdez et al., 2021). The analysis indicated that the contig count varied from 183 to 1216, with an average assembly size of 5.55 Mb ± 0.14. The GC content was found to be 57.23% ± 0.16%, with a N50 value of 169,445 ± 41,243. Additionally, the study identified a total of 5,135 ± 144 coding sequences (CDS) across the genomes.